BMJ Open

Proteostasis and ALS: protocol for a phase II, randomised, double-blind, placebo-controlled, multicentre clinical trial for colchicine in ALS (Co-ALS)

Jessica Mandrioli,[1] Valeria Crippa,[2] Cristina Cereda,[3] Valentina Bonetto,[4] Elisabetta Zucchi,[5] Annalisa Gessani,[1] Mauro Ceroni,[6,7] Adriano Chio,[8] Roberto D'Amico,[9] Maria Rosaria Monsurrò,[10] Nilo Riva,[11] Mario Sabatelli,[12,13,14] Vincenzo Silani,[15,16] Isabella Laura Simone,[17] Gianni Sorarù,[18] Alessandro Provenzani,[19] Vito Giuseppe D'Agostino,[19] Serena Carra,[20] Angelo Poletti[2]

For numbered affiliations see end of article.

Correspondence to
Dr Jessica Mandrioli;
mandrioli.jessica@aou.mo.it

ABSTRACT

Introduction Disruptions of proteasome and autophagy systems are central events in amyotrophic lateral sclerosis (ALS) and support the urgent need to find therapeutic compounds targeting these processes. The heat shock protein B8 (HSPB8) recognises and promotes the autophagy-mediated removal of misfolded mutant SOD1 and TDP-43 fragments from ALS motor neurons (MNs), as well as aggregating species of dipeptides produced in C9ORF72-related diseases. In ALS-SOD1 mice and in human ALS autopsy specimens, HSPB8 is highly expressed in spinal cord MNs that survive at the end stage of disease. Moreover, the HSPB8–BAG3–HSP70 complex maintains granulostasis, which avoids conversion of dynamic stress granules (SGs) into aggregation-prone assemblies. We will perform a randomised clinical trial (RCT) with colchicine, which enhances the expression of HSPB8 and of several autophagy players, blocking TDP-43 accumulation and exerting crucial activities for MNs function.

Methods and analysis Colchicine in amyotrophic lateral sclerosis (Co-ALS) is a double-blind, placebo-controlled, multicentre, phase II RCT. ALS patients will be enrolled in three groups (placebo, colchicine 0.01 mg/day and colchicine 0.005 mg/day) of 18 subjects treated with riluzole; treatment will last 30 weeks, and follow-up will last 24 weeks. The primary aim is to assess whether colchicine decreases disease progression as measured by ALS Functional Rating Scale - Revised (ALSFRS-R) at baseline and at treatment end. Secondary aims include assessment of (1) safety and tolerability of Colchicine in patiets with ALS; (2) changes in cellular activity (autophagy, protein aggregation, and SG and exosome secretion) and in biomarkers of disease progression (neurofilaments); (3) survival and respiratory function and (4) quality of life. Preclinical studies with a full assessment of autophagy and neuroinflammation biomarkers in fibroblasts, peripheral blood mononuclear cells and lymphoblasts will be conducted in parallel with clinic assessment to optimise time and resources.

Ethics and dissemination The study protocol was approved by the Ethics Committee of Area Vasta Emilia Nord and by Agenzia Italiana del Farmaco (EUDRACT N.2017-004459-21) based on the Declaration of Helsinki. This research protocol was written without patient involvement. Patients' association will be involved in disseminating the study design and results. Results will be presented during scientific symposia or published in scientific journals.

Trial registration number EUDRACT 2017-004459-21; NCT03693781; Pre-results.

Strengths and limitations of this study

► Amyotrophic lateral sclerosis (ALS) is a rare and devastating disease without an effective treatment so far; this urgent gap can be filled with a multi-centre randomised controlled trial (RCT) that can give reliable data on candidate molecules.

► Colchicine in ALS is going to be a randomised clinical trial using a drug (colchicine) that potentially targets multiple complex mechanisms involved in ALS, such as autophagy and inflammation, with the potential of slowing disease progression, but the possible limitation that we might not be able to understand which specific process prevaricates in disease initiation and then progression.

