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Full-length Article

Long-term survival of participants in a phase II randomized trial of RNS60 in amyotrophic lateral sclerosis

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ABSTRACT

Background: Positive effects of RNS60 on respiratory and bulbar function were observed in a phase 2 randomized, placebo-controlled trial in people with amyotrophic lateral sclerosis (ALS). Objective: to investigate the long-term survival of trial participants and its association with respiratory status and biomarkers of neurodegeneration and inflammation.

Study design and settings: A randomized, double blind, phase 2 clinical trial was conducted. Trial participants were enrolled at 22 Italian Expert ALS Centres from May 2017 to January 2020. Vital status of all participants was ascertained thirty-three months after the trial's last patient last visit (LPLV). Participants were patients with Amyotrophic Lateral Sclerosis, classified as slow or fast progressors based on forced vital capacity (FVC) slope during trial treatment. Demographic, clinical, and biomarker levels and their association with survival were also evaluated.

Results: Mean duration of follow-up was 2.8 years. Long-term median survival was six months longer in the RNS60 group ($p = 0.0519$). Baseline FVC, and rates of FVC decline during the first 4 weeks of trial participation, were balanced between the active and placebo treatment arms. After 6 months of randomized, placebo-controlled treatment, FVC decline was significantly slower in the RNS60 group compared to the placebo group. Rates of FVC progression during the treatment were strongly associated with long-term survival (median survival: 3.7 years in slow FVC progressors; 1.6 years in fast FVC progressors). The effect of RNS60 in prolonging

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long-term survival was higher in participants with low neurofilament light chain (NfL) (median survival: >4 years in low NfL − RNS60 group; 3.3 years in low NfL − placebo group; 1.9 years in high NfL − RNS60 group; 1.8 years in high NfL − placebo group) and Monocyte Chemoattractant Protein-1 (MCP-1) (median survival: 3.7 years in low MCP-1 − RNS60 group; 2.3 years in low MCP-1 − placebo group; 2.8 years in high MCP-1 − RNS60 group; 2.6 years in high MCP-1 − placebo group) levels at baseline.

Conclusions and relevance: In this post-hoc analysis, long term survival was longer in participants randomized to RNS60 compared with those randomized to placebo and was correlated with slower FVC progression rates, suggesting that longer survival may be mediated by the drug's effect on respiratory function. In these post-hoc analyses, the beneficial effect of RNS60 on survival was most pronounced in participants with low NfL and MCP-1 levels at study entry, suggesting that this could be a subgroup to target in future studies investigating the effects of RNS60 on survival.

Trial registration: Study preregistered on 13/Jan/2017 in EUDRA-CT (2016-002382-62). The study was also registered at ClinicalTrials.gov number NCT03456882.

1. Introduction

In a multicenter, randomized, double-blind, placebo-controlled, parallel-group, Phase II clinical trial, 147 people living with amyotrophic lateral sclerosis (ALS) were randomized to either RNS60 (n = 74) or placebo (n = 73) for 24 weeks (NCT03456882) (Beghi et al., 2023). 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 ALS and other models of neurodegeneration, neuroinflammation, and brain injury (Vallarola et al., 2018; Khasnavis et al., 2014; Rangasamy et al., 2020; Mondal et al., 2017).

The methods used in the trial were described elsewhere (Beghi et al., 2023). Briefly, this was a phase II, multicentre, randomized, double-blind, placebo-controlled, parallel-group trial. Participants diagnosed with definite, probable or probable laboratory-supported ALS were enrolled in 22 Italian Expert ALS Centers and assigned to receive RNS60 or placebo for 24 weeks intravenously (375 ml) once a week and via nebulization (4 ml/day) on non-infusion days, followed by an additional 24 weeks of off-treatment follow-up.

Participants were randomly assigned to receive either RNS60 or matching placebo whilst concomitantly taking riluzole. Treatment allocation was centrally managed using a computer generated, permuted block (with a block size of 4), 1:1 randomization scheme.

A total of 142 participants was required to detect with 80 % power a 44 % decrease in the rate of progression of peptidyl prolyl isomerase A (PPIA) at a two-sided 5 % level of significance, allowing for a 10 % drop-out rate over the entire study period. The number of participants required to detect effects on biomarkers ranged from 8 to 68. In addition, with this sample size the study was also powered to detect a 43 % decrease in the rate of progression in ALSFRS-R over 24 weeks.

