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Effect of RNS60 in amyotrophic lateral sclerosis:

a phase II multicentre, randomized, double-blind, placebo-controlled trial

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ABSTRACT

Background: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with limited treatment options. RNS60 is an immunomodulatory and neuroprotective investigational product that has shown efficacy in animal models of ALS and other neurodegenerative diseases. Its administration has been safe and well-tolerated in ALS subjects in previous early phase trials.

Methods: Phase II, multicenter, randomized, double-blind, placebo-controlled, parallel-group trial. Participants diagnosed with definite, probable, or probable lab-supported ALS were assigned to receive RNS60 or placebo administered for 24 weeks intravenously (375ml) once a week and via nebulization (4ml/day) on non-infusion-days, and followed by additional 24 weeks off-treatment. Primary objective: to measure effects of RNS60 treatment on selected biomarkers of inflammation and neurodegeneration in peripheral blood. Secondary objectives: to measure effect of RNS60 on functional impairment (ALSFRS-R), a measure of self-sufficiency, respiratory function (forced vital capacity [FVC]), quality of life (ALSAQ-40) and survival. Tolerability and safety were assessed.

Results: 74 participants were assigned to RNS60 and 73 to placebo. Assessed biomarkers did not differ between arms. The mean rate of decline in FVC and eating and drinking domain of ALSAQ-40 was slower in RNS60 arm (FVC: difference 0.41 per week; standard error 0.16; $p=0.0101$; ALSAQ-40: difference -0.19 per week; standard error 0.10; $p=0.0319$). Adverse events were similar in the two arms. In a post-hoc analysis, Neurofilament Light chain increased over time in bulbar onset placebo participants, while remained stable in those treated with RNS60.

Conclusions: The positive effects of RNS60 on selected measures of respiratory and bulbar function warrant further investigation.

Keywords: randomized, clinical trial, placebo-controlled, treatment, ALS

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a progressive and fatal neurodegenerative disease that affects motor neurons in the spinal cord, brainstem, and motor cortex resulting in muscle atrophy and paralysis. Respiratory complications are the most common cause of death and develop due to progressive paralysis of the diaphragm and the accessory muscles that support ventilation [1]. Riluzole [2] and edaravone [3] are the only two available disease-modifying medications on the market and they both have only modest effects on disease outcomes. Thus, there is an urgent need to develop more treatments for people living with ALS.

In recent years, multiple lines of evidence have supported a critical role for neuroinflammation in ALS pathophysiology including *in vivo* imaging studies in mouse ALS models, *ex vivo* analyses revealing astrocyte and microglia activation even in presymptomatic phases, and recent discoveries showing involvement of several genes directly linked to the immune response [4]. Defects in mitochondrial bioenergetics have also been implicated in the pathogenesis of ALS and are being considered as potential therapeutic targets [5-7].

RNS60 is an investigational product generated by using modified Taylor-Couette-Poiseuille flow under elevated oxygen pressure, which is hypothesized to generate oxygen-filled charge-stabilized nanostructures (O₂ nanobubbles). Although at a molecular level the mechanism of action of RNS60 has not been fully elucidated yet, its immunomodulatory and cytoprotective properties have been demonstrated in animal models of neuroinflammation, neurodegeneration and brain injury [8-11]

Importantly, in SOD1G93A transgenic mice, a widely used model of ALS, RNS60 administered at the onset of the disease delayed the neuromuscular deficit and paralysis in virtue of its effects on multiple disease mechanisms in motor neurons, glial cells, and peripheral immune cells [8]. RNS60 promotes mitochondrial function and biogenesis [12,13] and activates the PI3K-Akt pro-survival pathway in neurons [14]. Furthermore it reduces glial inflammation [9] and increases regulatory T cells (Tregs) in multiple sclerosis and ALS mouse models [8,11,15]. Moreover, RNS60 has shown to increase

neurotransmission and reduce fatigability of nerve stimulated muscles in a murine phrenic nerve-diaphragm ex-vivo preparation [16]. Clinically, long-term RNS60 administration has shown to be safe and well-tolerated in a pilot open-label trial in ALS patients. No serious adverse events related to RNS60 occurred and no participant withdrew from the trial due to drug-related adverse event [17]. Here we report the results of a phase II trial in people with ALS where we investigated the effects of the RNS60 on a number of candidate biomarkers and clinical endpoints. Biomarkers measured in this trial included markers known to be modified by RNS60 in preclinical studies [Interleukin-17 (IL-17), Tregs, and protein nitration] [8,11], markers implicated in ALS pathogenesis [Monocyte Chemoattractant Protein-1(MCP-1) and Cyclophilin A/Peptidyl Prolyl Isomerase A (PPIA)] [18,19], and Neurofilament Light chain (NfL), an established biomarker of neuroaxonal degeneration) [20].

METHODS

Trial Design

This was a multicenter, randomized, double-blind, placebo-controlled, parallel group, add-on trial. The primary study objective was to measure the effect of RNS60 treatment on candidate markers of inflammation and neurodegeneration in peripheral blood of people living with ALS. Candidate markers were MCP-1, PPIA, Tyrosine Nitrated-Actin (Actin-NT), 3-nitrotyrosine (3-NT), IL-17, NfL and Tregs (measured via FOXP3 and CD25 mRNA). Secondary objectives were to evaluate: the effect of RNS60 on functional impairment as measured by the ALS Functional Rating Scale – Revised (ALSFRS-R); the effect on respiratory function as measured by forced vital capacity (FVC % predicted of normal value); the impact on quality of life as measured by ALS Assessment Questionnaire-40 (ALSAQ-40) (5 domains: physical mobility, activities of daily living [ADL] and independence, eating and drinking, communication, emotional reactions); the effect on self-sufficiency defined as having a score of 3 or 4 on all three selected ALSFRS-R questions (specifically swallowing, cutting food and handling utensils, and walking); the effect on survival; tolerability and safety as measured by the identification of adverse events (AEs).

Study population

Trial participants were enrolled at 22 Italian Expert ALS Centers from May 2017 to January 2020. The trial enrolled adults (age: 18-80) with a diagnosis of definite, probable, or probable lab-supported ALS according to the revised El Escorial criteria [21] whose symptom onset had occurred 6 to 24 months prior to enrollment. Additional eligibility criteria included: geographic access to the enrolling center (so that they could go to the site on a weekly basis for intravenous infusions); self-sufficiency (defined as having a score of 3 or 4 on all three selected ALSFRS-R questions, specifically swallowing, cutting food and handling utensils, and walking); satisfactory respiratory function ($FVC \geq 80\%$ of predicted normal value); documented progression of ALS symptoms in the three months prior to screening as measured by a decrease of at least one point in the ALSFRS-R scale total score; stable treatment with riluzole at a dose of 50 mg twice a day.

