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LIST OF ABBREVIATIONS

3-NT	3-nitrotyrosine
AD	Alzheimer's disease
AE	Adverse event
Actin-NT	Nitrated actin
ALS	Amyotrophic lateral sclerosis
ALSFRS-R	ALS Functional Rating Scale-Revised
ALSAQ-40	ALS Assessment Questionnaire
API	Active pharmaceutical ingredient
BD	Budesonide
CBC	Complete Blood Count
CNS	Central nervous system
CRF	Case report form
CRO	Contract Research Organization
CSF	Cerebral Spinal Fluid
CSN	Charge-stabilized nanostructures
Cyp-A	Cyclophilin A
DAR	Drug Adverse Reaction
DAE	Drug Adverse Event
DM	Italian Ministry Decree
DMC	Data Monitoring Committee
EAE	Experimental autoimmune encephalomyelitis
EKG	Electrocardiogram
EMA	European Medicine Agency
FADD	Fas Associated Death Domain
FasL	Fas Ligand
FEV_1	Forced expiratory volume in one second
FoxP3	Forkhead box P3
FVC	Forced vital capacity
GCP	Good Clinical Practice
ICF	Informed consent form
ICH	International Conference on Harmonization
IEC	Independent Ethics Committee
IFN	Interferon
IGF-1	Insulin growth factor

IL	Interleukin
IMP	Investigational Medical Product
IND	Investigational new drug
iNOS	Inducible nitric oxide synthase
IRB/IEC/REB	Institutional Review Board/Independent Ethics Committee/Research Ethics Board
IRCCS	Istituto di Ricerca e Cura a Carattere Scientifico
IV	Intravenous
Kg	Kilogram
LMN	Lower motor neurons
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein -1
mL	Milliliter
MND	Motor neuron disease
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger RNA
NO	Nitric oxide
NS	Normal saline
3-NT	3-nitrotyrosine
PBMC	Peripheral Blood Mononuclear Cells
PD	Parkinson's disease
PP	Per protocol
QP	Qualified Person
RNA	RiboNucleic Acid
RP	Revalesio Pump
RRMS	Relapsing Remitting Multiple Sclerosis
SAE	Serious adverse event
SOD1	Superoxide dismutase 1
SOP	Standard Operating Procedure
SUSAR	Suspected unexpected serious adverse reactions
Th	T helper cell
TLR	Tool like receptor
TNF	Tumor necrosis factor
T reg	regulatory T lymphocytes
UMN	Upper motor neurons

1 INTRODUCTION

1.1 Background and Rationale

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease that affects motor neurons in the spinal cord, brainstem and motor cortex. Clinical features include fasciculations, muscle wasting and weakness, spasticity, and hyper- or hyporeflexia. Respiratory complications usually develop in patients with advanced disease, and the cause of death is generally the paralysis of the respiratory muscles and diaphragm (Beghi et al., 2007). Although the exact pathophysiological mechanisms underlying neurodegeneration in ALS remain uncertain, a common pathological hallmark is the presence of ubiquitin-immunoreactive cytoplasmic inclusions in degenerating neurons, followed by an inflammatory reaction (McGeer and McGeer, 2002; Bendotti et al. 2012; Malaspina et al., 2015). Prominent neuroinflammation can be observed in pathologically affected areas of the central nervous system (CNS) and in spinal cords from both human ALS patients and mouse models of the disease (McGeer and McGeer, 2002; Philips and Robberecht, 2011). Typically, inflammation in ALS is characterized by gliosis and the accumulation of large numbers of activated microglia and astrocytes. Activation of glia is marked by elevated production of potentially cytotoxic molecules such as ROS, inflammatory mediators such as COX-2, and proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 (McGeer and McGeer, 2002). Major histocompatibility complex molecules and complement receptors are highly expressed by reactive microglia in the primary motor cortex and in the anterior horn of the spinal cords of ALS patients (McGeer and McGeer, 2002; Sta et al., 2011). The major determinants of motor neuron death in ALS remain to be established.

A motor neuron-specific death pathway has been suggested for ALS based on the finding that motor neurons isolated from transgenic superoxide dismutase 1 (SOD1) mutant mice were more sensitive to Fas- or nitric oxide (NO)-triggered cell death than wild-type motor neurons (Raoul et al., 2002). Upon binding of FasL to the Fas receptor, the intracellular portion of Fas recruits the adaptor molecule FADD (Fas Associated Death Domain), which then activates a caspase cascade that culminates in the death of motor neurons. This pathway was also activated in presymptomatic ALS mice (Raoul et al., 2006). Given that astrocytes and microglia produce NO and that astrocytes from SOD1 mutant mice produce FasL (Barbeito et al., 2004), glial cells could directly kill motor neurons. Another member of the same receptor family, the p75 neurotrophin receptor, has also been implicated in ALS-dependent motor neuron death (Pehar et al., 2004). Specifically, nerve growth factor secreted by SOD1 mutant astrocytes induced the death of motor neurons expressing p75 by a mechanism involving the formation of NO and peroxinitrite (Pehar et al., 2004). Peroxynitrite

converts tyrosine to 3-nitrotyrosine (3-NT), leading to protein nitration. Interestingly, high levels of nitrated proteins were found in the spinal cord and peripheral blood mononuclear cells (PBMC) of mutant SOD1 animal models, already at a presymptomatic stage of the disease, and in PBMC of sporadic ALS patients (Casoni et al., 2005; Nardo et al., 2009; Nardo et al., 2011).

Even though motor neurons are the main cells affected in ALS, increasing evidence points to the involvement of neighboring glia cells during pathogenesis (Clement et al., 2003; Yamanaka et al., 2008a). Cell-specific deletion of mutant SOD1 in astrocytes and microglia reduced disease severity and boosted the survival of ALS mice (Boillee et al., 2006; Yamanaka et al., 2008a). Evidence for a role for non-neuronal cells has been obtained using chimeric mice selectively expressing SOD1 in neurons or in non-neuronal cells (Yamanaka et al., 2008b). In chimeric mice expressing high levels of mutant SOD1 in 100% of motor neurons and oligodendrocytes, the presence of wildtype neighboring support cells substantially delayed the onset of motor neuron degeneration. Gene expression profiling implies that inflammatory cascades are activated before the initiation of motor neuron degeneration, suggesting that inflammation could be involved in the presymptomatic phase of the disease (Vargas et al., 2008). Mutant SOD1, but not the wild-type protein, has been proposed to be secreted into the extracellular space via chromogranin vesicles, causing activation of microglia and resulting in motor neuron death in culture (Urushitani et al., 2006). Consistently, mutant SOD1 is present in the cerebrospinal fluid of ALS patients and is toxic to rodent spinal cord cultures (Tikka et al., 2002). Moreover, intracerebral infusion of mutant SOD1 into wild-type mice induced microglial activation and cytokine production (Kang and Rivest, 2007).

Emerging evidence points to an involvement of the adaptive immune response in ALS disease progression. In fact, increased levels of CD4+ and CD8+ T lymphocytes and dendritic cells were detected in close proximity to dying motor neurons in the spinal cords of SOD1 mutant mice (Chiu et al., 2008) and in the brain parenchyma of ALS patients (Kawamata et al., 1992; Henkel et al., 2004). Infiltrating T cells (Th2, Treg) can be neuroprotective after secreting IL-4, which signals reactive microglia to produce neurotrophic factors such as insulin growth factor (IGF-1) (Chiu et al., 2008). In the case of ALS, as in Alzheimer's disease (AD) and Parkinson's disease (PD) as well as other neurodegenerative diseases in which microglia and astrocytes are the primary contributors to inflammation, therapeutic approaches logically would aim to modulate the sensor/transducer/effector functions of the innate immune system, that is, TLRs, NF-κB, and TNF-α, respectively. These findings suggest that targeting inflammatory pathways might be effective in preventing disease progression.