► The absence of reliable biomarkers for disease progression and drug efficacy is an important target to be addressed in ALS; the study will give inference to possible biomarkers of disease progression (neurofilaments and neuroinflammation biomarkers) and will allow in vivo search of colchicine-driven modifications of disease (study of autophagy process, protein aggregation, exosomes and mRNA) compared to placebo arm, which can widen knowledge about ALS pathogenesis.

► Colchicine is an already approved drug, with known pharmacokinetics and safety, which is already available, and therefore there is significant possibility of transferability to patients and of a rapid translation to daily clinics.

► This short study will optimise time and resources to get reliable information for a following larger phase III RCT.

INTRODUCTION
Background and rationale

Amyotrophic lateral sclerosis (ALS) is characterised by progressive degeneration of motor neurons (MNs) in both brain and spinal cord leading to progressive muscle weakness, relentless disability and death within 3–5 years from symptom onset.[1] The entire motor system is involved, determining impairment of movement, communication, feeding and respiration, with patients needing ventilatory and nutritional support to survive. Disease usually starts focally in bulbar and spinal sites of onset and spreads to other body regions in a heterogeneous way among patients, according to different phenotypes having dissimilar prognosis.[2] Besides classic and bulbar ALS, flail arm, flail leg, upper motor neuron predominant (UMN-p) phenotypes are characterised by unusually long survival, compared with respiratory phenotype by a very short clinical course.

If considerable heterogeneity characterises disease progression, a prognostic biomarker of disease is still lacking. Riluzole, the first drug approved for the disease, prolongs survival by a mean of 3 months,[3] while edaravone, currently available in some countries, shows limited effects in slowing the pace of disease progression.[4] More than 50 randomised controlled trials (RCTs) of proposed disease-modifying drugs have failed to show positive results in the past half-century, and the reasons for this failure have been classified into three categories: trial rationale and preclinical study results, pharmacological issues, RCT design and methodological issues.[5] Revised Airlie House Consensus Guidelines for design and implementation of ALS clinical trials have been recently assembled.[6]

From a pathological point of view, protein aggregates into neurons, and glial cells are common features of the different ALS forms. Such aggregates include proteins encoded by genes that cause ALS when mutated (SOD1, TDP-43, FUS, SQSTM1/p62, VCP, UBQLN2, OPTN encoded by *SOD1, TARDBP, FUS, SQSTM1, VCP, UBQLN2* and *OPTN* genes, respectively). The protein quality control (PQC) system has a crucial role in dealing with the above-mentioned aggregates, in particular with TDP-43 proteinopathy, which is a hallmark of more than 95% of non-mutated ALS cases.[7]

The PQC system is based on chaperones and degradative pathways, which include the ubiquitin–proteasome systems (UPSs), the autophagy and the endoplasmic reticulum-associated degradation (ERAD). Disruption of autophagy in the brain results in inclusion bodies with ubiquitinated proteins and early neuronal death.[8] In ALS, several gene products have links with protein degradation pathways as they contribute to recruitment of ubiquitinated proteins to the autophagosome: UBQLN2, VCP, OPTN and SQSTM1/p62 function as adapters that deliver polyubiquitinated proteins to the proteasome or the autophagosome for degradation. OPTN serves as a receptor for autophagy, and VCP has a role in ERAD and sorting endosomal proteins, in autophagy and UPS.

Protein aggregation and autophagy inhibition may also induce clearance of pathological TDP-43 via secretion of exosomes, small extracellular vesicles, which may play a key role in TDP-43 aggregate disposal and/or the propagation of TDP-43 proteinopathy.[8]

Autophagy is also required for the removal of aberrant stress granules (SGs),[9 10] which have been involved in ALS pathology. Finally, in ALS models and patients, activation of inflammasome complexes in both astrocytes and microglia is critically involved in neuroinflammation.[11] There is a crosstalk between autophagy and neuroinflammation: autophagy downregulates inflammasome activity, which is activated in response to cellular inclusions formation,[12] and TBK1, OPTN and SQSTM1/p62 gene products converge on autophagy and neuroinflammation, suggesting that compounds addressing both pathways may be promising for ALS treatment.