Trial interventions and procedures

Participants were randomly assigned to receive either RNS60 or matching placebo while concomitantly taking riluzole. Treatment allocation was centrally managed using a computer-generated, permuted block (with a block size of 4), 1:1 randomization scheme. Any concomitant treatment could be continued as per medical indication. RNS60 or placebo were administered intravenously (375 mL infused at a rate of 700 mL/h) once a week as well as inhaled via nebulization (4 mL RNS60 or placebo) every morning (6 days a week on non-infusion days) for 24 weeks (on-treatment period). The IV dose was scaled from animal studies using conventional formulas, although the dosing in animal studies (intraperitoneal route) was more frequent. To minimize the need for more frequent IV infusions, the earlier pilot trial had employed daily inhalations to bridge the interval between the higher volume weekly IV doses. As that combination dose had been well tolerated in the pilot trial, we decided to use the same dose in this trial. Participants were also followed for an additional 24 weeks off-treatment period (through week 48) to investigate whether RNS60 effects persist after suspension of the treatment and, in case of a positive effect, to evaluate their duration. Adherence with the assigned home treatment was measured by counting the number of used/unused syringes returned by the participant at each visit.

Nebulizer machines, RNS60 and placebo for infusion and inhalation were supplied by Revalesio Corporation in identical IV bags and syringes, respectively. Both participants and study staff were blinded to the treatment assignment. Assigned box (infusion and nebulizer) treatment was shown by interactive web response system at each visit making investigators blinded for the entire study.

Data collection

Blood samples for biomarker analyses (RNA and proteins, for detailed methods see Supplementary Material) were collected on day 1 (before the first infusion) and at weeks 4, 12, 24 (on-treatment period), and 48 (off-treatment). Safety and efficacy were assessed by way of physical examination, vital signs, and adverse events (AEs). Changes in function and quality of life were assessed using the ALSFRS-R, FVC (on day 1 and at weeks 4, 12, 24, 36 and 48), and ALSAQ-40 (on day 1 and at weeks 4, 24 and 48).

Statistical analysis

Separate analyses were performed in: 1) All randomized participants (ITT population, primary analysis); 2) Study completers and compliers (which included participants who completed the 24-week on-treatment period and took at least 75% of the assigned dose); 3) Participants who did not have any major protocol deviation (PP population).

Descriptive statistics were performed comparing the two treatment groups. Continuous variables were described using mean and standard deviation or median and interquartile range; categorical variables were described as counts and percentages.

Pre-enrollment progression rate was calculated as follows: $(48 - \text{ALSFRS score at diagnosis}) / (\text{disease duration at diagnosis})$, where disease duration at diagnosis is the time from onset to diagnosis in months. Participants with a progression rate higher than or equal to 0.67 were classified as fast progressing, while those with a score lower than 0.67 were classified as slow progressing [22,23].

Biomarker Analyses

The mean changes in biomarker levels over the on-treatment period (week 0 to 24) and the follow-up, off-treatment period (week 24 to 48) were analyzed using repeated measures analysis of variance (ANOVA) with an unstructured variance-covariance matrix (see Supplementary Material for details).

The variation of the biomarkers over time was tested by the time effect, the difference between treatment groups at specific visit times was tested by the treatment main effect, and the difference between groups in the variation over time was tested by the treatment*time interaction term. In all models, time was expressed in weeks.

Clinical Outcome Analyses:

The mean changes in Forced Vital Capacity (FVC, % predicted), ALSFRS-R total score, and the 5 domains of the ALSAQ-40 scale were analyzed over the on-treatment period (week 0 to 24) and the follow-up, off-treatment period (week 24 to 48) using repeated measures linear mixed models with random intercept and slope and an unstructured variance covariance matrix (see Supplementary Material for details). In these models, the treatment effect was measured by the difference between the slopes of the two treatment arms, which is tested by the treatment*time interaction terms. Differences in baseline values between treatment groups are tested by the treatment main effect, while changes over time are tested by the time effect. In all models, time was expressed in weeks.

The cumulative proportions of participants remaining self-sufficient and survival were calculated in both treatment arms at 4, 12, 24, 36 and 48 weeks using Kaplan-Meier survival curves. Differences between survival curves were assessed with the Log-rank test.

Safety

Total, mean number (with standard deviation), and the proportion of patients with at least one AE were reported separately for different types of AEs (mild, moderate, severe, serious AEs (SAEs), AEs leading to treatment discontinuation, and drug-related AEs) at the end of the study (week 48) and during the on-treatment period at weeks 4, 12 and 24, in each study group. Differences between treatment arms were

tested with the Wilcoxon-Mann-Whitney test and the Fisher's exact test. For the classification of the different types of AEs, see Supplementary Material.

Significance level was set at 5% for all analyses and all tests were two-tailed. Missing data were handled including all available data, using maximum likelihood estimations in repeated measures ANOVA and mixed models. All analyses were performed with SAS 9.4 (SAS Institute, Cary, NC, USA).

Post-hoc analyses

FEV1 (a measure of the amount of air that a person can exhale within the first second of forced expiration) and the 3 ALSFRS-R subscores (bulbar, motor, respiratory) were evaluated using the same statistical methods used for FVC and ALSFRS-R.

Subgroup analyses were performed stratifying participants by site of onset (bulbar or spinal), pre-enrolment progression rate (fast or slow), and NfL level at baseline (below the median or above the median). The outcomes analysed included all biomarkers, FVC and ALSFRS-R. These analyses were performed using the same models used in the total sample, with the addition of the stratification variable main effect (in this case onset, and, separately, progression rate) and its interactions with all other effects included in the model. Least squares means were estimated separately for each category of the stratification variable (bulbar and spinal onset, fast and slow progressing patients) at each time point and in each treatment group. Differences between categories of the stratification variable were tested by the stratification variable main effect, the treatment*(stratification variable) and the time*(stratification variable) interaction terms. The difference in the treatment effect between categories of the stratification variable was tested by the treatment*time*(stratification variable) triple interaction.

Determination of sample size

The sample size was determined making different assumptions for each biomarker included as primary outcome. A total of 142 patients was required to detect with 80% power a 44% decrease in the rate of progression of PPIA at a two-sided 5% level of significance, allowing for a 10% drop-out rate over the entire study period. The number of patients required for other biomarkers ranged from 8 to 68. In

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addition, with this sample size the study was also powered (80% at a 5% two-sided level of significance) to detect a 43% decrease in the rate of progression in ALSFRS-R over 24 weeks, and to show an absolute 25% reduction in the proportion of patients becoming non self-sufficient at 24 weeks assuming that this proportion in the placebo arm was 85%. See Supplementary material for a detailed explanation of sample size calculation and relative assumptions.

Ethical Issues and quality assurance

The study and any amendments were approved by an Independent Ethics Committee of each participating center. This study was planned and performed according to the principles of Good Clinical Practice (ICH-GCP), the declaration of Helsinki, and the national laws and regulations about clinical studies. Eligible patients were included in the study only after written IRB/IEC/REB -approved informed consent, or, if incapable of doing so, after approval by a legally acceptable representative.

Data review and management were performed by “Mario Negri” IRCCS.

The study was registered in ClinicalTrials.gov number, NCT03456882; EudraCT number: 2016-002382-62.