In pre-specified analyses, the mean rate of decline in forced vital capacity (FVC) over 24 weeks was slower in the RNS60 arm (FVC, slope difference 0.41 per week, standard error 0.16, p = 0.0101). In addition, the decline in the eating and drinking domain of a quality of life measure favoured RNS60 over placebo (ALSAQ-40, slope difference −0.19 per week, standard error 0.10, p = 0.0319). Vital capacity, measured either as FVC or as slow vital capacity (SVC), has been correlated with survival in ALS patients (Czaplinski et al., 2006; Andrews et al., 2018; Pinto and de Carvalho, 2017). Adverse events were similar in the two arms. The mean changes in biomarker levels (MCP-1, PPIA, tyrosine-nitrated actin, 3-nitrotyrosine, IL-17, and Tregs, measured via FOXP3 and CD25 mRNA) over the on-treatment period (weeks 0–24) and the follow-up, off-treatment period (weeks 24–48) were previously reported and did not show differences between the active and placebo groups. Neurofilament light chain levels measured increased over the on-treatment period (weeks 0–24) in bulbar onset placebo participants whilst remaining stable in those treated with RNS60. No differences were detected in the total score nor in the subscore of the ALSFRS-R scale between treatment groups. The relatively short duration of the placebo-controlled clinical trial did not allow for a meaningful evaluation of survival, given the small number of deaths that occurred over the study period (Beghi et al., 2023). We therefore decided to assess survival in all trial participants after a longer follow-up period. We also sought to evaluate whether the effect of the treatment was different according to the rate of FVC decline or selected biomarkers levels.

2. Methods

First patient first visit and last patient last visit of the Phase II clinical trial occurred in May 2017 and December 2020, respectively.

All enrolled participants were entered in this post-hoc analyses (N = 147). Study-wide ascertainment of vital status occurred in September 2023. Vital status was ascertained by contacting the corresponding clinical centers. The date of death and tracheostomy placement were recorded. Survival analyses were performed using Kaplan-Meier survival curves, considering death or tracheostomy as the event variable (the event is the occurrence of the first of the two), and time from randomization to death or tracheostomy as the time variable. Differences in the survival curves between the two treatment groups were tested with the log-rank test.

The mean progression rate of FVC in the two treatment groups was estimated in the primary analysis of the trial using linear mixed models, where treatment was a fixed effect and participant was a random effect. With this model, participant-specific regression lines describing FVC decline were estimated for each participant. The median of the slopes of these regression lines was calculated over the treatment period (week 0–week 24).

Participants were classified as slow or fast progressors based on whether their FVC slope was above or below the median between week 0 and week 4 (at a time when the drug was not expected to have a significant effect on outcomes), and between week 0 and week 24 (at the end of treatment). The number and percentage of participants classified as slow or fast progressors, over the two different periods, was then compared between the treatment groups to: 1. verify if they were balanced before treatment effect (week 0-week 4); 2. confirm if, after treatment, FVC progression rates were slower in participants randomized to RNS60. Kaplan-Meier survival curves were reported in slow and fast progressors to assess the association between FVC rate of decline (assessed from week 0 to week 24) and survival. Differences in survival curves between slow and fast FVC groups were evaluated with the log-rank test. The FVC slope was also included as continuous variable in a Cox proportional hazard model, to evaluate the association between the individual FVC rates of progression (slopes) and mortality.

To detect different treatment effects on survival between subgroups defined by demographic and clinical variables, separate Cox proportional hazards models, including treatment, the demographic or clinical variable and their interaction term as independent variables, were assessed. Variables evaluated were the following: age (<60 vs. ≥60

years; median age = 60 years); sex at birth (males vs. females); site of onset (bulbar vs. spinal); disease duration (time from disease onset to randomization, ≤12 months vs. >12 months); FVC% at baseline (above vs. below the median = 100 %); progression rate (as measured by the ALSFRS-R scale, fast vs. slow); FVC progression rate (fast vs. slow) at baseline; levels (above vs. below the median) at baseline of all biomarkers evaluated in the trial (MCP-1, PPIA, tyrosine-nitrated actin, 3-nitrotyrosine, IL-17, NfL and Tregs, measured via FOXP3 and CD25 mRNA). In the presence of a significant interaction of the demographic or clinical variable with treatment, a subgroup analysis with Kaplan-Meier survival curves was also performed, comparing treatment arms within subgroups defined by the corresponding demographic or clinical variable.

In all Cox models the assumption of proportionality of the hazards was verified for each effect included in the model by testing for a non-zero slope in a generalized linear regression of the scaled Schoenfeld residuals on function of time. In case non-proportionality was detected (significant test for non-zero slope) for a covariate, the corresponding time-dependent covariate was added in the model, to account for non-proportionality. For the primary analysis of the trial, NfL plasma levels were measured at baseline and at the end of the treatment period (week 24). For these post-hoc analysis NfL was measured at an additional time point, at week 48 (in the phase II trial, participants were followed for an additional 24 weeks off study drug). NfL plasma concentration was measured using a Simoa® kit on the Quanterix SR-XTM platform, as described (Beghi et al., 2023). Repeated measures ANOVA with an unstructured variance covariance matrix was used to compare the mean levels of this biomarker at the available time points (week 0 – baseline, week 24 – end of treatment, week 48 – end of study) between the two treatment groups.