Preliminary data

With our study, we aim to assess the role of colchicine as a therapeutic agent for ALS. Colchicine is a Food and Drug Administration-approved drug that we identified in a high-throughput screening performed by using the promoter region of the gene encoding for a specific chaperone, the heat shock protein B8 (HSPB8).[13]

HSPB8 acts in conjunction with the co-chaperone Bcl2-associated athanogene 3 (BAG3), and the HSPB8–BAG3–HSP70 complex enhances the intracellular clearance of all motor neuron disease-associated misfolded proteins tested so far.[14–16]

The role of HSPB8 in the stress response in ALS has been elucidated in animal models and humans, indicating that HSPB8 is upregulated in the spinal cord of patients with ALS and in surviving MNs of ALS mice.[15 17]

In mutant models of ALS, HSPB8 recognises and promotes the removal of the misfolded mutant SOD1 and TDP-43 fragments, as well as aggregating dipeptides produced in C9ORF72-related neurodegenerative diseases, by promoting their autophagic removal from MNs.[18–20]

As for sporadic ALS, HSPB8 counteracts accumulation of TDP-43 and its C-terminal fragment of 25 KDa (TDP-25), which is highly aggregation-prone due to the presence of a prion-like domain.[13 19 20] In stress conditions, caspase cleavage of TDP-43 generates TDP-35 and TDP-25 fragments. TDP25 loses one of the RNA recognition motifs and the nuclear localisation sequence (NLS), but retains the nuclear exportation sequence, and as a consequence, it mislocaliszes into the cytoplasm, inducing the formation of p62 bodies, a characteristic of sporadic ALS.[21] The overexpression of HSPB8 promotes the clearance of TDP-43 and both TDP disease-associated fragments, and induces a decrease of cytoplasmic TDP-25 aggregates, while its silencing had opposite effects.[13 21 22] Upregulation of the fly homologue of human HSPB8 protected against the toxicity mediated by TDP-35 and by an NLS-mutant form of TDP-43 that mislocaliszes into the cytosol in *Drosophila melanogaster.*[19]

Finally, HSPB8-BAG3-HSP70 maintains the so-called 'granulostasis', a surveillance mechanism that avoids the conversion of dynamic SGs into aggregation-prone assemblies.[1 23 24]

Collectively, these data establish that HSPB8 has a crucial activity for MN function and exerts a protective role against misfolded protein accumulation in cells.

Colchicine is an inexpensive drug with strong anti-inflammatory effects, approved for the treatment of gout and familial Mediterranean fever. In addition, colchicine is used for the treatment of several other diseases, including Behçet's disease, primary biliary cirrhosis and pericarditis.[25] It has been proposed that colchicine interferes with intracellular assembly of inflammasome complex present in neutrophils and monocytes by inhibiting the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 and decreasing the serum levels of functional interleukin (IL)−1b and IL-2.[26]

Recent studies show that colchicine induces HSPB8 mRNA and protein expression levels, independently from the heat shock factor 1 (HSF1) and from BAG1 and BAG3.[13] Colchicine could also upregulate other proteins involved in autophagy, including the master regulator transcription factor EB (TFEB), the TFEB regulated adaptor protein SQSTM1/p62 and autophagy player microtubule-associated protein 1A/1B-light chain 3 (LC3). TFEB activates autophagy but does not induce HSPB8 expression, which is thus upregulated by colchicine in a TFEB-independent manner.

These proteins act synergistically, as TFEB stimulates autophagy, while HSPB8 (with BAG3) recognises misfolded proteins facilitating SQSTM1/p62-mediated insertion into newly formed LC3-II-activated autophagosomes. The concomitant induction of TFEB and HSPB8 mediated by colchicine might thus lead to enhanced clearance of misfolded species of TDP-43 and its 25 KDa fragment, whose accumulation is involved in sporadic forms of ALS.[13]

Colchicine may thus exert protective effects by simultaneously acting on inflammation and autophagy. Based on these premises, we are going to perform a clinical trial with colchicine, which enhances the expression of HSPB8 and of autophagy players,[13] thereby blocking TDP-43 accumulation in neuronal cells. Our study will contribute to the pharmacological research in ALS and will elucidate the implication of changes in autophagy and inflammation during disease, through a full assessment of biological markers of autophagy and inflammation.