RESULTS

A total of 147 participants were enrolled and randomly assigned to RNS60 (74 participants) or placebo (73 participants). Results are reported for the Intention-To-Treat (ITT) population that included all randomized participants. The study flow-chart is illustrated in Figure 1.

Demographic and other baseline characteristics

Baseline characteristics of the ITT population are shown in Table 1. The baseline characteristics of the two treatment groups were fairly similar.

Biomarker Levels

The levels of MCP-1 (p value=0.0162), NfL (p=0.0373), FOXP3 mRNA (p=0.0067) changed significantly over the course of study participation in both treatment arms. In both arms, MCP-1 and

NfL levels increased over time, while FOXP3 mRNA decreased, without significant differences between the two arms, as indicated by the treatment main effect and treatment*time interaction effect that were not statistically significant. PPIA, Actin-NT, 3-NT, IL-17, CD25 mRNA did not significantly change over time, and no significant differences in their levels between treatment groups were observed. The mean estimates at each visit time for all analysed biomarkers and their graphical representation are shown in Supplementary Table 1, Figure 2, and Supplementary Figure 1.

Clinical Outcomes

FVC and FEV1

The RNS60 and the placebo groups showed similar FVC values at baseline. FVC decreased significantly over time in both arms ($p < 0.0001$) (Table 2 and Figure 3A). During the on-treatment period (baseline to week 24), the estimated mean rate of change was lower in the RNS60 arm (-0.46 per week) as compared to the placebo arm (-0.87 per week) ($p = 0.0101$). During the off-treatment follow-up period (week 24 to week 48), no differences were observed (-0.45 per week for RNS60; -0.54 per week for placebo; $p = 0.5924$).

In a post-hoc analysis, Forced Expiratory Volume 1 (FEV1) was also evaluated. A significant difference in the rate of decrease of FEV1 between the two treatment groups was observed (-0.45 per week for RNS60; -0.52 per week for placebo; $p = 0.0146$) during the on-treatment period, with the RNS60 group showing a slower decline, in line with the findings on FVC (Table 2 and Figure 3B). There were no significant differences between groups during the follow-up, off-treatment period.

ALSFRS-R

The ALSFRS-R scores were not significantly different between groups at baseline. The ALSFRS-R total score declined significantly over time in both groups ($p < 0.0001$) (Table 2 and Figure 3C). The rate of change over time in ALSFRS-R scores did not differ between treatment arms.

ALSAQ-40

There were no significant differences between treatment groups at the baseline visit for any of the domains of the ALSAQ-40 (Supplementary Table 2). The mean scores for each domain increased significantly over time ($p < 0.0001$), corresponding to a decrease in each aspect of quality of life. The mean rate of change in the eating and drinking domains of the ALSAQ-40 were significantly slower in the RNS60 arm (difference, -0.19 per week; SE 0.1 , $p = 0.0319$). (Figure 3D). No significant differences between treatment groups were detected for the other four domains of the ALSAQ-40 scale.

Self-sufficiency and survival

No significant differences in percent of participants who remained self-sufficient were seen in the treatment arms throughout the study period. The cumulative probability of remaining self-sufficient in the RNS60 arm was 62% at 4 weeks, 29% at 12 weeks, 15% at 24 weeks and 7% at 48 weeks. The corresponding probabilities in the placebo group were 62%, 38%, 23%, 0% (Supplementary Figure 2A).

Survival was not different between treatment arms. The cumulative survival probability was 99% in both arms at 24 weeks, 88% in the RNS60 arm and 89% in the placebo arm at 48 weeks (Supplementary Figure 2B).

Completers and compliers and PP population

Data for completers and compliers ($n = 110$; $n = 60$ in the RNS60 group; $n = 50$ in the placebo group) and PP populations ($n = 47$; $n = 25$ in the RNS60 group; $n = 22$ in the placebo group) are shown in the supplementary material.

Results of the biomarkers' analyses are shown in Supplementary Table 3 and 4. For MCP-1, FOXP3 mRNA, and NfL a significant change over the course of the treatment and follow-up periods was confirmed in completers and compliers. For MCP-1 and FOXP3 mRNA this effect was confirmed also in the PP population. A significant treatment main effect, entirely explained by differences at the baseline visit, was found for IL17-A only in the completers and compliers population. A significant difference in the evolution over time between treatment groups was found for PPIA only in the PP

population. A change over time of borderline significance was found only in the PP population for Actin-NT. No other significant effects were found for any biomarker in the completers and compliers nor in the PP population.

Results of the analysis of clinical outcomes are shown in Supplementary Tables 5 and 6. The evolution over time was consistently significant in all clinical continuous outcomes (decrease of FVC, decrease of ALSFRS-R, increase of all five domains of the ALSAQ-40), both in the completers and compliers and in the PP population.

The significant difference between treatment groups in the evolution over time found in the ITT population for FVC was not confirmed in completers and compliers, nor in the PP population. The difference between treatment groups in the evolution over time for the eating and drinking domain of the ALSAQ-40 was consistently significant in completers and compliers and PP population. A difference in the evolution over time for the emotional reactions domain of the ALSAQ-40 was found only in the PP population, with the RNS60 group showing a slower increase. No other significant effects were found for the clinical outcomes in the completers and compliers nor in the PP population.

Adverse events

The total number of AEs and the proportion of participants experiencing at least one AE were similar in the two groups, except for a higher number, and respectively, proportion, of moderate AE and AE leading to study discontinuation in the placebo group (Table 3). The list of all AEs in each treatment group is shown in Supplementary Table 7.

Post-hoc subgroup analyses

Bulbar and spinal onset subgroup analyses

A significant difference in the evolution over time of NfL was found between bulbar and spinal onset in different treatment arms (significant triple interaction) (Figure 4A, Supplementary Table 8). When considering participants with spinal onset, the baseline levels and the change over time was similar

between the two treatment arms. When considering participants with a bulbar onset, those in the RNS60 arm showed higher values than those in the placebo arm at baseline; NfL levels were stable over the 24 weeks of treatment in the RNS60 arm, while in the placebo arm their levels increased. However, the difference between treatment arms in the bulbar onset subgroup was not statistically significant, due to small sample size. A significant difference between the bulbar and spinal onset subgroups was also found for FVC: bulbar onset participants had lower FVC values and a more rapid decrease over time, as compared to spinal onset participants. A slower decrease of FVC in the RNS60 arm compared with placebo arm was also observed in both subgroups, particularly in bulbar onset, however this difference was not statistically significant (Figure 4B Supplementary Table 8).

Fast and slow progressing sub-groups

A significant difference in the evolution over time of NfL was found between fast and slow progressing participants included in different treatment arms (significant triple interaction). When considering participants with a slow progression rate, the baseline values, and the change over time (increase) was similar between the two treatment arms. When considering participants with a fast progression rate, those in the RNS60 arm showed values that were higher than those in the placebo arm, and the change over time was different between the two treatment groups: participants in the RNS60 arm showed an increase in the first four weeks that was followed by a decrease between week 4 and week 24; in the placebo arm a progressive increase between baseline and week 24 was observed (Figure 4C, Supplementary Table 9).