The significance level was set at 0.05. All analyses were performed using the SAS statistical package, version 9.4 (SAS Institute, Cary, NC, USA).

The study was pre-approved by the Italian drug Agency (Competent Authority) on 28/oct/2016 and subsequently by all independent ethics committee of each participating centre. Eligible patients were included in the study only after written IRB/IEC/REB-approved informed consent and data protection informed consent, if incapable of doing so, after approval by a legally acceptable representative.

The study was carried out in accordance with the Declaration of Helsinki, GCP, and local laws and institutional guidelines.

3. Results

All randomized participants were included in these post-hoc analyses: vital status was available for all as of September 2023. Baseline characteristics of the ITT population are shown in Supplementary Table 1 and study flow chart is available in Supplementary Fig. 1. Mean follow-up duration at study-wide vital status ascertainment was 2.8 years (SD 1.5), with a maximum of six years of follow-up. The number of deaths was 40 (54.1 %) in the group randomized to RNS60 and 46 (63.0 %) in the group randomized to placebo. A total of 13 tracheostomies were placed in the RNS60 group (17.6 %) vs. 15 (20.6 %) in the placebo group. In the RNS60 group, the cumulative survival probability was 87 % at one year, 69 % at two years, 51 % at three years, and 37 % at four years. The corresponding numbers in the placebo group were 88 %, 58 %, 38 % and 22 %. Median survival was 3.0 years (95 % CI 2.5–3.7) in the RNS60 group and 2.4 years (95 % CI 1.9–3.0) in the placebo group (logrank test for the difference between the two survival curves: p = 0.0519) (Fig. 1, Table 1). The median of the FVC slopes (week 0-week 24), used to classify participants in fast or slow progressors, was −0.5. FVC% at baseline and FVC slope between week 0 and week 4 (before a measurable treatment effect can be expected) were balanced between the active and placebo groups: 40/71 participants (43.7 %) in the RNS60 group and 42/73 participants (43.7 %) in the placebo group were classified as slow progressors. Three participants in RNS60 arm were excluded from this analysis because FVC values were not available at week 4. Between baseline and week 24, a higher number of participants in the RNS60 group was classified as slow progressor (n = 44, 59 %) compared to placebo (n = 30, 41 %) (p = 0.0260).

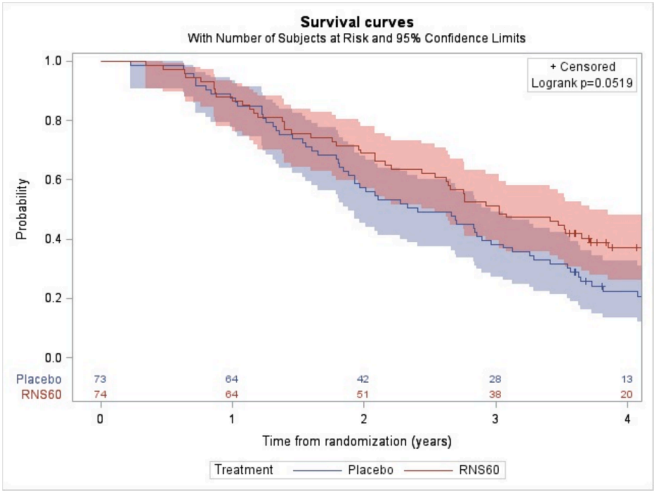

Fig. 1. Kaplan-Meier survival curves in RNS60 and placebo arm.

A strong inverse association between the rate of FVC decline and survival was detected. Median survival was 3.7 years (95 % CI 3.3 – not estimable) in slow FVC progressors and 1.6 years (95 % CI 1.3–1.9) in fast FVC progressors (p < 0.0001, Fig. 2). A significant inverse association between individual FVC slopes and mortality was also detected in the Cox model. The assumption of proportionality of the hazards was violated, suggesting that the hazard ratio for a one-unit increase in FVC slopes is time dependent.