METHODS AND ANALYSIS
This multicentre, randomised, double-blind, placebo-controlled, phase II study is meant to compare the efficacy, biological effects and safety of colchicine in combination with riluzole for a 30-week treatment period in patients with ALS.

Study design
The trial was designed following the guidelines on clinical investigation of medicinal products for the treatment of ALS provided by the European Medicines Agency and adopted by the Agenzia Italiana del Farmaco (AIFA), according to Standard Protocol Items for Randomized Trials statement guidelines. Protocol (version 2, 26 June 2018) was approved by competent authorities (Comitato Etico Area Vasta Emilia Nord in September 2018 and AIFA in August 2018).

Nine specialised ALS centres will recruit patients for the trial and four laboratories will be in charge for biological outcome measures (Table 1).

Figure 1 shows the design of the study.

Eligibility criteria
Study population will include patients with a probable laboratory-supported, clinically probable or definite ALS according to revised El Escorial criteria (sporadic and unmutated).[27]

To reduce phenotypic variability, we will exclude from the study flail arm, flail leg, UMN-p, respiratory and primary lateral sclerosis, and progressive muscular atrophy, which are characterised by a longer survival, and we will include only bulbar or classic phenotypes in an attempt to reduce disease progression heterogeneity.

The following definitions according to Chiò et al[28] should be considered for classic and bulbar phenotypes:
1. Classic phenotype is characterised by onset of symptoms in the upper or lower limbs, with clear but not predominant pyramidal signs associated with lower MN signs.
2. Bulbar phenotype patients have a bulbar onset with dysarthria and/or dysphagia, tongue wasting, fasciculation and limited spinal involvement for the first months after symptom onset. Pyramidal signs are not required to be evident at the beginning but need to be evident thereafter.

To further reduce disease progression heterogeneity, we will stratify patients according to progression rate (ΔFS > or <0.7).[29] Patients' inclusion and exclusion criteria are shown in table 2.

Use of highly effective contraceptive measures according to Clinical Trial Facilitation Group (CTFG) criteria (http://www.hma.eu/ctfg.html) is mandatory both for men and women.

Randomisation
Eligible patients will be sequentially allocated to the three arms with a 1:1:1 ratio by using a computer-generated list of random numbers that will be centrally generated in the statistical unit of the University of Modena. After the investigators obtain the person's consent, they will connect to a website dedicated to the study, in which the randomisation sequence is concealed.

The randomisation unit will share the randomisation list exclusively to an authorised company (Eclisse—Euromed