A significant difference between fast and slow progressing participants was found in the rate of decrease of FVC, with fast progressing participants showing a more rapid decrease, as compared to slow participants, however in the absence of significant differences between treatment groups (Figure 4D, Supplementary Table 9).

A significant difference between fast and slow participants was observed in the progression rate of ALSFRS-R score, with fast progressing patients showing lower values. However, no significant differences between treatment groups were found (data not shown).

NfL levels at baseline

A significant difference in the rate of change of FVC, ALSFRS-R and all ALSAQ-40 domains, with the exception of the emotional reaction domain, was found between patients with high (above the median) or low (below the median) baseline NfL levels. Participants with high baseline NfL levels showed a faster decline, compared to patients with low baseline levels. However, no significant differences in RNS60 treatment effects were detected between the two subgroups (data not shown).

DISCUSSION

In this phase 2 randomized, double-blind, placebo-controlled trial, administration of RNS60 showed no effects on a number of biological markers of inflammation and neurodegeneration.

The biomarkers that were investigated in this trial were chosen based on previous preclinical data and/or relevance to ALS pathophysiology. IL-17 was chosen because increased serum and CSF levels of IL-17 have been observed in ALS patients [24] and, in a mouse model of multiple sclerosis, RNS60 treatment was associated with reduced expression of IL-17 [15]. Yet, measurement of IL-17 is limited by the fact that there is no prior information about the longitudinal pattern of IL-17 in ALS patients and by the very low levels of IL-17 in the blood (< 1 pg/ml). FOXP3 mRNA was measured because, in a mouse model of multiple sclerosis and in SOD1G93A mice, RNS60 upregulated FOXP3 mRNA [8,15]. FOXP3 mRNA is low in rapidly progressing ALS patients and its levels inversely correlate with progression rates [25]. 3-NT was evaluated because, in a previous study conducted in our lab, RNS60 reduced 3-NT levels in the spinal cord of the SOD1G93A mouse model, suggesting a possible role of RNS60 in suppressing Nitric Oxide (NO) production [8]. Increased levels of 3-NT and actin-NT were found in PBMCs of ALS patients [26-28], implicating inflammation and oxidative/nitrative stress in disease pathogenesis. Decreased levels of PPIA in PBMCs of ALS patients have been associated with an early

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onset of the disease [29] and with a short disease duration, [30] indicating that reduced chaperone/foldase activity may be at the basis of a more severe phenotype. Increased levels of MCP-1 have been found in central and peripheral nervous system of the SOD1G93A mouse model [19] and in serum and CSF of ALS patients [31-34], indicating central and peripheral immune activation. NfL levels were measured as there is growing evidence supporting their prognostic and pharmacodynamic potential [20]. In our study, NfL values were significantly higher in participants with bulbar onset, in agreement with recent studies showing an association between higher NfL serum levels and bulbar onset in ALS [35].

Although RNS60 did not affect NfL levels in the whole population of the study, a statistically different trajectory of NfL was found by the post-hoc analysis in patients with bulbar onset, in which RNS60 seems to have a stabilizing effect.

While RNS60 administration did not affect the levels of candidate biomarkers of neuroinflammation and neurodegeneration, encouraging results were obtained on selected clinical outcomes. Specifically, RNS60 administration was associated with slower rates of decline in respiratory function and in the bulbar domains of the ALSAQ-40 compared to placebo over a 24-week treatment period. These effects were not accompanied by positive effects on global function or survival, though longer treatment periods may be needed to detect such changes. During the off-treatment follow-up period (weeks 24-48), the difference in the two treatment arms persisted, suggesting a protective effect of early treatment with the drug rather than a transient symptomatic effect. These findings were similar in post-hoc analyses showing differences in FVC% decline when comparing bulbar and spinal ALS onset, though differences did not attain statistical significance probably due to the small sample size of the subgroups. RNS60 treatment slowed the decline of FVC in patients with bulbar onset and stabilized levels of NfL. Indirect evidence of a possible beneficial effect of the active treatment comes from the difference in the drop-out rate between active treatment and placebo (18.9% vs, 31.5%, respectively) over 48 weeks. The lower drop-out rate in the RNS60 arm could be explained by possible subjective perception of a positive effect of the treatment. It is worth noting that there was a steep rise of drop-outs in both treatment arms

towards the end of follow-up. This might reflect the absence of treatment and perhaps disease progression in the absence of treatment during the second semester of the study. Our study also confirmed the benign safety profile of RNS60 expanding on previous open label observations [17].

The positive effects of RNS60 on respiratory function are supported by an exploratory study on the functional consequences of tissue oxygenation with O₂ nanobubbles in murine phrenic nerve-diaphragm ex-vivo preparations [16]. Alternatively, there may be a symptomatic effect of the nebulized treatment, which may be particularly evident in bulbar patients. Future trials focused on clinical efficacy of RNS60 may need to include additional measures of respiratory and bulbar function. Spirometry maneuvers in the present study were performed in the seated position. Future studies may include supine FVC which may be a more sensitive measure of decline in diaphragm function. Measures of orthopnoea and sleep disruption may also be considered. Hence we think that a larger clinical trial focused on clinical efficacy of inhaled/nebulized administration of RNS60 with respiratory and functional endpoints in well selected populations of patients is necessary to conclude whether if RNS60 can be intended used for the treatment of respiratory symptoms or preventing and delaying disease progression [16]. Treatment with RNS60 for 24 weeks did not affect disease progression as measured by the ALSFRS-R or mortality.

However, another compound with anti-inflammatory properties like palmitoylethanolamide (PEA) was able to improve the pulmonary function in ALS patients without modifying the ALSFRS-R scale; the effect was attributed to a direct action of PEA on neuromuscular junction of diaphragmatic muscle which are the last muscles to be paralyzed [36]. RNS60 may also have directly affected the diaphragm muscle.

These results are in contrast with the positive effects of RNS60 reported in the animal model of ALS, however in those mice the drug was administrated intraperitoneally before overt symptom onset [8]. The motor deficit-slowing effect observed in RNS60-treated SODG93A mice was accompanied by partial prevention of lumbar spinal motor neuron loss and NMJ denervation of skeletal muscle. Interestingly, the clinical studies with tofersen (VALOR, NCT02623699, NCT02623699) [37,38], an antisense oligonucleotide targeting SOD1, have shown that those who started tofersen earlier had better

outcomes than those who started later (<https://investors.biogen.com/static-files/4e9393b8-0f67-47a3-a331-05ef3ac7fc63>). A therapeutic intervention in earlier phases of ALS is under investigation in the ATLAS study (NCT04856982), in which tofersen is initiated in clinically presymptomatic SOD1 variant carriers [39]. It should be noted that in the six-month Phase 3 study there were positive results only with regard to neurofilaments and not to ALSFRS-R and survival, as occurred in the present study.