The only drug showing to improve survival in patients with ALS is riluzole (Miller et al, 2007). However, the benefits consist in a three-month delay of death while disability and other outcome measures are virtually unaffected. This highlights the need to test novel approaches to slow the progression of the disease.

1.2 Pre-clinical Data

RNS60 is a novel, anti-inflammatory and cytoprotective drug that has shown remarkable efficacy in animal models of neuroinflammation and neurodegeneration. In multiple *in vitro* and *in vivo* models of different inflammatory diseases, treatment with RNS60 has shown favorable effects ranging from reduced levels of pro-inflammatory cytokines to improved functional outcomes. With direct relevance for this study, intraperitoneal and intravenous (IV) injections of RNS60 in multiple rodent EAE models showed a remarkable improvement of clinical scores, reduction in the expression of inflammatory molecules such as IL-17 and iNOS, reduced gliosis and CNS infiltration by inflammatory cells, and finally reduced demyelination. Moreover, intraperitoneal injection of RNS60 caused significant down regulation of proinflammatory molecules in the brain, and neuronal protection in mouse models of AD and PD. RNS60 inhibited microglial inflammation via type 1A phosphatidylinositol-3 kinase-Akt-CREB-mediated upregulation of IκBα and inhibition of NF-κB activation in cultured glial cells (Khasnavis, S. *et al,*2012). Similarly, RNS60 suppressed NF-κB activation through differential modulation of type 1A PI(3)K pathways in a mouse model of PD, and thereby protected mice against neurodegeneration (Khasnavis S *et al*, 2014). In a rodent EAE model, RNS60 treatment achieved a remarkable improvement of clinical scores, reduction in the expression of inflammatory molecules such as IL-17 and iNOS, reduced CNS infiltration by inflammatory cells, and finally reduced demyelination (Mondal, S. *et al.* 2012).

In both, *in vitro* and *in vivo* models of familial ALS carrying SOD1G93A mutation, we recently observed a reduced up-regulation of iNOS induced by lipopolysaccharide (LPS) in astrocytes (Fig 1.A) and a protective effect of RNS60 on motor neurons. In an *in vitro* model of ALS, consisting of co-cultures of astrocytes and spinal neurons expressing human SOD1 with G93A mutation, RNS60 led to a reduced spontaneous and selective loss of large mutant motor neurons (Fig 1.B). *In vivo*, the intraperitoneal treatment of SOD1G93A mice with RNS60 at the onset of the disease (evaluated at their body weight peak) caused a significant delay in the appearance of motor dysfunction (Fig.1C) and a partial but significant preservation of lumbar spinal cord motor neurons (Fig 1.D) and neuromuscular junctions (Fig 1.E). Moreover, RNS60 treatment led to a small but significant activation of the neuroprotective PI3K-Akt signaling pathway, as demonstrated by the increased phosphorylation of Akt in lumbar spinal cord (Fig 1.F). This demonstrates that the same pathways are modulated by RNS60 across preclinical models of different neurodegenerative diseases.

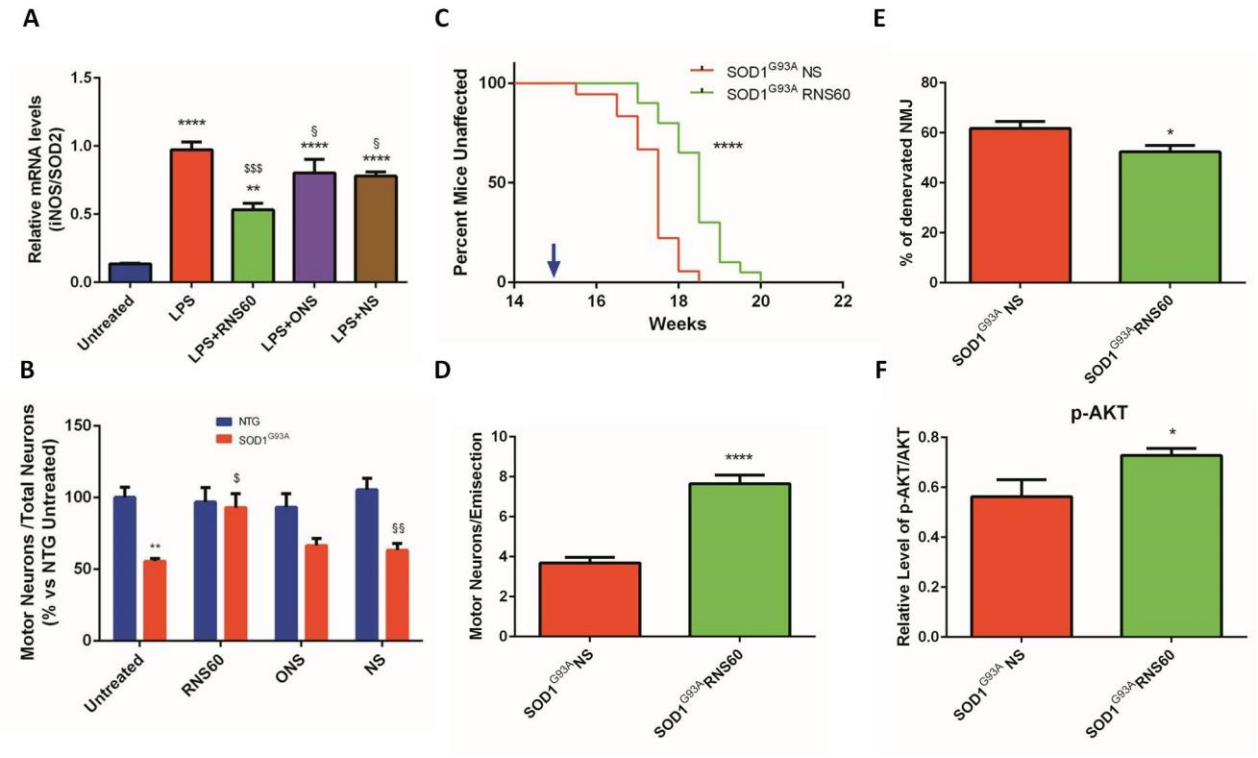


Figure 1. Effect of RNS60 in SOD1^{G93A} linked ALS models *in vitro* and *in vivo*. A) RNS60 reduces LPS-induced iNOS in astrocytes cultures. The iNOS mRNA is normalized to SOD2 mRNA levels. Data are mean ± SEM (n=4 independent experiments) and analyzed by One Way ANOVA (p value<0.001), Tukey Test: ****p<0.001 and **p<0.01 vs. untreated, $^{\$\$\$}$p<0.005 vs. LPS and $^{\$}$p<0.05 vs. LPS+RNS60. **B) RNS60 protects motor neurons in astrocyte-spinal neuron co-cultures from SOD1^{G93A} mice.** Data are mean ± SEM (n=5 independent experiments) and analyzed using Two Way ANOVA (int. p <0.04), Tukey Test: **p<0.01 vs. NTG untreated, $^{\$}$p<0.05 vs. SOD1^{G93A} untreated and $^{\$\$}$p<0.01 vs. NTG NS. **C) RNS60 treatment delays the onset of neuromuscular symptoms in SOD1^{G93A} mice**. The age of onset is considered as the age at which the mouse fails the grip strength test for the first time (grip latency <90 sec). The curves are evaluated by the Log-rank (Mantel-Cox) test (Chi square= 17.10, p <0.001). **D) RNS60 treatment protects motor neurons in SOD1^{G93A} mice**. Motor neuron count from spinal cord L2-L5 segment (10 sections/mouse). The bar graph represents mean ± SEM (n=3 mice per group). Data are analyzed using t-test (p<0.0001). **E) RNS60 slightly protects neuromuscular junctions from denervation.** Data represent denervated plaques as the percentage of total plaques counted in ten frames per section, five sections for each mouse, at 20X-magnification (n=5). At symptomatic stage, SOD1^{G93A} mice treated with NS show significant denervation (61.76%), which is slightly reduced by the treatment with RNS60 (52.35%). Histograms show the mean ± SEM of 5 mice per group. Data are analyzed using t-test (p=0.0237). **F) RNS60 activates PI3K-Akt signaling in the lumbar spinal cord**. Western blot of lumbar spinal cord proteins show that the level of phosphorylated Akt (p-Akt) is higher in SOD1^{G93A} mice treated with RNS60 as compared to NS-treated controls. The bar graph represents mean ± SEM (n=5 mice per group). Data are analyzed using t-test (p=0.05).