Table 1 Centres and facilities involved in the study

Centre	Role and activity	PI responsible	Work package
Azienda Ospedaliero Universitaria dì Modena (sponsor)	ALS centre, coordinating centre and enrolling centre	Dr Jessica Mandrioli	Coordination of the trial through monitoring of the centers' activities, facilitating communication, promoting exchange of ideas and methodological approach, and stimulating analysis and integration of results; organisation of kick-off meeting and regular meetings with partners; submission to ethical committees; selection of an independent contract research organisation for study monitoring; case report form (CRF) creation with statistics unit; establishment of an independent DSMB. Patients' enrolment and follow-up as specified for enrolling centres (see row below), and presentation and dissemination of results
University of Turin	ALS centre and enrolling centre	Professor Adriano Chiò	Each centre is expected ► To randomise at least six patients fulfilling including and excluding criteria in a period of 12 months and to administer the treatment for 30 weeks. ► To provide one PI and one neurologist to evaluate including and excluding criteria, administer treatment, and assess primary and secondary outcomes. ► To formally adhere to the practice parameters of the European Federation of Neurological Societies concerning the standardisation of the management of the patient in terms of ventilatory support and nutrition. ► To process, store and send biological samples to facilities as established by the protocol.
Istituto Auxologico, University of Milan	ALS centre and enrolling centre	Professor Vincenzo Silani	
IRCCS Mondino Foundation	ALS cCentre and enrolling centre	Professor Mauro Ceroni	
University of Bari	ALS centre and enrolling centre	Professor Isabella Laura Simone	
IRCCS San Raffaele Institute of Milan	ALS centre and enrolling centre	Dr Nilo Riva	
Neuromuscular OmniCentre, Rome, Catholic University, Rome	ALS centre and enrolling centre	Dr Mario Sabatelli	
Universita' dì Napoli 'L. Vanvitelli'	ALS centre and enrolling centre	Dr Maria Rosaria Monsurro	
University of Padova	ALS centre and enrolling centre	Dr Gianni Soraru	
University of Modena and Reggio Emilia, Modena	Statistics unit	Professor Roberto D'Amico	CRF creation with coordinating unit, randomisation, data extraction, statistical analysis, periodical reports to DSMB; presentation and dissemination of results
Center of Excellence on Neurodegenerative Diseases, University of Milan	Laboratory of experimental biology	Professor Angelo Poletti	Autophagy process (mRNA and protein levels of p62, LC3, TFEB, ATGs, HSPB8, BAG3, BAG1, HSP70 and HSF1) in lymphoblasts, fibroblasts and muscle cells; levels and relative ratio between soluble and insoluble species of TDP-43 and TDP-43 fragments, SQSTM1/p62, UBQLN and OPTN in lymphoblasts and fibroblasts
IRCCS Mondino Foundation, Pavia	Genomics and postgenomics centre	Dr Cristina Cereda	Sample collection and preparation for other laboratories, study of colchicine effects on RNA profile on PBMCs and fibroblasts of patients, study of colchicine effects on extracellular vesicles and biomarkers of neurodegeneration (phosphorylated neurofilament heavy chain) in plasma

Continued

Table 1 Continued

Centre	Role and activity	PI responsible	Work package
University of Modena andReggio Emilia, Modena	Laboratory of molecular biology	Professor Serena Carra	Identification of changes in stress granule response and composition in patients' fibroblasts and lymphoblasts; identification of changes in proteotoxic stress response and accumulation of aggregated proteins in the cytoplasm and nucleus, including compartmentalisation and clearance of defective ribosomal products
Istituto di Ricerche Farmacologiche Mario Negri, IRCCS, Milan	Laboratory of translational biomarkers	Dr Valentina Bonetto	Samples collection, study of colchicine effects on extracellular vesicle secretion in cerebrospinal fluid (CSF) using biochemical profiling, analysis of biomarkers of neurodegeneration (phosphorylated neurofilament heavy chain in CSF) and neuroinflammation (IL-18, IL-18BP, MCP1 and IL-17 in plasma and CSF)

ALS, amyotrophic lateral sclerosis; CRF, case report form; CSF, cerebrospinal fluid; DSMB, data and safety monitoring board; HSF1, heat s hock f actor 1; HSP, heat shock protein; IL, interleukin; IRCCS, Istituto di Ricoveroe Cura a Carattere Scientifico; LC3, light chain 3; PBMC, peripheral blood mononuclear cell; PI, principal investiga tor; TFEB, transcription factor EB.

Clinical Supply Services Srl, Cantù (Como), Italy; http://www.css.euromed.it/en/) responsible for the preparation of investigational drug and placebo.

All patients will receive a unique patient identification number at the screening visit when signing the informed consent and before any study procedures are performed. This number will be used to identify the patient throughout the study. The patient identification number will remain constant throughout the entire study.

Fifty-four patients will be randomised in the following three groups:
► Eighteen patients will receive colchicine 0.01 mg/kg/day+riluzole.
► Eighteen patients will receive colchicine 0.005 mg/kg/day+riluzole.
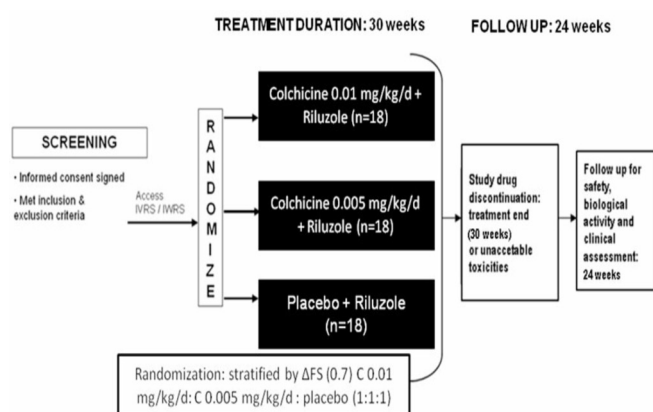► Eighteen patients will receive placebo+riluzole.