When analyzing the effect of demographic characteristics, clinical variables, and biomarkers on survival in the Cox proportional hazards models, no significant interaction terms with treatment were detected, suggesting that the effect of treatment on survival was not affected by these variables. The only exceptions were for NfL (p for treatment*time interaction = 0.0403) and MCP-1 (p for treatment*time interaction = 0.0462) levels at baseline. NfL and MCP-1 mean value and Standard Error (SE) during the on-treatment period (week 0–24) and off-treatment period (week 24–48) are showed in Supplementary Table 2. When subgroups defined by NfL levels at baseline (below vs. above the median = 64.03 pg/ml; 74 subjects with NfL<64.03 pg/ml – low NfL category; 73 with NfL ≥ 64.03 – high NfL category) were evaluated, we observed a significant treatment effect on survival in the low NfL subgroup (Fig. 3A, p = 0.0120) but not in the high NfL subgroup (Fig. 3B). The median survival in the low NfL subgroup was >4 years in the RNS60 (60 % of participants were still alive in this subgroup at 4 years, therefore the median was not estimable) and 3.3 years (95 % CI 2.7–4.1) in the placebo arm. The corresponding numbers in the high NfL subgroup were 1.9 years (95 % CI 1.2–2.6) and 1.8 years (95 % CI 1.3–2.1). When comparing participants with low vs. high NfL levels at baseline, we observed that those in the low NfL subgroup had longer disease duration (17 months vs. 12 months in the high subgroup, p < 0.0001) and were less frequently bulbar onset (5 % vs. 22 %, p = 0.0069).

Similarly, a treatment effect on survival was observed only in the subgroup with low MCP-1 levels at baseline (below vs. above the median = 40.72 pg/ml; 74 participants with MCP-1 < 40.72 pg/ml – low MCP-1 category; 73 with MCP-1 ≥ 40.72 – high MCP-1 category) (Fig. 4 A and B). The median survival in the low MCP-1 subgroup was 3.7 years (95 % CI 1.9 – not estimable) in the RNS60 and 2.3 years (95 % CI 1.8–3.1) in the placebo arm, while the corresponding numbers in the high MCP-1 subgroup were 2.8 years (95 % CI 2.4–3.4) and 2.6 years (95 % CI 1.6–3.4). No differences in demographic and clinical variables were detected between participants with low or high MCP-1 levels, with

Table 1
Survival probabilities in the RNS60 and placebo arms with number of events, number at risk and censored observations.

Treatment	Time (years)	Survival probability	Died or tracheostomized	Alive (without tracheostomy) and still on follow-up	Censored
RNS60	1	0.86	10	64	0
(N = 74)	2	0.69	23	51	0
	3	0.51	36	38	0
	4	0.37	46	20	8
	5	0.35	47	10	17
Placebo	1	0.88	9	64	0
(N = 73)	2	0.58	31	42	0
	3	0.38	45	28	0
	4	0.22	56	13	4
	5	0.19	58	4	11

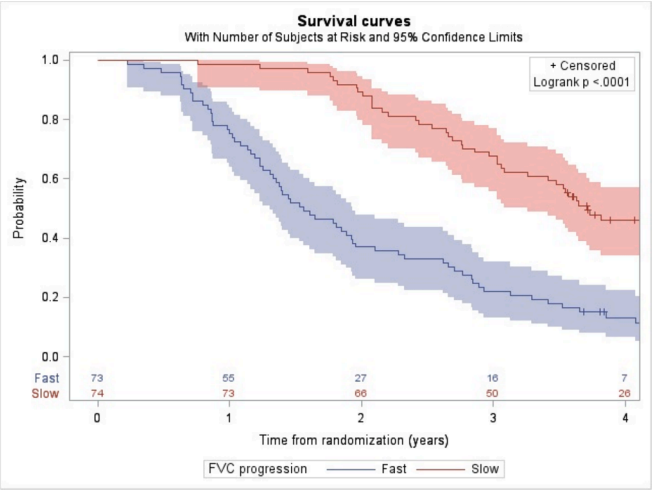

Fig. 2. Kaplan-Meier survival curves in slow and fast FVC progressing participants.

the exception of age: those with low MCP-1 levels were younger (median 55.3 years) than those in the high subgroup (60.1 years) (p = 0.0020).

Given the significant effect of treatment on survival in participants with low NfL and in those with low MCP-1, the correlation between the two biomarkers was evaluated. Pearson's correlation coefficient was 0.05, indicating absence of correlation. In addition, the proportion of participants with low or high levels of NfL was similar between those low or high MCP-1 levels: 39 participants (26.5 %) had low NfL and low MCP-1, 35 (23.8 %) low NfL and high MCP-1, 33 (23.8 %) high NfL and low MCP-1, 38 (25.9 %) high NfL and high MCP-1. The distribution among these 4 categories was similar in the two treatment groups, without significant differences. A variable defined by the four categories above was included in a Cox model to assess its interaction with treatment, that resulted significant (p = 0.0385). A significant effect of treatment with RNS60 in prolonging survival was observed only in the subgroups with low NfL and low MCP-1 at baseline (Fig. 5). The median survival in the low NfL and low MCP-1 subgroup was >4 years in the RNS60 (77 % of participants were still alive in this subgroup at 4 years, therefore the median was not estimable) and 3.1 years (95 % CI 1.9–4.1) in the placebo arm. The corresponding numbers were 3.5 years (95 % CI 2.8 – not estimable) and 3.7 years (95 % CI 2.7 – not estimable) in the low NfL and high MCP-1 subgroup, 1.6 years (95 % CI 0.9–2.2) and 1.8 years (95 % CI 1.3–2.8) in the high NfL and low MCP-1 subgroup, 2.3 years (95 % CI 1.0–2.8) and 1.5 years (95 % CI 1.0–2.3) in the high NfL and high MCP-1 subgroup.