Therefore, RNS60 presents itself as a promising candidate for the treatment of ALS. Its exceptional safety profile, demonstrated both in preclinical toxicology studies and Food and Drug Administration (FDA)-approved clinical phase I studies upon inhaled and IV administration, supports testing of RNS60 in clinical phase II studies in ALS patients.

1.3 Pre-clinical Toxicology Data

Extensive *in vitro* and *in vivo* preclinical testing of RNS60 has been completed at multiple institutions and in various models. RNS60 did not show any concerning signs of toxicity in any of the *in vitro* cell culture studies performed to date or in the animal toxicology studies conducted in accordance with FDA guidelines for Investigational New Drug (IND) Applications. The administration of the maximum deliverable dose of RNS60 by bolus injections (10 mL/kg) or IV infusion for 6 (48 mL/kg) or 24 hours (96 mL/kg) daily for 28 days was well tolerated in albino rats. There were no RNS60-related mortalities, clinical observations, ocular observations, abnormal laboratory results (serum chemistry, hematology and urinalysis), organ weight changes, macroscopic or microscopic observations or genotoxic activity. The administration of the maximum deliverable dose of RNS60 by bolus injections (10 mL/kg) or IV infusion for 6 (48 mL/kg) or 24 hours (96 mL/kg) once daily for 28 days was well tolerated and did not reveal toxicity in any target organs in cynomolgus monkeys.

In the bacterial reverse mutation assay (Ames assay) using Salmonella typhimurium strains (TA98, TA 100, TA 1535 and TA 1537) and Escherichia coli WP uvrA in the presence and absence of S9 metabolic activation, RNS60 caused neither an increase in the number of revertant colonies nor a dose-related response in any of the tested strains at any dose level. In the mouse lymphocyte assay, RNS60 similarly demonstrated minimal cytotoxicity and did not increase the mutation frequency relative to the vehicle controls in either the presence or absence of metabolic activation. Therefore, it was concluded that RNS60 was not mutagenic.

1.4 Clinical Data to Date

RNS60 has been well tolerated in human safety studies, both after IV infusion and when administered by inhalation. In a Phase I safety and tolerability study (Clinicaltrials.gov # NCT01264783), RNS60 was administered to 9 healthy subjects as continuous IV infusion at 3 subsequently increasing rates of 100 mL/h, 150 mL/h, and 200 mL/h over 48 hours at each rate. No serious adverse events (SAEs) were reported in this study and no subjects were discontinued from the study due to an adverse event (AE). Furthermore, no clinically relevant changes were noted in clinical laboratory tests, vital signs, physical examinations, oral fluid intake or urine output. The most frequently reported AE was mild headache, which occurred most often in the highest infusion

rate of 200 mL/h, and was considered possibly related to the treatment. However, there was an increase in overall incidence with the increased infusion rate for both placebo and RNS60-treated subjects, with 55% of subjects in the RNS60 group and 33% of subjects in the placebo group reporting mild headache. Since headache is a common symptom of fluid (volume) load it is probable that the headaches were primarily a volume-related effect of the infusion. Other AEs reported as treatment related were a single incidence each (11.1%) of mild arthralgia, mild limb discomfort, and moderate musculoskeletal pain. Cardiac monitoring showed only mild, sporadic and infrequent Electrocardiogram (EKG) changes from baseline; however these changes were not attributed to use of RNS60.

In a second Phase I study (Clinicaltrials.gov # NCT01057498), RNS60 administered to 16 subjects with mild to moderate asthma by inhalation through a nebulizer, at a 4-mL dose twice a day for 28 days was well tolerated. No SAEs were reported in this study and no subjects were discontinued from the study due to an AE. Furthermore, no clinically relevant changes were noted in clinical laboratory tests, vital signs, or physical examinations. The most commonly reported treatment related AE was mild oropharyngeal pain, reported by 2 subjects (12.5%). One subject (6.3%) reported a single incidence of moderate nasal mucosal disorder. Other AEs reported as treatment related were all mild, and were single incidences (6.3%) of eye allergy, ocular hyperemia, chest discomfort, increased blood pressure, increased appetite, headache, somnolence, allergic sinusitis, cough, dyspnea, nasal congestion, throat irritation, and pruritus. Additionally, RNS60 treatment did not cause bronchoconstriction, but rather demonstrated positive effects on forced expiratory volume in 1 second (FEV_1) and peak expiratory flow measurements, as well as improved quality of life scores.

In a third Phase I study (Clinicaltrials.gov # NCT01511302), RNS60 administered by nebulization in combination with a budesonide (BD) dose of either 0.25 mg or 0.5 mg for 28 days, to subjects with mild to moderate asthma who were previously taking a prescribed corticosteroid, was also well tolerated. 57 subjects were treated with the combination of RNS60 and BD (RNS60-BD). No SAEs were reported and no discontinuation of subjects occurred that were related to use of RNS60-BD. AEs attributed to the use of RNS60-BD were sporadic, with single incidences (3.4%) of moderate migraine, moderate wheezing, moderate asthma prophylaxis, mild chest discomfort, and mild increase in hepatic enzymes. Furthermore, there were no significant findings noted in clinical laboratory tests, vital signs, or physical examinations. Additionally, use of RNS60-BD did not cause any incidence of clinically significant bronchoconstriction; rather it demonstrated positive

trends in quality of life improvements and reduced use of rescue inhalers. For details of these studies refer to the Investigator's Brochure (IB) for RNS60.

An ongoing interventional Phase II trial with a weekly IV infusion of RNS60 in relapsing/remitting multiple sclerosis (RRMS) patients is currently enrolling subjects at the University hospitals in Zurich and Innsbruck.

An ongoing single center, open label, pilot trial of RNS60 in ALS patients is enrolling at Massachusetts General Hospital. Subjects are receiving weekly IV infusion of RNS60 in addition to daily inhalation of RNS60 for six months while being monitored for disease progression and microglial activation by PET imaging.

1.5 Description of RNS60

Chemically, RNS60 consists of 0.9% sodium chloride and dissolved oxygen. RNS60 is manufactured by electrokinetic processing in Revalesio's patented Revalesio Pump (RP). During operation of the RP, the fluid is exposed to very strong hydrodynamic, rotational, vortical and longitudinal shear forces, resulting in forceful dispersion of the oxygen. The combined effect of these forces alters the physical relationship between gas and liquid, and has a concomitant effect on the electrical properties of the fluid caused by a reorganization of ionic charges and the formation of associated electrical double layers. RNS60 does not contain a chemical Active Pharmaceutical Ingredient (API).