Moreover, recent clinical trial on high caloric nutrition diets has yielded positive results for fast-progressing patients only [40], possibly because the duration of the investigation was also too short to detect effects in slower progressing patients, which could have been the case in our study, too.

Therefore, it is possible that earlier and/or longer treatment with RNS60 might have a better clinical benefit in people living with ALS, which warrants further investigation.

The strengths of the study include the analysis of multiple candidate biomarkers by a centralized laboratory at the Mario Negri Institute-IRCCS using standard operation procedures, thereby limiting analytical variation. Thus, this trial provided an excellent opportunity to characterize the pattern and longitudinal behaviour of these markers in the context of a well-defined trial population. Tertiary sites with broad national distribution were included to increase access and support the generalizability of study results. FVC measurements were performed using the same instrument at each site provided by the Study Sponsor. Finally, the study was sufficiently powered to detect clinically meaningful differences in measures of functional impairment.

The study was limited by the COVID-19 outbreak that started in Italy in February 2020. The pandemic caused restrictions in terms of participant access to trial sites and limitations in the performance of respiratory function tests. This context led to missing visits and missing data.

Also, a total of 155 protocol deviations occurred during the study (71 RNS60 group, 84 placebo group) in 100 patients (49 RNS60 group, 51 placebo group), of which 22 (10 RNS60 group, 12 placebo group) were due to the COVID-19 pandemic. The description of all protocol deviations that occurred during the study, with number of occurrences in each treatment group is reported in Supplementary Table 10. The

third limit was the drug administration route that required weekly visits to the participating center, with significant discomfort for disabled patients. For this reason, testing the efficacy of a full treatment via inhalation route only at the patient's home would have been preferred.

CONCLUSION

In conclusion, administration of RNS60 to people living with ALS for 24 weeks showed no effects on candidate biomarkers of inflammation and neurodegeneration, ALSFRS-R scores, or survival. Positive effects of RNS60 on selected measures of respiratory and bulbar function warrant further investigation, which might include earlier treatment, longer follow-up, additional measures of respiratory and bulbar function, and focus on dosage and route of administration.

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AUTHOR CONTRIBUTIONS (see Appendix)

CONFLICT OF INTEREST

E. Beghi reports grants from Italian Ministry of Health, grants from SOBI, personal fees from Arvelle Therapeutics, outside the submitted work. E. Beghi reports grants from American ALS Association for the conduction of the present study.

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M. Filosto serving on a scientific advisory board for Amicus, Sanofi, Sarepta; Honoraria for speaking engagements for Sanofi, Alnylam. All of them are outside the submitted work.

A. Padovani serving on a scientific advisory board: Director of School of Neurology-University of Brescia); President-elect of the National Society of Neurology SIN; GE Healthcare, Lilly and Actelion Ltd Pharmaceuticals. Travel funded by a commercial entity:Roche, Ely-Lilly, Biogen, Zambon, Nutricia, Lundbeck. Serving as a journal editor, associate editor, or on an editorial advisory board: MDPI. Patents held or pending: Method of generation of a diagnostic index for Alzheimer's disease, electronic device for implementing the method and system, 2016. Honoraria for speaking engagements: He has received honoraria from Nutricia, PIAM, Langstone Technology, GE Healthcare, Lilly and Chiesi. Commercial research support: Advisory and served on the Scientific Advisory Board for GE Healthcare, Lilly and Actelion Ltd Pharmaceuticals. Government research support (including funding organization, grant number, and role): He has participated as PI in more than 30 research projects funded by the Ministry of Health and MIUR. Support from a non-profit foundation or society: CHDI Foundation, Fondazione Cariplo. All of them are outside the submitted work.

G. Logroscino received funds from Roche, Amplifon; he received travel funds for participation in a congress as speaker. All of them are outside the submitted work.

C. Lunetta received compensation for consulting services from Neuraltus, Cytokinetics, Mitsubishi Tanabe Pharma Europe and Italfarmaco and has received funds from ARISLA and Italian Ministry of Health. All of them are outside the submitted work.

J. Mandrioli received research grants from Fondazione Italiana di Ricerca per la Sclerosi Laterale Amiotrofica (ARISLA), the Agenzia Italiana del Farmaco (AIFA), the Italian Ministry of Health, Emilia Romagna Regional Health Authority, Roche, Pfizer. All of them are outside the submitted work.

F. Trojsi serving as a journal editor, associate editor, or on an editorial advisory board: Associate Editor of *Frontiers in Neuroscience/ Frontiers in Neurology* (section Neurodegeneration) (2017 - today) and *Orphanet Journal of Rare Diseases* (BMC/ Springer Nature) (2021); Academic Editor of *Brain Sciences* (MDPI Ed.) (2021). Fee for consultant: Alnylam Italy Srl (2020). All of them are outside the submitted work.

All other authors report no disclosures.

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DATA AVAILABILITY STATEMENT

Anonymized data are available from the corresponding author upon request.

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Appendix 1 (continued)

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Appendix 1 *(continued)*

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Appendix 1 *(continued)*

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Table 1. Demographic and clinical characteristics of the trial population at baseline (ITT population)

	RNS60 (N=74)		Placebo (N=73)		p-value
	Mean	SD	Mean	SD	
Age	59.3	10.4	56.0	10.0	0.0534
Disease duration (months)	15.5	5.8	14.3	5.8	0.2172
Diagnostic delay (months)	9.8	5.1	8.8	4.9	0.2292
BMI	25.0	3.3	24.8	4.1	0.7878
FVC (% of predicted normal value)	102.7	18.2	103.3	16.1	0.8317
ALSFRS-R total score	41.6	3.2	41.4	3.6	0.6871
ALSAQ-40					
Physical mobility	32.1	21.3	33.2	23.1	0.7540
ADL and independence	36.8	27.8	32.9	26.1	0.3762
Eating and drinking	14.5	23.9	13.5	23.9	0.8015
Communication	19.3	25.5	18.6	28.6	0.8742
Emotional reactions	33.6	18.5	33.1	22.2	0.8825
	n	%	n	%	
Sex					0.4467
Male	52	70.3	47	64.4	
Female	22	29.7	26	35.6	
Site of Onset					0.6778
Bulbar	11	14.9	9	12.5	
Spinal	63	85.1	63	87.5	
Pre-enrollment progression rate					0.7232
Fast	18	27.3	18	27.3	
Slow	48	72.7	48	72.7	
Unknown	8		7		

*BMI=Body Mass Index; FVC= Forced Vital Capacity; ADL=Activities of Daily Living; SD=Standard Deviation.