Figure 1 Design of the study. FS, progression rate; IVRS, interactive voice response system; IWRS, interactive web response system.

Stratification

Patients will be stratified according to ΔFS ($</\geq 0.7$).[29] The investigator will randomise patients directly online. In case of discontinuation from the study, the randomisation number will not be reused.

Experimental drug preparation, dispensation and blinding procedures

The investigational medical product (IMP) will be packaged and labelled according to current Good Manufacturing Procedure guidelines, Good Clinical Practice (GCP) guidelines and national legal requirements by an authorised company (Eclisse). The package given to the patient will have a tear-off part that will be removed at the time of dispensation by the investigator and attached to the study documents. Treatment and placebo will be made indistinguishable by Eclisse.

The investigator will be provided with technical options and password information to selectively break the code for an individual patient by telephone, or through electronic message transfers. The premature breaking of the code will be confined to emergency cases in which knowledge of the administered drug is necessary for adequate treatment. Should any code be broken, the respective patient will be withdrawn from further participation in the study.

The investigator or pharmacist will receive numbered treatments and will be responsible for safe and proper handling and storage of the IMP at the investigational site. All remaining IMPs, used and unused, will be collected and returned for destruction during the study, and Eclisse will be responsible for IMP manufacture and logistics services, including preparation of randomisation list, envelopes, packaging and labelling, storage, shipment and destruction of the product.

Table 2 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
► Patients diagnosed with a laboratory-supported, clinically 'probable' or 'definite' ALS according to the Revised El Escorial criteria. ► Sporadic ALS. ► ALS phenotypes: classic or bulbar. ► Female or male patients aged between 18 and 80 years old. ► Disease duration from symptom onset no longer than 18 months at the screening visit. ► Patients treated with a stable dose of riluzole (100 mg/day) for at least 30 days prior to screening. ► Patients with a weight of >50 kg and a BMI of ≥18. ► Patients with an FVC ≥65% predicted normal value for gender, height and age at the screening visit. ► Patients able and willing to comply with study procedures as per protocol. ► Patients able to understand and capable of providing informed consent at screening visit prior to any protocol-specific procedures. ► Use of *highly effective* contraception for both men and women.	► Prior use of colchicine. ► Prior allergy/sensitivity to colchicine. ► Receiving colchicine or other anti-inflammatory drugs (such as corticosteroids, methotrexate, antineoplastic, interleukin 1–1b antagonist, tumor necrosis factor-alpha inhibitor). ► Receiving food or comedications, such as strong–moderate cytochrome P450 3A4 inhibitors that will result in elevated plasma levels of colchicine. ► Inflammatory disorders (systemic lupus erythematosus, rheumatoid arthritis and connective tissue disorder), chronic infections (HIV and hepatitis B or C infections) or significant history of malignancy. ► Severe renal (estimated Glomerular Filtration Rate <30 mL/min/1.73 m^2) or liver failure or liver aminotransferase (aspartate transaminase/alanine transaminase >2× upper limit of normal). ► Existing blood dyscrasia (eg, myelodysplasia). ► White blood cells <4 x 10^9/L, platelets count of <100 x 10^9/L and hematocrit level of <30%. ► Severe comorbidities (heart, renal and liver failures), autoimmune diseases or any type of interstitial lung disease. ► Patients who underwent non-invasive ventilation, tracheotomy and/or gastrostomy. ► Women who are pregnant or breastfeeding. ► Participation in pharmacological studies within the last 30 days before screening. ► Patients with the following ALS phenotypes: flail arm, flail leg, UMN-p, respiratory PLS and progressive muscular atrophy. ► Patients with familial ALS defined as the presence of at least one first-degree family member (parents/son/daughter/brother/sister) affected by ALS. ► Patients with known pathogenic mutations (SOD1, TARDBP, FUS and C9ORF72).