The observed bioactivity of RNS60 is, instead, based on the presence of charge-stabilized nanostructures (CSN) generated by the electrokinetic processing in the RP. The CSNs consist of an oxygen core surrounded by layers of separated positive and negative electrical charges. The electrical charges associated with the core oxygen stabilize the layers of separated electrical charges and form CSN-associated electrical fields in RNS60.

1.6 Tolerability

As stated in the previous sections referring to preclinical toxicology work, minimal adverse effects were seen with the treatment of RNS60. Section 1.4 explains how RNS60 was safe and well tolerated in multiple human safety studies. RNS60 has demonstrated safety when administered to subjects via inhalation as well as when administered intravenously.

1.7 Dose Rationale

The IV and inhaled doses of RNS60 have been selected based on previous experience in clinical studies. Because no safety concerns have been identified to date, these doses are expected to be well

tolerated by the subjects and provide appropriate exposure to test the hypothesis of the study. The importance of remaining within the margin of safety in dose selection has been taken into account while maximizing dosing frequency without infringing on practicality for subjects.

1.8 Pharmacokinetics

The observed bioactivity of RNS60 is not based on an API, as RNS60 contains neither a chemical drug substance nor a biologic. Instead, the bioactivity is caused by a physical property of RNS60 that is associated with the presence of CSNs generated by the electrokinetic processing in the RP. The CSNs consist of an oxygen core surrounded by layers of separated positive and negative electrical charges. Preclinical studies have conclusively demonstrated that the oxygen alone is not responsible for RNS60's bioactivity, but that it is the combination of oxygen with the processing in the RP that results in a physical quality of the fluid that is able to modulate the stress response of cells. As it is currently impossible to directly image CSNs *in vivo,* traditional pharmacokinetic evaluations are not applicable to RNS60. Preclinical data clearly demonstrate, however, that RNS60 is able to modulate the type 1A PI(3)K signaling pathway within the CNS after systemic administration. RNS60 induced the activation of type IA phosphatidylinositol-3 kinase (PI3K) in the substantia nigra of healthy as well as MPTP-intoxicated mice within 3 hours post intraperitoneal injection, and the effect was sustained until 6 hours post injection (Khasnavis S *et al*, 2014). Thus, RNS60 is expected to mediate its effects both on peripheral immune cells as well on CNS targets, as reported earlier (Modi, KK. *et al.* 2014; Khasnavis, S. et at. 2014; Mondal, S. *et al.* 2012).

2 STUDY OBJECTIVES AND OUTCOMES

2.1 Primary Objective

To measure the effect of RNS60 treatment on selected pharmacodynamic biomarkers in ALS patients concurrently treated with riluzole. Candidate markers include:

1. T-reg (measured via FOXP3 and CD25 mRNA);
2. Cyp-A;
3. 3-NT;
4. Actin-NT;
5. MCP-1;
6. IL-17.

2.2 Secondary Objectives

1. The preliminary efficacy of RNS60 on functional disability, as measured by the ALSFRS-R scale;
2. The preliminary efficacy of RNS60 in prolonging survival (or time to tracheostomy, whichever comes first);
3. The preliminary efficacy of RNS60 in slowing the decline of forced vital capacity (FVC) from baseline;
4. The tolerability and safety of RNS60 through the identification of unexpected adverse events;
5. The impact of RNS60 on quality of life as measured by ALSAQ-40 scale.

2.3 Primary Endpoint

The mean change over time in the proportion of T-reg (measured via FOXP3 and CD25 mRNA); the mean change over time in the plasma concentrations of Cyp-A, 3-NT, Actin-NT, MCP-1, IL-17 in the two treatment arms, during the entire treatment period (from baseline to 24 weeks).

2.4 Secondary Endpoints

1. The mean change in the proportion of T-reg (measured via FOXP3 and CD25 mRNA); the mean change over time in the plasma concentrations of Cyp-A, 3-NT, Actin-NT, MCP-1, IL-17 during the follow up period (from 24 to 48 weeks);
2. The cumulative proportion of subjects becoming no longer self-sufficient at 4, 12, 24, 36 and 48 weeks in the two treatment arms (defined as those scoring 2 or lower on at least one of the ALSFRS-R items for swallowing, cutting food and handling utensils, or walking);
3. The mean change of ALSFRS-R total score in the two treatment arms during the treatment and follow up period;
4. The cumulative proportion of deaths or tracheostomies in the two treatment arms during the entire study including follow-up;
5. The mean change of FVC score in the two treatment arms during the treatment and follow up period;
6. The total number of subjects in the two treatment arms experiencing at least one AE leading to treatment discontinuation at 4, 12 and 24 weeks;
7. The mean number of AEs per treatment arm at 4, 12, 24 and 48 weeks;

8. The mean change of ALSAQ-40 score measuring quality of life at the completion of the treatment and follow up period.

3 STUDY DESIGN

The study is a multicenter, randomized, double-blind, placebo-controlled, parallel group, add-on pilot trial in patients taking riluzole. Patients diagnosed with ALS who meet the study's inclusion and exclusion criteria and sign the Informed Consent Form (ICF) will be enrolled. There will be two groups making up a total of 142 subjects: Group A (71 subjects) and Group B (71 subjects). Subjects will be randomly assigned to receive treatment with either RNS60 or placebo while concomitantly taking riluzole (50 mg twice a day). RNS60 or placebo will be administered intravenously once a week as well as inhaled via nebulization every morning (6 days a week on non-infusion days) for 24 weeks. All visit windows (± 1 or + 4 days according to study procedure) are consecutive calendar days and are calculated from the day the participant starts study treatment (the day of the Randomization-Baseline Visit).

Blood samples will be collected five times for enrolled subjects to measure biomarkers. The first blood sample collection will be at baseline before the first infusion dose. Safety and efficacy will be assessed by way of physical exam, vital signs and AEs. Changes in disability and quality of life will be assessed using the ALSFRS-R scale, FVC and ALSAQ-40 scale. The overall treatment duration will be 24 weeks. Each subject will be followed up for a maximum period of 48 weeks (24-week treatment + 24-week follow up) or until death or tracheotomy, whichever occurs first.

4 STUDY POPULATION

4.1 Disease definition

[….]

Included in the study may be cases of bulbar-onset and spinal-onset ALS. Cases with only bulbar signs or symptoms for the first three months from onset, with spinal symptoms or signs occurring later, are considered bulbar onset ALS. Cases in which any spinal symptoms or signs appeared in the first three months are considered spinal onset ALS. Bulbar onset and spinal onset ALS will be considered separately at the analysis stage.

Patients not fulfilling the diagnostic criteria as indicated in the <u>protocol</u> cannot be enrolled.

4.2 Inclusion Criteria

1) Age 18 through 80 years inclusive;

2) Geographically accessible to the site and able to come to the site center once a week for 24 weeks;
3) "Definite" or "Probable" or "Probable laboratory-supported" ALS diagnosis according to the revised El Escorial criteria;
4) Disease duration 6 to 24 months from symptom onset to study entry;
5) Self sufficiency: Satisfactory bulbar and spinal function (score 3+ on the ALSFRS-R for swallowing, cutting food and handling utensils, and walking);
6) Satisfactory respiratory function (FVC ≥80% of predicted);
7) Documented progression of symptoms in the last three months as measured by the ALSFRS-R scale (decrease of at least one point);
8) Ability to understand and comply with the study requirements;
9) Ability to give written informed consent personally or, as an alternative, via a legally authorized representative;
10) Treatment with riluzole 50 mg twice/day for at least 1 month prior to screening visit;

4.3 Exclusion Criteria

1) History of HIV, clinically significant chronic hepatitis, antecedent polio infection, or other active infection;
2) Motor neuron disease (MND) other than ALS;
3) Involvement of other systems possibly determining a functional impairment (as measured by the end-points) for the entire duration of the study;
4) Other severe clinical conditions (e.g., cardiovascular disorders, neoplasms) with impact on survival or functional disability in the next 12 months;
5) Renal insufficiency as defined by a serum creatinine > 1.5 times the upper limit of normal
6) Poor compliance with previous treatments;
7) Other experimental treatments in the preceding 3 months prior to screening visit;
8) Women who are lactating or able to become pregnant (e.g. who are not post menopausal, surgically sterile, or using inadequate birth control) and men unable to practice contraception for the duration of the treatment and 3 months after its completion;
9) Unwillingness or inability to take riluzole;
10) Poor compliance with an inhalation device;
11) Abnormal liver function defined as AST and/or ALT > 3 times the upper limit of the normal.