When analyzing NfL levels longitudinally until 48 weeks, mean NfL plasma levels showed an increase in the first 24 weeks of observation (on-treatment period) and a decrease in the subsequent 24 weeks (off-treatment period) in both treatment groups. Baseline NfL values were

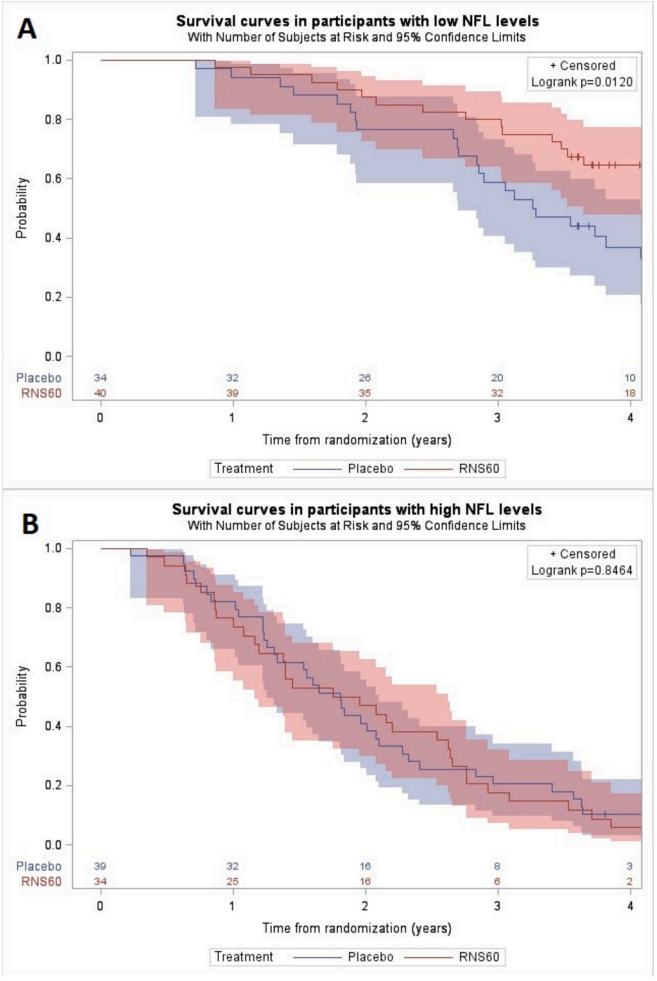

Fig. 3. Kaplan-Meier survival curves in RNS60 and placebo arm by NfL levels at baseline.

comparable in participants randomized to RNS60 and those randomized to receive placebo (estimated mean 81.1 in RNS60 and 76.5 in placebo arm). Repeated measures ANOVA detected a significant variation in NfL levels over time (p for time effect <0.0001), while no significant differences were detected between treatment groups (treatment effect and treatment*time interaction were not significant). The mean estimated NfL levels are shown in Fig. 6.

NfL plasma levels were analysed in subgroups defined according to sex (n = 99 males, 48 females), site of onset (n = 20 bulbar, 126 spinal) and progression rate (n = 36 fast, 96 slow).