Table 2. Global tests of fixed effects, means and contrasts for FVC, FEV1, ALSFRS-R at different visit times in each treatment arm (ITT population)

FVC									
Tests of fixed effects									
Effect	Week			Treatment			Week*treatment		
p-value	<0.0001			0.9928			0.0101		
Effect	Follow-up			Follow-up*treatment					
p-value	0.1387			0.1734					
Estimated means and contrasts									
Visit time (week)	RNS60			Placebo			Difference (RNS60-Placebo)		
	Mean	SE	p	Mean	SE	p	Mean	SE	p
Treatment period									
0	102.6	1.9	<0.0001	102.6	2.0	<0.0001	-0.02	2.8	0.9928
4	100.7	2.1	<0.0001	99.1	2.1	<0.0001	1.6	2.9	0.5778
12	97.0	2.5	<0.0001	92.1	2.5	<0.0001	4.9	3.5	0.1647
24	91.4	3.5	<0.0001	81.6	3.5	<0.0001	9.8	4.9	0.0474
Slope (treatment period)	-0.46	0.11	<0.0001	-0.87	0.1	<0.0001	0.41	0.16	0.0101
Follow-up period (off treatment)									
36	86.0	3.7	<0.0001	75.1	3.8	<0.0001	10.9	5.3	0.0394
48	80.6	4.4	<0.0001	68.6	4.6	<0.0001	12.0	6.3	0.0571
Slope (follow-up period)	-0.45	0.11	<0.0001	-0.54	0.13	<0.0001	0.09	0.18	0.5924
FEV1									
Tests of fixed effects									
Effect	Week			Treatment			Week*treatment		
p-value	<0.0001			0.8723			0.0146		
Effect	Follow-up			Follow-up*treatment					
p-value	0.4219			0.1148					
Estimated means and contrasts									
Visit time (week)	RNS60			Placebo			Difference (RNS60-Placebo)		
	Mean	SE	p	Mean	SE	p	Mean	SE	p
Treatment period									
0	96.7	2.2	<.0001	97.2	2.2	<.0001	-0.5	3.1	0.8723
4	95.2	2.2	<.0001	94.1	2.2	<.0001	1.1	3.1	0.7107
12	92.3	2.5	<.0001	87.9	2.5	<.0001	4.4	3.5	0.2128
24	87.9	3.4	<.0001	78.6	3.5	<.0001	9.3	4.9	0.0563
Slope (treatment period)	-0.36	0.1	0.0022	-0.77	0.1	<.0001	0.41	0.2	0.0146
Follow-up period (off treatment)									
36	82.6	3.7	<.0001	72.3	3.8	<.0001	10.3	5.3	0.0522
48	77.2	4.3	<.0001	66.0	4.4	<.0001	11.2	6.1	0.0678
Slope (follow-up period)	-0.45	0.1	<.0001	-0.52	0.1	<.0001	0.07	0.1	0.5758
ALSFRS-R									
Tests of fixed effects									
Effect	Week			Treatment			Week*treatment		
p-value	<0.0001			0.9076			0.5725		
Effect	Follow-up			Treatment*follow-up					
p-value	0.3181			0.3363					

Estimated means and contrasts									
Week	RNS60			Placebo			Difference (RNS60-Placebo)		
	Mean	SE	p	Mean	SE	p	Mean	SE	p
Treatment period									
0	41.4	0.4	<0.0001	41.3	0.4	<0.0001	0.1	0.6	0.9076
4	40.4	0.5	<0.0001	40.2	0.5	<0.0001	0.2	0.7	0.8010
12	38.3	0.6	<0.0001	37.9	0.6	<0.0001	0.4	0.9	0.6877
24	35.1	0.9	<0.0001	34.5	1.0	<0.0001	0.6	1.3	0.6280
Slope (treatment period)									
	-0.26	0.03	<0.0001	-0.28	0.03	<0.0001	0.02	0.04	0.5725
Follow-up period (off treatment)									
36	32.0	1.1	<0.0001	31.6	1.1	<0.0001	0.4	0.6	0.8048
48	28.9	1.3	<0.0001	28.8	1.3	<0.0001	0.1	1.9	0.9493
Slope (follow-up period)									
	-0.26	0.03	<0.0001	-0.24	0.03	<0.0001	-0.02	0.04	0.5728
ALSAQ-40 (Eating and drinking)									
Tests of fixed effects									
Effect	Week			Treatment			Week*treatment		
p-value	<0.0001			0.5022			0.0319		
Estimated means and contrasts									
Week	RNS60			Placebo			Difference (RNS60-Placebo)		
	Mean	SE	p	Mean	SE	p	Mean	SE	p
0	15.9	2.8	<0.0001	13.3	2.8	<0.0001	2.6	3.9	0.5022
24 (end of treatment)	20.4	3.2	<0.0001	22.4	3.2	<0.0001	-2.0	4.5	0.6524
48 (follow-up)	24.9	4.1	<0.0001	31.6	4.2	<0.0001	-6.7	5.8	0.2521
Slope									
	0.19	0.1	0.0032	0.38	0.1	<0.0001	-0.19	0.1	0.0319

*FVC=Forced Vital Capacity; FEV= Forced Expiratory Volume; SE=Standard Error

Table 3. Adverse events occurred during the on-treatment period (up to week 24) and the entire study period (from baseline to week-48) (ITT population)

AEs occurred during the on-treatment period (up to week 24)												
	Total number of AE							Patients with at least one AE				
	RNS60			Placebo			p-value	RNS60		Placebo		p-value
	Sum	Mean	SD	Sum	Mean	SD		n	%	n	%	
All AEs	128	1.7	2.6	144	2.0	3.6	0.6477	42	56.8	47	64.4	0.3442
Mild AEs	117	1.6	2.5	120	1.6	3.3	0.7427	42	56.8	40	54.8	0.8107
Moderate AEs	6	0.1	0.3	18	0.2	0.5	0.0166	5	6.8	15	20.6	0.0147
Severe AEs	3	0.04	0.2	6	0.1	0.3	0.4520	3	4.1	5	6.9	0.4940
SAEs	5	0.1	0.3	5	0.1	0.3	0.9882	4	5.4	4	5.5	0.9842
AEs leading to study/treatment discontinuation	0	0.0	0.0	3	0.04	0.2	0.0826	0	0.0	3	4.1	0.1199
Drug related AEs	8	0.1	0.6	1	0.01	0.1	0.3140	3	4.0	1	1.4	0.6198
AEs occurred through week 48												
	Total number of AEs							Patients with at least one AE				
	RNS60			Placebo			p-value	RNS60		Placebo		p-value
	Sum	Mean	SD	Sum	Mean	SD		n	%	n	%	
All AEs	142	1.9	2.8	158	2.1	3.6	0.6084	44	59.5	48	65.8	0.4304
Mild AEs	128	1.8	2.8	129	1.8	3.4	0.8810	42	56.8	40	54.8	0.8107
Moderate AEs	7	0.1	0.4	20	0.3	0.6	0.0101	5	6.8	16	21.9	0.0086
Severe AEs	7	0.1	0.3	9	0.1	0.4	0.8563	7	9.5	6	8.2	0.7912
SAEs	6	0.1	0.3	6	0.1	0.3	0.4122	5	6.8	5	6.9	0.9822
AEs leading to study/treatment discontinuation	0	0	0	5	0.1	0.3	0.0243	0	0.0	5	6.9	0.0224
Drug related AEs	10	0.1	0.7	1	0.01	0.1	0.1728	4	5.4	1	1.4	0.3664