4.4 Participating Centers

Patients will be recruited by tertiary ALS centers representative of all Italian regions and by the Massachusetts General Hospital (MGH) in Boston (USA).

5. BIOMARKERS

Six candidate pharmacodynamic markers of treatment efficacy with RNS60 have been selected because they have been previously associated with ALS, they are modulated by neuroinflammation, and because some of them have been shown to be modified by RNS60 or by other compounds with anti-inflammatory properties in *in vitro* and *in vivo* models of different inflammatory conditions (see Table 1).

Table 1. Pharmacokinetic biomarkers used in this study

Type	Marker	In ALS Patients	Effect of RNS60
RNA	FoxP3 /CD25 (T-reg)	T-regs are lower in rapidly progressing ALS patients and inversely correlate with progression rates (Henkel et al., 2013).	RNS60 upregulates FoxP3 and enriches T-regs in experimental autoimmune encephalomyelitis (EAE), mouse model of multiple sclerosis (Mondal et al., 2012).
Protein	Cyp-A	Increased levels in PBMC of ALS patients and associated with disease progression (Nardo et al., 2011)	Not known
Amino acid	3-NT	Increased levels in PBMC of ALS patients (Nardo et al., 2009)	Not known, decreased by compounds with anti-inflammatory properties (Hooper et al., 2000; Yip et al., 2013)
Protein	Actin-NT	Increased levels in PBMC of ALS patients (Nardo et al., 2011)	Not known
Protein	IL17	Increased serum and CSF levels of ALS patients (Rentzos et al., 2010)	Reduced in RNS60 treated MOG and T cell transfer induced models of EAE in rats and mice (Mondal et al., 2012). Reduced in combination with IFN-γ in asthma patients after 4 weeks of treatment (Revalesio, unpublished data).
Protein	MCP1	High levels in CSF and serum of ALS patients (Kuhle et al., 2009; Baron et al., 2005).	Reduced in allergen challenge model of asthma in rats after RNS60 treatment (Ghosh et al.,

			2011).

5.1 Blood sample collection

Blood samples of a total volume of 21 ml will be collected from each subject five times during the entire trial. Fasting is not needed, but the patient is encouraged to have a light breakfast before blood collection. Blood for biomarker assessments will be collected in two 4-ml EDTA vacutainers (BD cat. no. 367861) for plasma samples, two 2.5-ml PAXgene tubes (BD cat. no.762165) for RNA samples and two 4-ml Vacutainer® CPT™ Cell Preparation Tube (BD cat. no. 362781) for PBMC samples at scheduled visits:

- At the day 1 visit (baseline - randomization), blood samples will be collected prior to first IV RNS60/placebo dose (pre-dose, off drug blood sample).
- At the week 4, 12 and 24 study visits, the blood sample will be collected just prior to drug administration.
- An additional blood sample will be obtained at the week 48 visit (last study visit).

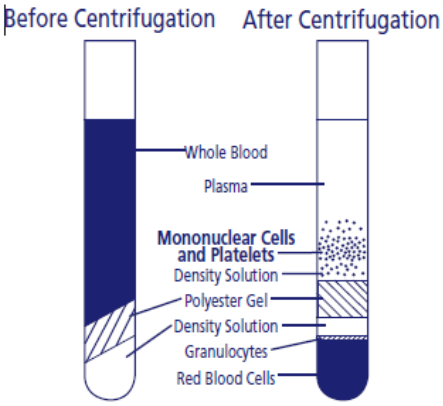
5.2 Blood sample handling

The time of sample collection and the start time of the processing for plasma and PBMC samples will be recorded. Samples should be processed as soon as possible and within 2 hours after collection.

Plasma samples: Invert EDTA vacutainers several times (at least 5 times) after blood draw. Centrifuge the blood samples in the vacutainers at 3000xg for 20 minutes at room temperature (18 to 25°C). Aliquot the plasma into two 1.8-ml Nunc cryo tubes labeled per vacutainer (4 samples). Freeze plasma samples at -70°C as soon as possible.

Blood for RNA samples: Invert PAXgene tubes several times (at least 5 times) after blood draw. Samples must stay upright at room temperature for a minimum of 24 hours and no more than 72 hours before being frozen. Freeze the whole blood sample at -20°C as soon as possible.

PBMC: Invert the Vacutainer® CPT™ Cell Preparation tubes gently 10 times and centrifuge at room temperature (18 to 25°C) for 30 minutes at 1500xg (RCF). After centrifugation, mononuclear cells and platelets will be in a whitish layer just under the plasma layer (see Figure).



For recovering the separated mononuclear cells, resuspend the cells into the plasma by inverting the un-opened BD Vacutainer® CPT™ Tube gently 5 to 10 times. To collect the cells, open the BD Vacutainer® CPT™ Tube and pipette the entire contents of the tube above the gel into a separate vessel. Add PBS to bring volume to 15 mL. Cap tube. Mix cells by inverting the tube 5 times gently. Centrifuge for 15 minutes at 300xg (RCF). Aspirate as much supernatant as possible without disturbing cell pellet. Freeze plasma samples at -70°C as soon as possible.

A specific procedure will be adopted by the US Center in keeping with the study's laboratory manual.

5.3 Sample labels

[…..]

5.4 Sample numbering

[….]

5.5 Sample Storage

In each study center as well as at the "Mario Negri" IRCCS the samples will be stored in a dedicated room to be accessed only by authorized persons. For each sample the storage, usage and transfers will be performed according to the requirements aimed at preserving quality, integrity, accessibility and availability of the sample.

5.6 Sample shipment

[…]

5.7 Analytical method

Analysis of RNA, Cyp-A, 3-NT, Actin-NT in PBMC samples are described in the study's laboratory manual.

6 STUDY TREATMENTS

6.1 Study Drug Administration

RNS60 or placebo supply will be sent to site by ALMAC group and should be kept refrigerated in the Hospital Pharmacy with access limited to those directly involved in the study.

RNS60 or placebo will be administered weekly intravenously (starting on Day 1) at the clinic site by properly trained staff designated by the investigator. Subjects will receive 375 mL RNS60 or placebo infused at a rate of 700 mL/hr. Infusion documentation should include recording infusion start and stop times, volume infused, and any interruptions to the infusion using an ad-hoc form.

During the remaining days of the week, treatment with nebulized RNS60 or placebo will be performed at home in the morning on non-infusion days. The subject will remove the kit box of 2 syringes from refrigeration, and visually inspect the syringe to ensure that the plunger and tip cap have remained intact during storage. If either the plunger or the tip cap is loose, the faulty syringe shall be set aside and returned to study staff on the next infusion. Any faulty syringes will be sent back to Revalesio for investigation. Subjects will receive a number of syringes that will guarantee a sufficient number of doses between visits. To dispense a syringe, subjects should first remove the tip cap of the syringe, then gently push the plunger down to dispense the full 2 mL of RNS60/placebo from the syringe into the nebulizer dosing cup. The procedure is then repeated with a second syringe to get to the full daily dose of 4 mL. The dose should be nebulized immediately thereafter until the contents of the nebulizer cup are empty (approximately 15 minutes). Subjects should try to dose at a similar time each morning. Subjects will be provided with and trained to use a Pari Mini system and LC Sprint nebulizers (Italian subjects) or Pari Trek system and LC Sprint nebulizers (US patients). Dosing records for nebulization will be completed at home at the time of each daily nebulization. The dosing record will include the date and time of nebulization as well as the number of syringes used.