Females, bulbar onset and fast progressing participants showed

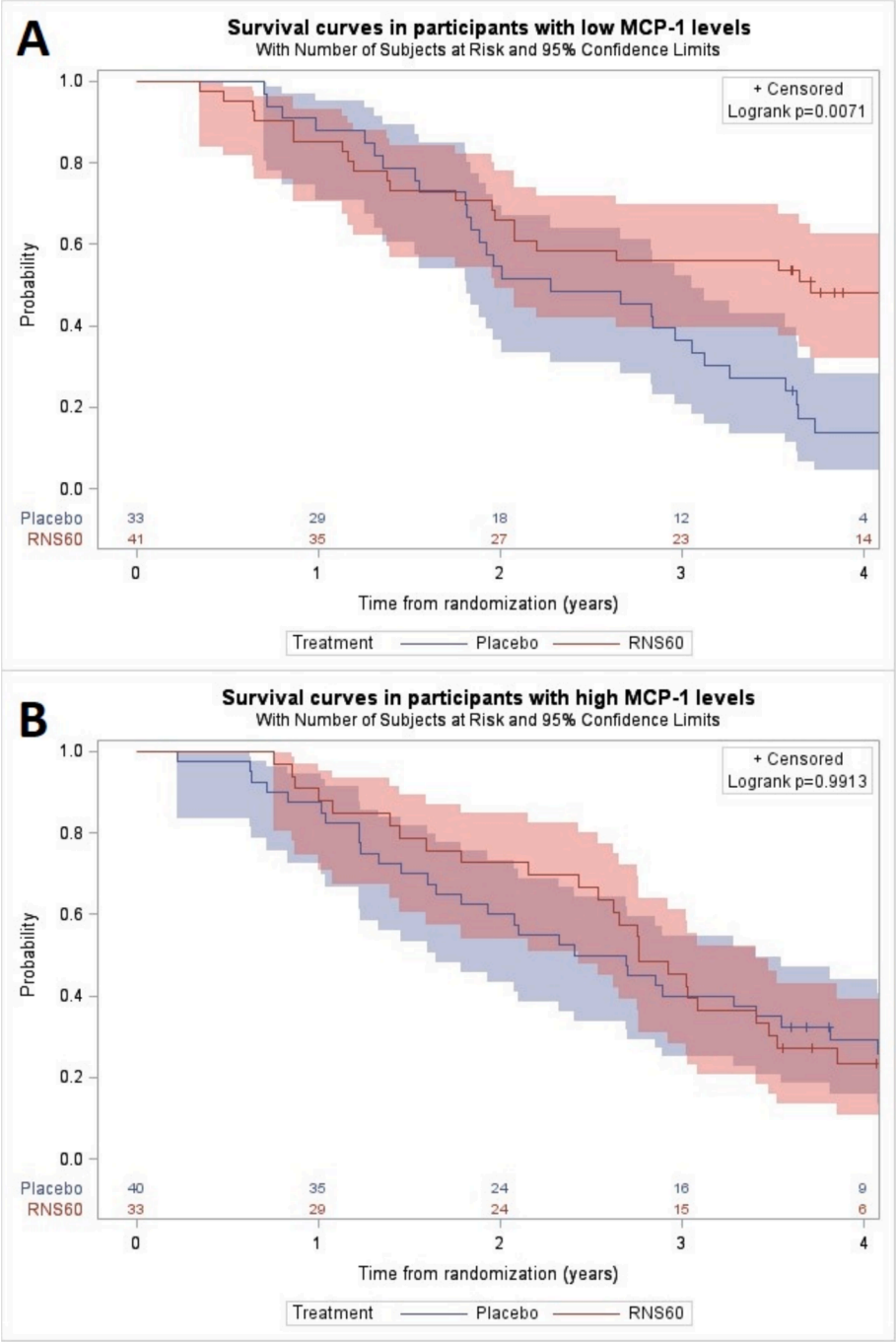

Fig. 4. Kaplan-Meier survival curves in RNS60 and placebo arm by MCP-1 levels at baseline.

higher values, however the difference was significant only for site of onset ($p = 0.0026$ for onset main effect), but without significant differences by treatment group within the two site of onset categories (Fig. 7).

4. Discussion

Administration of RNS60 for 24 weeks in a previously completed phase II ALS trial resulted in positive effects on measures of respiratory and bulbar function, but no effects on candidate biomarkers and other clinical parameters (Beghi et al., 2023). Respiratory function is known to be associated with long-term survival in ALS. However, survival evaluation during the trial was limited by the relatively short treatment duration and low number of events. Here we expand on our previous findings and report the results of a post-hoc long-term survival analysis in all trial participants. This additional analysis demonstrated a trend for longer survival in participants who were randomized to RNS60, with a median survival that was six months longer than in the placebo arm. Of note, administration of RNS60 lasted only 24 weeks and it is possible that longer treatment might result in stronger effects on survival.

The rate of respiratory function decline, as measured by FVC during the trial, was strongly associated with long-term survival. This finding suggests that the survival results might be mediated by RNS60′s respiratory effects, though the presence of residual confounding due to unmeasured or unmeasurable factors cannot be completely excluded.