ALS, amyotrophic lateral sclerosis; BMI, body mass index; FVC, forced vital capacity; UMN-p, upper motor neuron predominant; PLS, primary lateral sclerosis.

Study treatment administration

Subjects enrolled will receive a total daily dose of 1 or 0.5 or 0.25 mg colchicine, depending on body weight (cut-off: 70 kg) and treatment arm, or a matching placebo, to be taken in the morning and in the evening at fasting (>3 hours), as indicated in table 3, a and b. Patients will be instructed to bring their used and unused IMP at each visit. Compliance will be assessed by the investigator by

Table 3 Doses of colchicine according to treatment arm and weight: (a) treatment arm colchicine 0.01 mg/kg/day, (b) treatment arm colchicine 0.005 mg/kg/day and (c) dose reduction steps

Patient's weight (kg)	Daily dose (mg)	Before breakfast	Before dinner
	0.01 mg/kg/day		
≤70	0.5 mg	1 tablet (0.5 mg) of AT	1 tablet (0.5 mg) of P
>70	1 mg	1 tablet (0.5 mg) of AT	1 tablet (0.5 mg) of AT
	0.005 mg/kg/day		
≤70	0.25 mg	1 tablet (0.5 mg) of AT every other day	1 tablet (0.5 mg) of P
>70	0.5 mg	1 tablet (0.5 mg) of AT	1 tablet (0.5 mg) of P

Starting dose (mg/day)	First dose reduction (mg/day)	Second dose reduction (mg/day)	Third dose reduction (mg/day)
1	0.5	0.25	Stop
0.5	0.25	Stop	
0.25	Stop		

AT, active treatment; P, placebo.

counting the remaining tablets returned by the patient. Dose reductions may be necessary in case of toxicities or overdoses, which will be monitored at each visit; this request will be made by online CRF through extradispensation or at drug dispensation. The system will automatically perform dose escalation according to the initial dose of colchicine or matching placebo, as depicted in table 3, c.

Primary objective

The primary objective is to assess whether different colchicine doses decrease disease progression, measured through ALS Functional Rating Scale—Revised (Italian version), in patients with ALS compared to the control arm and measured at baseline and at week 30 (treatment end).

Secondary objectives

Colchicine safety and tolerability in ALS

It will be assessed by periodic monitoring of possible adverse events (AEs), the most common being diarrhoea, abdominal cramping and pain, nausea and vomiting. These events are often the first signs of toxicity and may indicate the need for dose reduction or therapy discontinuation; a plan for dose reduction will be shared by all clinicians. Other known AEs that will be monitored are pharyngolaryngeal pain (reported in 1%–10%); blood cells alterations (<0.1%); peripheral neuropathy, myopathy, rhabdomyolysis, elevated creatine phosphokinase (CPK) and muscle pain (<0.1%); alopecia, delayed corneal wound healing, hypersensitivity and azo-/oligospermia (<0.1%); and rashes, urticaria, dermatitis, renal damage, hepatic damage and loss of appetite (<0.01%). Death from any cause or tracheotomy will be considered as a serious adverse event (SAE).

Biological assessment

We expect to find that colchicine increases the expression of inducers, markers and chaperones of the autophagy process, with a consequent reduction of accumulation of TDP-43; ALS-associated TDP-43 fragments; insoluble forms of SQSTM1/p62, UBQLN and OPTN; changes in extracellular vesicle secretion, as well as a decrease in the stress response. Colchicine action will be studied in fibroblasts, peripheral blood mononuclear cells (PBMCs) and lymphoblasts.[30–32]

Specific biological targets to be looked for between colchicine and placebo arms at baseline and at the end of treatment include

► Quantification of mRNA and protein levels of p62, LC3, TFEB, ATGs, HSPB8, BAG3, BAG1, HSP70 and HSF1 in patients' PBMCs, lymphoblasts and fibroblasts (transcriptome profile)[33]; we will test this profile also in available muscle biopsy (optional for patients); particular attention will be paid to possible formation of autophagic vacuoli, which may resemble those identified in inclusion body myopathy, Paget disease of bone with Frontotemporal Dementia (IBMPFD) and ALS possibly associated with autophagy hyperactivation.[34]

► Identification of changes in SG response and composition in patients' fibroblasts and lymphoblasts.