All subjects will concurrently take 50-mg riluzole twice a day during the entire treatment period. According to the European Medicine Agency (EMA) guidelines, riluzole can be discontinued when the plasma transaminase levels exceed by fivefold the baseline levels.

The first dose of riluzole should be taken each day at the end of infusion or nebulization. The second dose should be taken after 12 ± 2 hours.

6.2 Investigational Product(s)

[…]

RNS60

RNS60 for infusion, i.e. in the IV bags, is produced using 0.9% Sodium Chloride for injection. RNS60 for inhalation, i.e. in the syringes, is produced using 0.9% Sodium Chloride for irrigation. Both RNS60 for infusion and RNS60 for inhalation are processed in the Revalesio Pump with elevated levels of oxygen. RNS60 is supplied for single-use only and contains no preservatives.

Syringes and IV bags are to remain refrigerated at 2 to 8°C (36 to 46°F) when not in use. RNS60 meets its stability specification for 12 months.

Placebo

Normal saline (NS) for infusion, i.e. in the IV bags, is packaged 0.9% Sodium Chloride for injection. NS for inhalation, i.e. in the syringes, is packaged 0.9% Sodium Chloride for irrigation. NS does not require refrigerated storage for use. However, for blinding purposes refrigeration is required before distributing to subjects. NS meets stability specifications for 24 months.

6.3 Drug Accountability

[…]

6.4 Storage and Handling of RNS60 and Placebo

[…]

6.5 Blinding

[…]

6.6 Randomization

All eligible subjects will be randomized to receive either RNS60 or placebo (in both IV and inhalation modes of administration) for 24 weeks. Treatment allocation will be centrally managed by the study statistician using a computer-generated, permuted block (with a block size of 4), 1:1 randomization scheme. A computer-generated randomization list will be provided to each investigational site.

Individual treatment disclosure envelopes (code break) will also be provided to the investigative site.

6.7 Treatment Arms

142 patients with ALS will be randomized in 2 parallel groups, in a ratio of 1:1. One group will be treated with RNS60 and one group will be treated with placebo.

Group A	RNS60 375 mL IV once a week + RNS60 4 mL inhaled once a day (six days/week) + Riluzole 50 mg twice a day
Group B	NS 375 mL IV once a week + NS 4 mL inhaled once a day (six days/week) + Riluzole 50 mg twice a day

6.8 Treatment Assignment and Subject Numbering

[…]

6.9 Concomitant Medications and Prohibited Treatment

Any concomitant treatment can be continued as per medical indication.
Except for other experimental drugs, there are no restrictions for concomitantly used medications. However, the subjects and investigators must keep records of the drugs on the concomitant medication section of the e-CRF along with daily dose and duration of use.

6.10 Study Drug Discontinuation

If study treatment is discontinued before the defined treatment duration period ends, the subject will be considered to have prematurely withdrawn from the study. Subjects lost to follow up should be recorded as such on the e-CRF. Subjects who discontinue study drug before completing the study should be scheduled for a visit as soon as possible, at which time all the assessments listed for the study completion visit will be performed for safety evaluations during the 30 days following the last dose of study drug. Subjects who discontinue the study drug should be considered withdrawn from the study after the final visit assessments are performed or when it is clear that the subject will not return for assessment.

6.11 Premature Subject Withdrawal

The duration of the study includes 24 weeks of treatment plus 24 weeks of follow up. Subjects may voluntarily withdraw from the study or can be dropped at the discretion of the investigator at any time. Subjects must be withdrawn from the study if any of the following occurs:

- Unwillingness to comply with procedures as outlined in the study protocol;
- Withdrawal of consent;
- Loss to follow-up;
- Repeated non-compliance to dosing regimen or visit schedule (intake of 50% of recommended dose or less in three consecutive intervals between visits);
- Intolerable adverse events which may or may not be related to study medication and, if deemed drug-related, non remitting with down titration;
- Death/Tracheostomy;
- Requirement of medication doses greater than those allowable in the study;
- Abnormal laboratory value(s) deemed of medical concern;
- Administrative problems;
- Female becoming pregnant;
- Severe protocol deviations (at the Study Coordinator judgment).

If a subject withdraws or fails to return for visits, the investigator must determine the primary reason for the subject's premature withdrawal from the study, and record this information on the study-completion visit. Subjects must return all unused/remaining study drug to the investigative site. For subjects who are lost to follow up, the investigator should show "due diligence" by documenting in the source documents the steps taken to contact the subject, e.g., dates of telephone calls, registered letters, etc. Subjects who are prematurely withdrawn from the study will not be replaced.

Discontinued use of riluzole during the treatment period implies the discontinuation of the experimental drug. The subject will undergo a final safety evaluation and data collected up to the time of drug discontinuation will be used in the data analysis.

6.12 Modification of Study Intervention/Investigational Product for a Subject

Any dosage adjustment, including the reason for and dates of adjustment, will be documented in the CRF for each subject requiring this manipulation. The PIs or licensed physician Sub-Investigator may reduce the dosage of study drug or discontinue the study drug in its entirety for AEs thought to be related to the study drug or for other reason during the trial (the reason for, and dates of

suspension or dose reduction must be documented). If the AE is mild or moderate, the dosage may be reduced until the event improves. The PIs may then choose to resume the higher dosage or maintain the subject at a reduced dosage. The option to continue with administration by IV only or inhalation only is allowable.

If the event is serious or life threatening, and deemed to be definitely drug related, the study drug will be discontinued immediately. Study subjects must remain off the study drug permanently. Subjects may not resume study drug. All AEs will be followed to resolution.

6.13 Wash-out Period for Eligibility

If a subject is receiving treatment with another experimental drug, this subject requires a 3-month wash-out period before participating in the screening visit.

6.14 Treatment Compliance

[…]