*AE=Adverse Event; SAE=Serious Adverse Event; SD=Standard Deviation

Figure 1. Study flow-chart: screening, randomization, and follow-up

Figure 2. Effect plots for MCP-1, IL-17, NfL, FOXP3 mRNA at different visit times (in weeks) in each treatment arm (ITT population)

Figure 3. Effect plots for FVC, FEV1, ALSFRS-R, ALSAQ40 (eating and drinking) at different visit times (in weeks) in each treatment arm (ITT population)

Figure 4. Effect plots for NfL and FVC at different visit times in each treatment arm by site of onset (ITT population)

Supplementary material (text)

Supplementary Table 1. Global tests of fixed effects, means and contrasts for biomarker values at different visit times (in weeks) in each treatment arm (ITT population)

Supplementary Table 2. Means and contrasts for ALSAQ-40 domains at different visit times (in weeks) in each treatment arm (ITT population)

Supplementary Table 3. Global tests of fixed effects, means and contrasts for biomarker's values at different visit times (in weeks) in each treatment arm (completers and compliers population)

Supplementary Table 4. Global tests of fixed effects, means and contrasts for clinical continuous outcomes (FVC, ALSFRS-R and the 5 domains of the ALSAQ-40) at different visit times (in weeks) in each treatment arm (completers and compliers population)

Supplementary Table 5. Global tests of fixed effects, means and contrasts for biomarker's values at different visit times (in weeks) in each treatment arm (Per Protocol population)

Supplementary Table 6. Global tests of fixed effects, means and contrasts for clinical continuous outcomes (FVC, ALSFRS-R and the 5 domains of the ALSAQ-40) at different visit times (in weeks) in each treatment arm (Per Protocol population)

Supplementary Table 7. Adverse events (AE) list with frequencies and number of patients experiencing each event

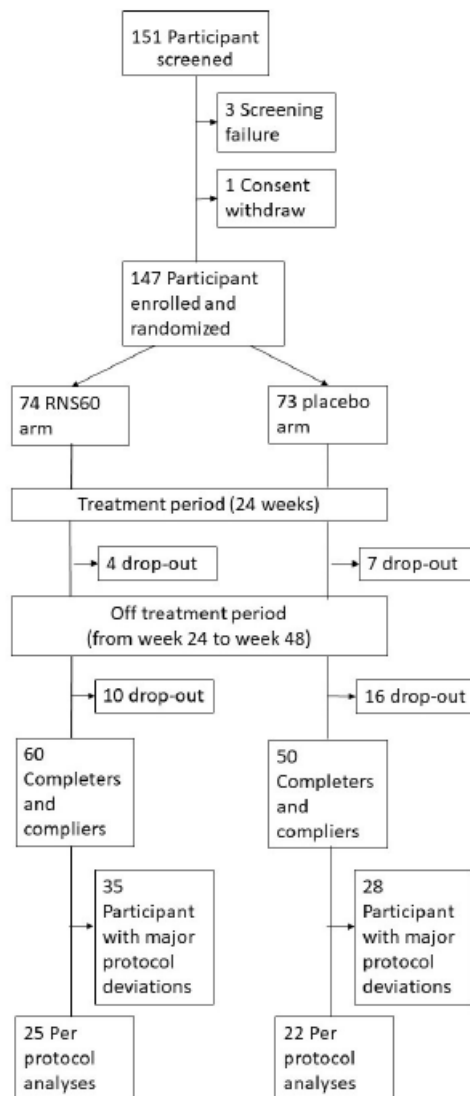
Supplementary Table 8. Global tests of fixed effects, means and contrasts for NfL and FVC at different visit times (in weeks) in each treatment arm by site of onset (ITT population)

Supplementary Table 9. Global tests of fixed effects, means and contrasts for NfL and FVC at different visit times (in weeks) in each treatment arm by progression rate (ITT population)

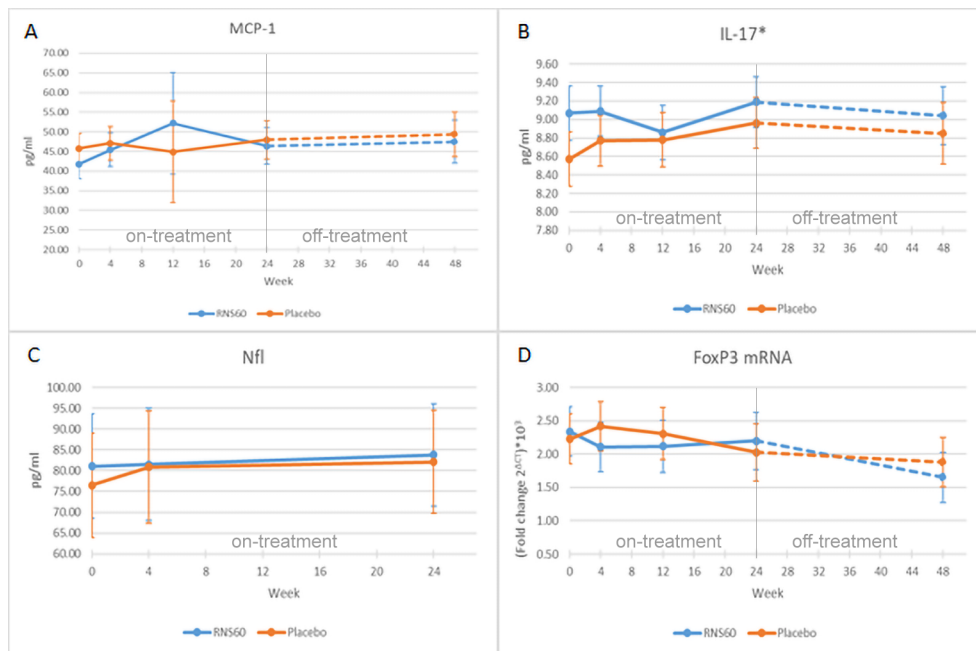
Supplementary Table 10. Protocol deviations by category

Supplementary Figure 1. Effect plots for PPIA, Actin-NT, 3-NT, CD25 mRNA at different visit times (in weeks) in each treatment arm (ITT population)