The beneficial effects of RNS60 are consistent with preclinical evidence. RNS60 was shown to slow down disease progression in ALS mouse models acting on multiple mechanisms in motor neurons, glial

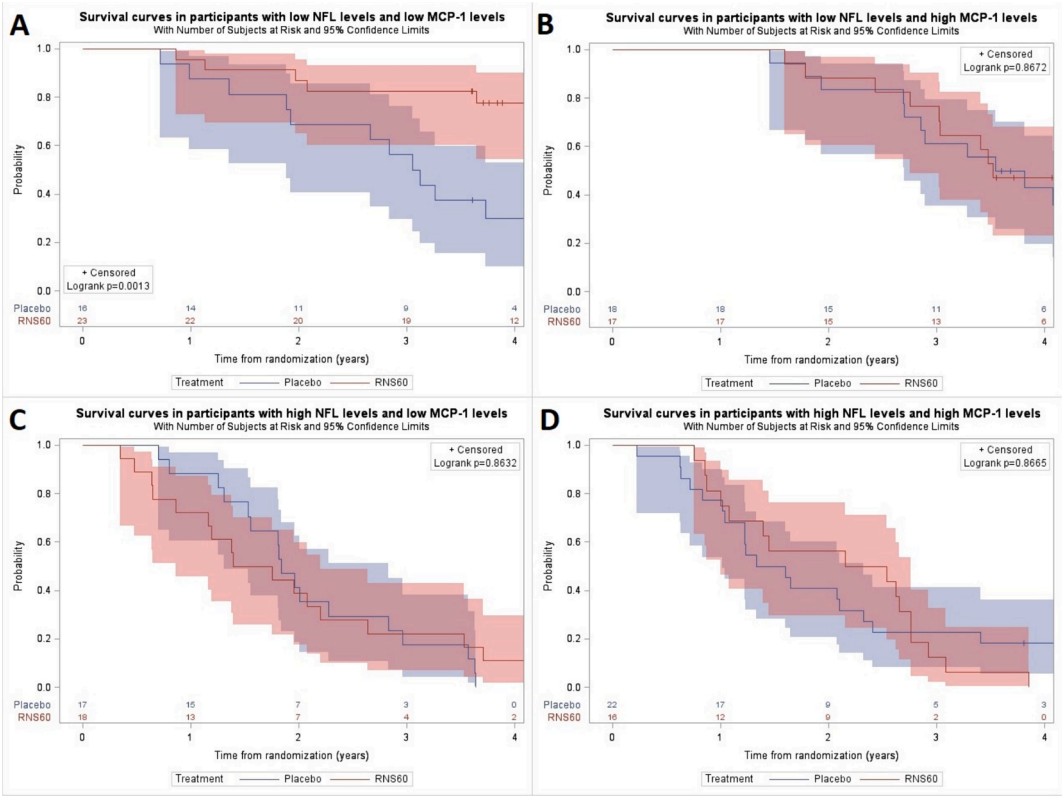

Fig. 5. Kaplan-Meier survival curves in RNS60 and placebo arm by NfL and MCP-1 levels at baseline.

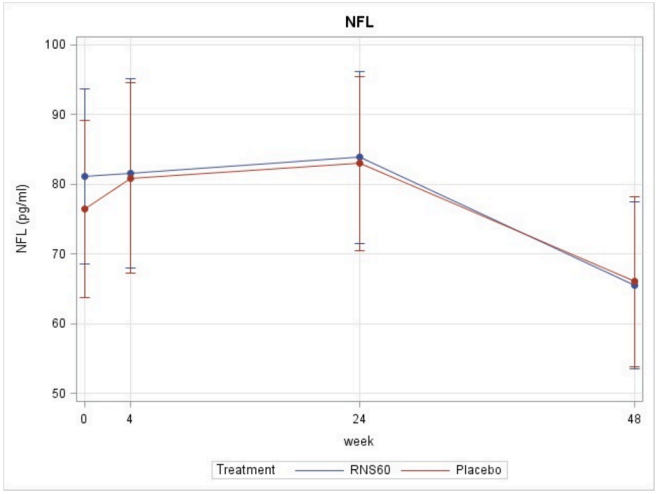

Fig. 6. Mean plasma NfL levels in RNS60 and placebo arms.