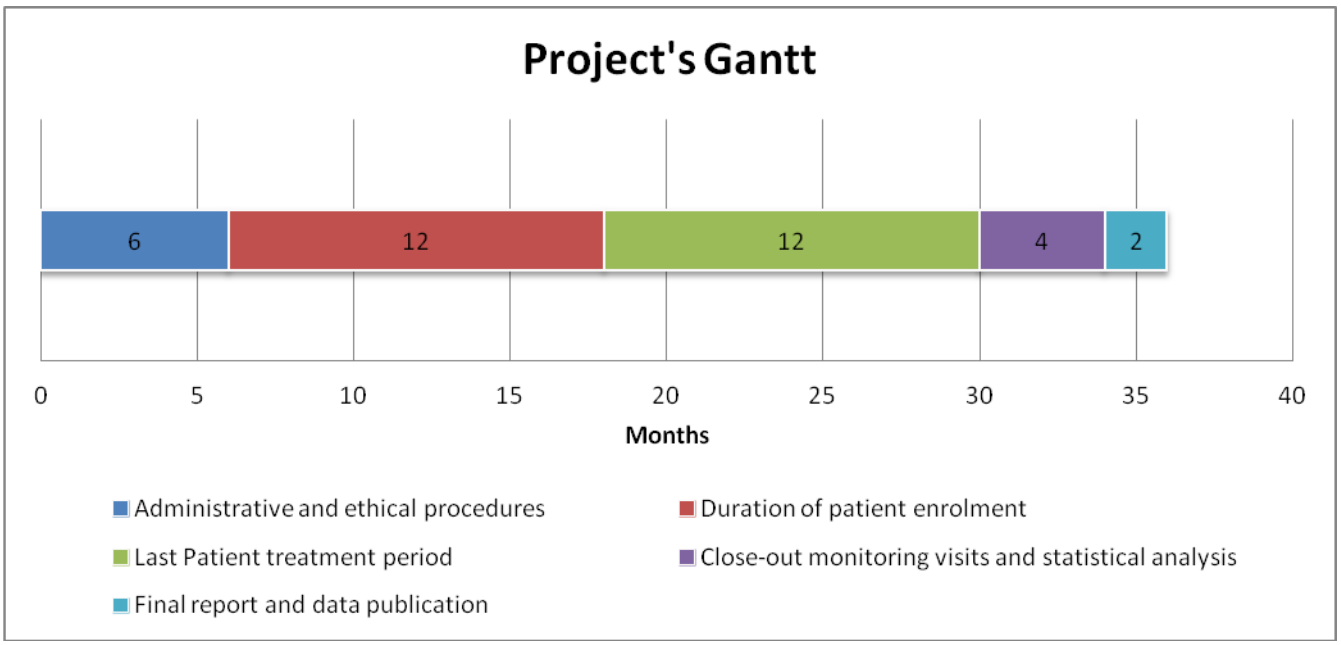
7 STUDY PROCEDURES

[…]

7.9 Study Duration

The overall duration of the study will be approximately 36 months as indicated below:

- Administrative and ethical procedures: 6 months
- Duration of subject enrolment: 12 months
- Study drug administration: 24 weeks/subject
- Follow-up period: 24 weeks/subject
- Close-out monitoring visits and statistical analysis: 4 months
- Final report and data publication: 2 months


Project's Gantt
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Months
Administrative and ethical procedures
Duration of patient enrolment
Last Patient treatment period
Close-out monitoring visits and statistical analysis
Final report and data publication

Table 2. SCHEDULE OF PROCEDURES

		Treatment Period							**Follow-up Period**		
	Screening (-28 days)	**Randomization – baseline Day 1**	**Weeks 1 - 3**	**Week 4**	**Weeks 5 - 11**	**Week 12**	**Weeks 13-23**	**Week 24**	**Week 36**	**Week 48**	**End of study (only for drop outs)**
Informed consent	x										
Patient's numbering assignment	x										
Date of visit	x	x	x	x	x	x	x	x	x	x	x
Demographic data	x										
Blood sample collection (biomarkers)		x		x		x		x		x	x
Personal and clinical data	x										
Physical exam	x	x									
Neurological exam	x	x		x		x		x	x	x	x
Medical history/ Concomitant medical disorders	x	x									
Concomitant medications	x	x	x	x	x	x	x	x	x	x	x
Inclusion/exclusion criteria	x	x*									
Randomization		x									
Study drug infusion		x	x	x	x	x	x				
Study drug management for nebulization		x	x	x	x	x	x	x			x
Study drug dispensing		x	x	x	x	x	x				
Vital signs, weight	x	x	x	x	x	x	x	x	x	x	x
Height	x	x									
ALSFRS-R scale	x	x		x		x		x	x	x	x
FVC	x	x		x		x		x	x	x	x
ALSAQ-40 scale		x						x		x	x

Clinical lab tests**	x	x		x		x		x			x
Pregnancy test		x						x			x
Compliance check (nebulization)			x	x	x	x	x	x			x
AE assessment		x	x	x	x	x	x	x	x	x	x
Reason of discontinuation											x

* Certain eligibility criteria are assessed at Day 1, prior to randomization

** According to the physician judgment. Clinical lab test needed for inclusion criteria could be valid if performed up to 6 months before randomization visit

7 STUDY ADMINISTRATION

8.1 Resource Utilization

[…]

8.2 Regulatory and Ethical Compliance

This study was planned and shall be performed according to the principles of Good Clinical Practice (ICH-GCP), the declaration of Helsinki, and the national laws and regulations about clinical studies. The study will not start without written approval of an Independent Ethics Committee (IEC) and the written informed consent of the patients. This study was designed and shall be implemented and reported in accordance with the protocol.

8.3 Responsibilities of the Investigator and IRB/IEC/REB

[…]

8.4 Informed Consent

Eligible patients can be included in the study only after proving written the IRB/IEC/REB - approved informed consent, or, if incapable of doing so, after such consent has been provided by a legally acceptable representative. In cases where the patient's representative gives consent, the patient should be informed about the study to the extent possible given his/her understanding. The physician will obtain a signed informed consent form (ICF) from the subject prior to any procedures. The signed and completed ICF will be accessible in the patient's records on site. A copy of the signed ICF will be given to each subject.

The process of obtaining informed consent should be documented in the patient source documents.

Women of child bearing potential should be informed that taking the study treatment may involve unknown risks to the fetus if pregnancy were to occur during the study and agree that in order to participate in the study they must adhere to the contraception requirement for the duration of the study.

8.5 Amendments to the Protocol

Any change or addition to the protocol can only be made in a written protocol amendment. Only amendments that are required for patient safety may be implemented prior to IRB/IEC/REB approval in accordance with the Italian legislation. Notwithstanding the need for approval of formal protocol amendments, the investigator is expected to take any immediate action required for the safety of any patient included in this study, even if this action represents a deviation from the

protocol. In accordance with the GCP rules and the Italian legislation, the acceptance of an amendment of the study protocol by each participating institution is subjected to the local IRB/IEC/REB approval or notification.

8.6 Publication of Study Results

The results of the study will be published whether or not the experimental drug is considered superior to placebo.

9 SAFETY MONITORING

Safety monitoring for all patients enrolled in the study will include laboratory safety assessments and clinical evaluations, as shown in Table 2. All AEs and SAEs will be recorded. Subject diaries about AEs and/or concomitant medication will not be copied but will be considered source documents. AE documentation by the investigator will include the date of onset and duration of the AE, as well as the severity and causality of each AE, and the actions taken, including the discontinuation of the experimental drug, where required.

9.1 Emergency Unblinding of Treatment Assignment

[…]

9.2 Adverse Events

The Investigator is responsible for recording all AEs observed during the study.

For expected (listed) AEs of RNS60, see the current version of the IB.

All AEs will be collected starting from the signing of informed consent through the infusion visits and the follow up. During this period the investigator is responsible for reporting to the sponsor all non-serious AEs, SAEs occurring in subjects treated by the investigator in the clinical trial.
AEs (but not SAEs) occurring before starting study treatment but after signing the informed consent form are recorded on the Medical History/Current Medical Conditions Case Report Form.

[…]

Investigators must code the grade of all AEs occurring during the trial. For more detail about grade criteria, refer to “Common Terminology Criteria for Adverse Events (CTCAE)” Version 4.0 Published: May 28, 2009 (v4.03: June 14, 2010) from the U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES National Institutes of Health National Cancer Institute.

9.3 AE Reporting

All AEs, regardless of severity and whether or not they occurred during the treatment or follow-up period, are to be recorded on the appropriate AE pages in the e-CRF. The investigator will include the date of onset and duration of the AE, as well as the severity and causality of each AE, and the actions taken. Each event should be recorded separately.

[…]

In case of patients needing a Non Invasive Ventilation (NIV), it is necessary to record the procedure into the AE form.

The investigator shall report all AEs and DAEs into the e-CRF, without reporting to "Mario Negri" IRCCS.