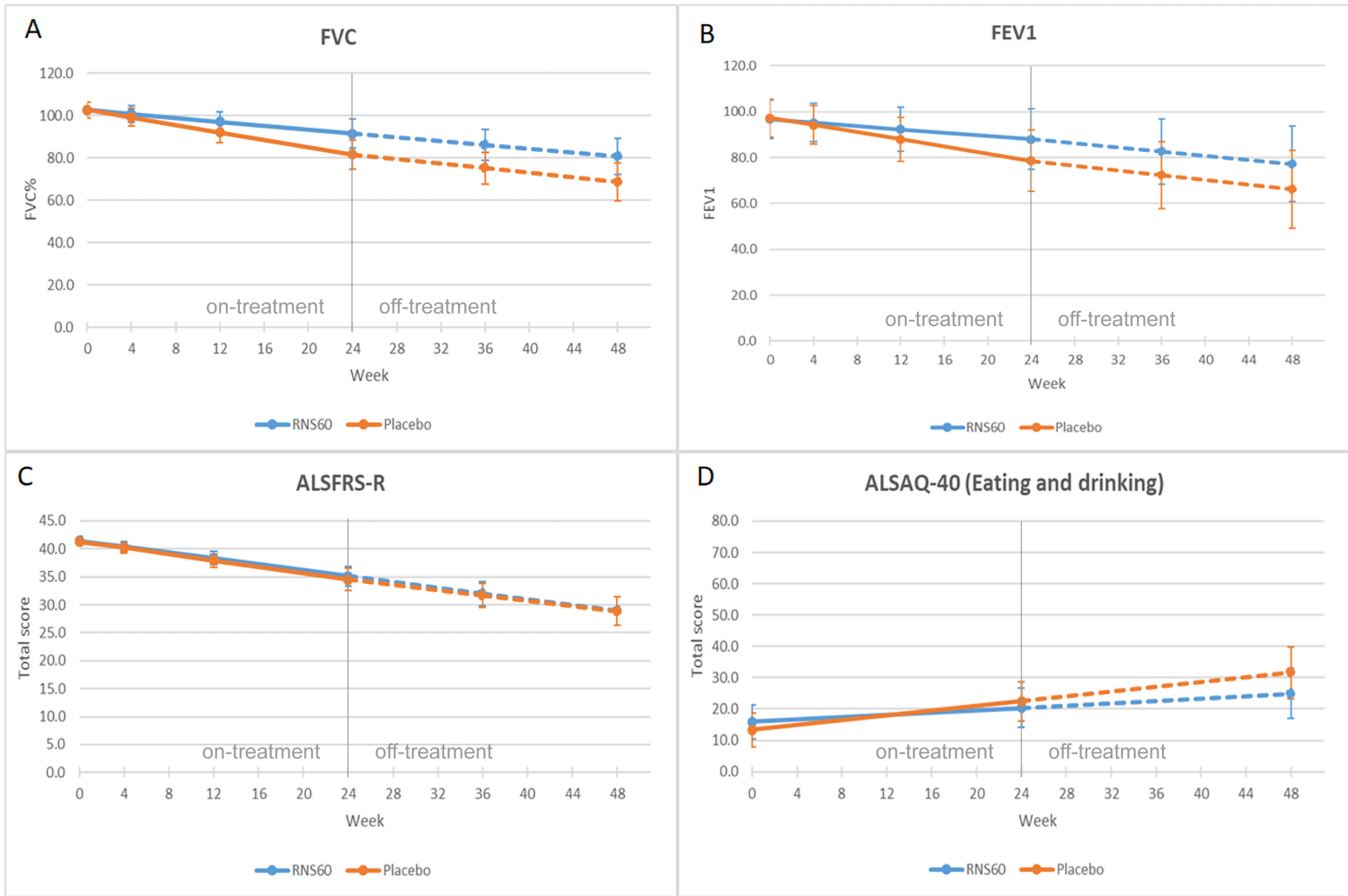
Supplementary Figure 2. Cumulative probability of remaining self-sufficient and survival in the two treatment arms (ITT population)



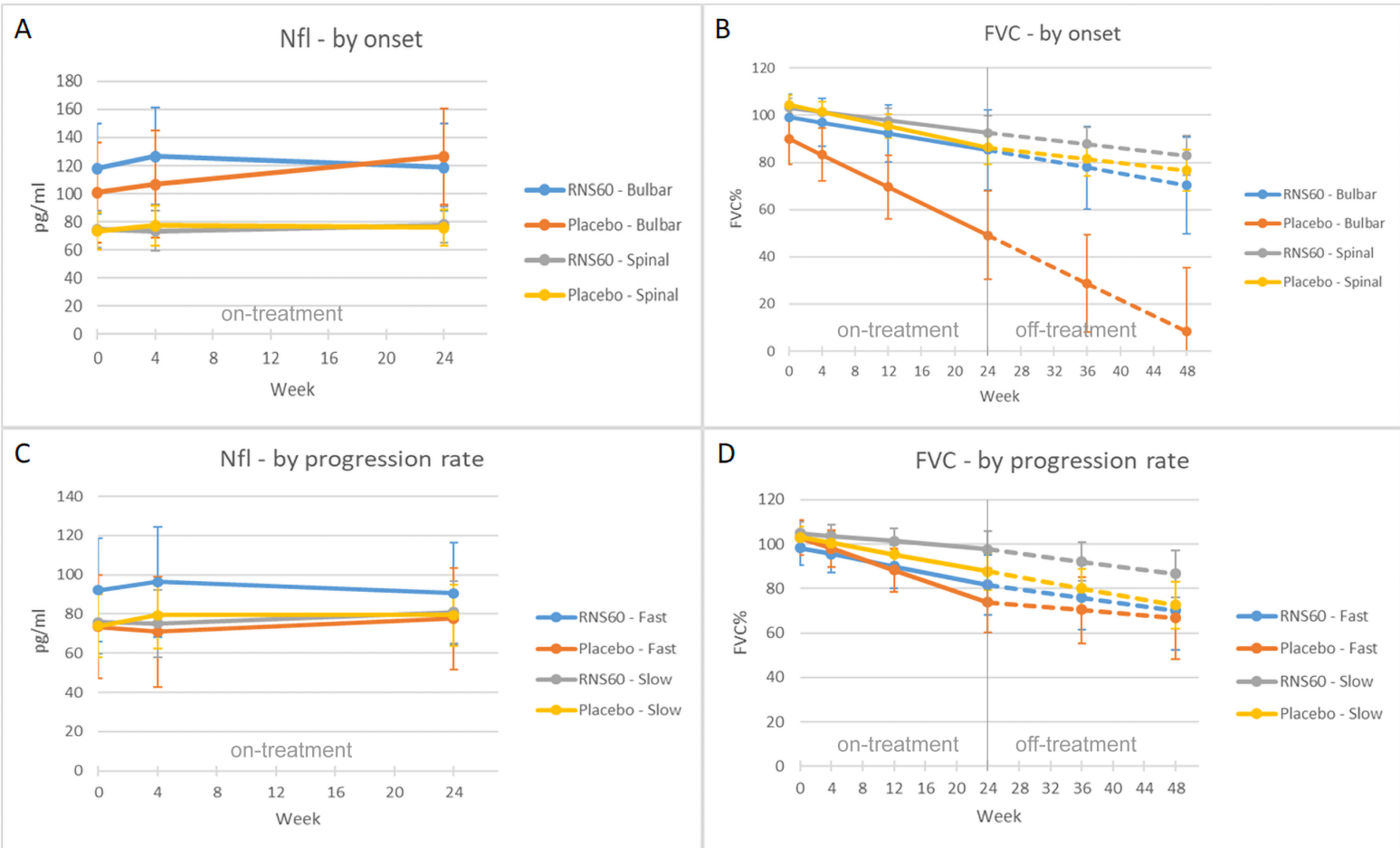
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