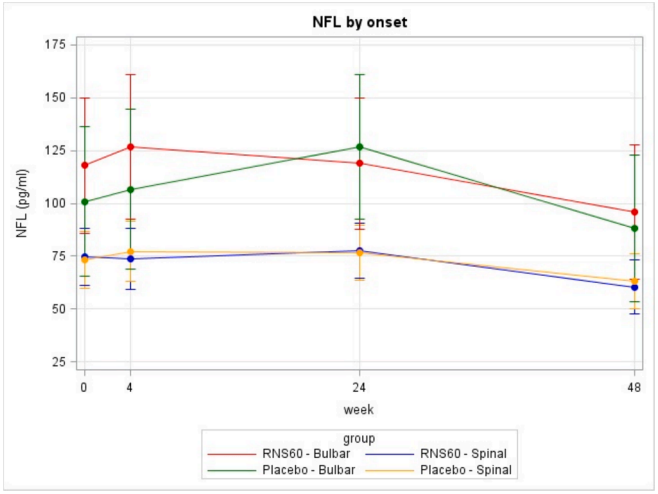

Fig. 7. Mean plasma NfL levels in RNS60 and placebo arms by site of onset.

cells and peripheral immune cells leading to a reduction in lumbar spinal motor neuron loss and neuromuscular junction (NMJ) denervation of skeletal muscle (Vallarola et al., 2018). RNS60 was also shown to increase neurotransmission and reduce fatigability of murine phrenic nerve diaphragm neuromuscular junctions ex vivo (Ivannikov et al., 2017), supporting the positive effects of RNS60 on respiratory function. Therefore, we hypothesize that the observed effects in ALS patients may result from a local effect of the nebulized treatment. Although RNS60 did not impact NfL plasma levels in the overall cohort, an effect of the treatment on NfL was observed in a subgroup of patients, as previously reported (Beghi et al., 2023). NfL levels measured during the on-treatment, placebo controlled randomized period (weeks 0–24) increased over time in bulbar onset placebo participants whilst remaining stable in those treated with RNS60 (Beghi et al., 2023).

RNS60′s effects were more pronounced in the subgroup with low NfL and MCP-1 levels at baseline. NfL is a marker of neuroaxonal damage and its blood level strongly correlates with progression rate at baseline (Lu et al., 2015). MCP-1 is an inflammatory marker that gradually increases as the disease progresses (Beghi et al., 2023; Huang et al., 2020). Thus, the effect of RNS60 may have been stronger in participants with overall slower disease progression. It is interesting to note that these results are consistent with preclinical evidence indicating a favourable impact of RNS60 on ALS mice with slow disease progression, rather than those with rapid progression (Vallarola et al., 2018). A limitation of the present study is that the evaluation of long-term survival in subgroups was underpowered due to the small sample size of the subgroups. In

addition, potential interaction effects that could not be detected with the available power may have been missed.

While the small sample size available in subgroups of participants with specific baseline characteristics limited the evaluation of variables that may affect the effect of RNS60, these data may help guide patient selection in future trials of RNS60. No effect of the treatment was detected on the levels of NfL over 48 weeks. The significant decrease in both treatment groups after a long period of observation, reported also in Mandrioli et al. (2023), may be caused by the exhaustion of the pool of degenerating neurons contributing to NfL plasma levels.

5. Conclusions

In summary, preliminary evidence suggests a possible role for RNS60 in the treatment of ALS. A Phase 3 clinical trial of RNS60 is warranted to evaluate whether longer administration of this investigational product is associated with slower decline in respiratory function and improved survival in people living with ALS. In addition, it would be interesting to investigate the effects of RNS60 in subgroups that might be more likely to respond, such as those with low MCP-1 and NfL levels at baseline who have a less aggressive disease progression. Finally, future studies may include additional biomarkers such as cardiac troponin T, a recently discovered marker of compromised respiratory function (Koch et al., 2024) which would add to our understanding of the effects of RNS60 on respiratory function.

6. Contributors

Pupillo E: study conception, study management, first and final draft; Bianchi E: study conception, statistical analysis, first and final draft; Bonetto V: Centralized laboratory management and sample analyses, first and final draft; Pasetto L: centralized laboratory management and sample analyses; Bendotti C: centralized laboratory management and sample analyses, first and final draft; Paganoni S: study conception, revision of first and final draft; Mandrioli J: data collection at study site, revision of first and final draft; Mazzini L: study conception, project coordinator, first and final draft.

All authors had complete and direct access to the entire dataset and verified the data reported in the present manuscript, contributed to the revision of the manuscript and accepted the final version.

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CRediT authorship contribution statement

Elisabetta Pupillo: Writing – original draft, Supervision, Conceptualization. **Elisa Bianchi:** Writing – original draft, Formal analysis, Data curation. **Valentina Bonetto:** Writing – original draft, Supervision. **Laura Pasetto:** Validation, Data curation. **Caterina Bendotti:** Writing – original draft, Validation, Data curation. **Sabrina Paganoni:** Writing – original draft, Conceptualization. **Jessica Mandrioli:** Writing – original draft, Data curation. **Letizia Mazzini:** Writing – original draft, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Anonymized data are available from the corresponding author upon request. The dataset generated in this study is available in the Zenodo repository https://zenodo.org/communities/irfmn-irccs?q=&l=list&p=1&s=10&sort=newest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2024.08.044.

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