9.4 Reporting of SAEs and SUSARs

[…]

The following serious events should not be recorded as SAE due to the usual progression of the disease and the investigator judgment:

- Death (only if caused by the disease progression);
- Tracheotomy;
- PEG/RIG placement.

If these events occur, the Investigator must record them into the AE form.

9.5 Follow up of AE/SAE/DAR/DAE/SUSAR

Any AE/SAE/DAR/DAE/SUSAR observed from randomization up to the end of the study will be followed up to resolution. Resolution means that the subject has returned to a baseline state of health or the Investigator does not expect any further improvement or worsening of the AE.

9.6 Data Monitoring Committee

According to the EMEA GUIDELINE ON DATA MONITORING COMMITTEES **(**January 2006) a Data Monitoring Committee (DMC) is an external group of independent experts assessing the progress, safety data and efficacy endpoints of this clinical study. […]

10 QUALITY CONTROL

[…]

11 STATISTICS

11.1 Sample Size Calculation

We analyzed preliminary data on Cyp-A which showed an increase of 0.27 over six months (Nardo, G. et al. 2011). With 142 patients enrolled, we will have 80% power to detect a 44% decrease in the rate of progression in Cyp-A at a two sided p=0.05 significance level. This assumes a drop-out rate of 1.75% per month (10% over the study). The calculation is based on the method shown in (http://hedwig.mgh.harvard.edu/biostatistics/node/42). For the other biomarkers we only had data on the between patient variation and not on the within patient variation which could be used for calculating sample size for the proposed study. Usually the within patient variation is less than the between patient variation, so power calculated using the between patient variation is conservative. The T-reg values were assessed in slowly progressing vs. rapidly progressing ALS43 and the estimated difference was used as the difference expected to slow the progression of the disease in patients receiving RNS60. A total of 128 patients is needed to detect a difference in means of 0.25 units between the two treatment arms at 24 weeks with a standard deviation (SD) of 0.5. Similar assumptions were made for the other biomarkers for which, except for Actin-NT (that would require 1542 patients), numbers range from 8 to 68 (Nardo, G. et al. 2009; Nardo, G. et al. 2011; Henkel, J. et al. 2013; Chiò A. et al., 2011):

- MCP-1: a total of 16 patients is needed to detect a difference in means of 220 units between the two treatment arms at 24 weeks with a standard deviation (SD) of 140;
- Actin-NT: a total of 1542 patients is needed to detect a difference in means of 0.1 units between the two treatment arms at 24 weeks with a standard deviation (SD) of 0.7;
- 3-NT: a total of 24 patients is needed to detect a difference in means of 50 units between the two treatment arms at 24 weeks with a standard deviation (SD) of 40;
- Cyp-A: a total of 68 patients is needed to detect a difference in means of 1.4 units between the two treatment arms at 24 weeks with a standard deviation (SD) of 2.0.

For ALSFRS-R, the software http://hedwig.mgh.harvard.edu/biostatistics/node/42 shows the variance parameters which are from the Celebrex Study.

The variance of the random effects is the matrix 31.8 (intercept), 0 .6687 (slope) with a covariance of 0.8527. The error has a variance of 2.7.

With 142 patients enrolled, we will have 80% power to detect a 43% decrease in the rate of progression in ALSFRS over 6 months, at a two sided p=0.05 significance level. The study is also powered (in this case p=0.05 two sided) to show an absolute 25% reduction in the proportion of patients becoming non self-sufficient at 24 weeks, using a validated outcome measure with strong clinical significance (Marin, B. et al. 2015). In two studies from our group (Beghi, E. et al 2013; Chiò, A. et al. 2010) the average proportion of patients becoming non self-sufficient after 24 weeks in the placebo arm was 85%.

11.2 Statistical Analysis Plan

The study will use a modified intent to treat approach with all patients included who took at least on dose of the study medication or placebo. The primary analysis, described below, is valid as long as the probability of non-observation of a data point is independent of the value that would have been observed conditional on the previous observed data. In the case of ALSFRS this method has been shown to be robust (Ref http://www.ncbi.nlm.nih.gov/pubmed/22987690). Separate analyses will be performed in: 1. All randomized subjects receiving at least one dose of study medication (Intention-to-treat population); 2. Study completers: subjects completing the 12-month follow-up; 3. Study completers and compliers: subjects completing the 12-month follow-up and taking at least 75% of assigned dose. 4. All randomized subjects excluding protocol deviations (Per protocol, PP population). Descriptive statistics will be performed comparing the two treatment groups and safety of RNS60 and placebo. Continuous variables will be described using mean and standard deviation or median and interquartile range; categorical variables will be described as counts and percentages.

<u>Biomarkers analysis:</u>

The primary analysis will be a repeated measures analysis of variance (ANOVA) with a treatment variable that is the same for all treatments at baseline and then differs at later observation times. We will use an unstructured variance covariance matrix. The contrast of interest will depend on the outcome. Note that this is more general than a random effects model in situations where the observation times are fixed. For biomarkers that progress over of time such as Cyp-A, the primary analysis will compare the rate of change in the biomarker over time in the placebo and active treatment groups. In biomarkers that do not progress but rather are elevated in patients, the primary analysis will be a comparison in the treatment effect. Both of these analyses are contrasts in a repeated measures ANOVA, the former by treatment X time, and the latter being treatment X (time>0). Each biomarker will be analyzed separately.

<u>Efficacy analysis:</u>

The numerical change of FVC, ALSFRS-R and ALSAQ-40 during the treatment and follow-up period will be assessed using repeated measures ANOVA, with an unspecified variance covariance matrix. The hypothesis will be tested using the time*treatment interaction which measures differences in the slope of the ALSFRS-R. Survival (time to death or tracheostomy) and time to functional disability (i.e., to reach a non self-supporting status or death or tracheostomy) will be assessed using actuarial methods with death and non-self supporting status as end-points. Survival will be described with the Kaplan-Meier method. Differences between the two treatment groups will be compared using the log-rank test. The statistical significance will be set at the 0.05 level for a two-tailed test.
The repeated measures ANOVA will use all available data, using Maximum Likelihood methods as suggested by (http://www.nap.edu/read/12955/chapter/1).

Safety analysis will be performed in all subjects receiving at least one dose of the experimental drug. All AEs, SAEs and AEs leading to treatment discontinuation will be recorded, listed and compared in the two treatment arms at any follow-up visit and at the end of the study. The number of patients reporting at least one AE or SAE will be compared between the treatment arms using the fisher exact test. The total number of AE and SAE will be compared between the treatment arms using a poisson model. MedDRA code system will be used for all recorded AE/SAE.

Given the exploratory value of this study, multiple comparison adjustments for Type I errors will not be considered as a more conservative significance level would lead to miss any potential treatment effect. Furthermore, if there is a suggestive benefit ($p<0.1$) in the clinical measures and several of the biomarkers, this will be considered sufficient evidence for further study.
If at the end of the patient enrolment period less than 50% of patients are enrolled, an interim-analysis will be performed to decide whether or not to continue the trial.

11.3 Compliance

The compliance with the assigned at-home treatment will be measured by counting the number of used/unused syringes returned by the patient at each visit. A subject will be considered non-compliant if the amount of unused drug exceeds 25% of the assigned drug. The intake of less than 50% of the total amount of nebulized drug for 3 consecutive visits or in the event that the infusion is not performed for 2 consecutive visits implies withdrawal from the study.

11.4 Dictionary

Concomitant medications entered into the database will be coded using the World Health Organization drug reference list (http://www.whocc.no), which employs the anatomical therapeutic chemical classification system. Medical history/current medical conditions and adverse events will be coded using the medical dictionary for regulatory activities (MedRA) terminology (MedRA, 2011).

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