► Assessment of the overall levels and the relative ratio between soluble and insoluble species of TDP-43, TDP-43 fragments, SQSTM1/p62, UBQLN and OPTN in fibroblasts, PBMCs and lymphoblasts.

► Determination, using buffers with increasing detergent power (phosphate-buffered saline, Triton-X100, sodium dodecyl sulfate and formic acid), of which form of the insoluble species of the proteins tested above is detectable after treatment.

► Study of colchicine effects on extracellular vesicle secretion in blood and cerebrospinal fluid (CSF). Analysis of TDP-43, hyperphosphorylated TDP-43, SQSTM1/p62, UBQLN and OPTN in extracellular vesicles derived from plasma and CSF.[35]

► Colchicine effects on peripheral and CSF biomarkers: creatinine, albumin, CK and vitamin D in plasma as markers of disease severity; phosphorylated neurofilament heavy chain in CSF and plasma as marker of neurodegeneration; plasma/CSF IL-18, its endogenous inhibitor IL-18BP, MCP1 and IL-17 as markers of inflammation.

If the data will reveal the existence of highly responsive patients (with an ALS Functional Rating Scale - Revised (ALSFRS-R) decline of ≤2 points in 30 weeks), the analysis of the effect of colchicine will then be replicated in induced pluripotent stem cells (iPS) cells derived from a selected subset of patients enrolled in the study (either from PBMCs or fibroblasts). The iPS cells will be differentiated to motoneuronal cells to determine whether the effects of colchicine evaluated in blood and skin cells will be replicated in 'bona fide' MNs.

Clinical assessment

Periodic clinical assessment will be performed at defined time points by

► Overall survival from randomisation to date of documented death or tracheostomy or non invasive ventilation (NIV)>22 hours/day.

► Survival rate at weeks 30, 42 and 54.

► Forced vital capacity score from baseline to weeks 8, 18, 30, 42 and 54.

Quality of life assessment

Determination of quality of life as perceived by patients with ALS will be investigated by comparing ALS assessment questionnaire (ALSAQ-40) from baseline to weeks 8, 30 and 54.

Table 4 shows study procedures to be undertaken (study flow chart).

Sample size calculation

ALS progression will be assessed through the ALSFRS-R. It has been reported that ALS caring neurologists consider of clinical significance a difference of 4 points

Table 4 Study flow chart

Examinations	Pre-treatment		Treatment				Treatment end	Follow-up		Study end
	Screening (VS)	Baseline (W0)	W1	W2, W6, W10, W14, W16	W4, W8, W12	W18, W24	W30	W36	W42	W54
Time window		<1 week from screening	±1 day	±1 day	±2 days	±2 days	±3 days	±5 days	±5 days	±7 days
Informed consent	x									
Medical history	x									
Inclusion exclusion criteria	x									
Patient able to understand and follow the patient card procedures	x									
Phone call				x						
Clinical assessment										
Neurological examination	x	X	x		x	x	x	x	x	x
ALSFRS-R	x	X	x		x*	x	x	x	x	x
FVC	x	X			x*	x	x		x	x
MRC	x	X			x*	x	x		x	x
BMI	x	X	x		x	x	x	x	x	x
Safety assessment										
Adverse events		X	x	x	x	x	x	x	x	x
Vital signs	x	X	x		x	x	x	x	x	x
Physical examination	x	x	x		x	x	x	x	x	x
Chest X-ray	x									
ECG	x									
Abdominal US	x									
Hematology	x		x		x*	x	x		x	x
Biochemistry	x		x		x*	x	x		x	x
Urinalysis	x						x			x
Pregnancy test	x			X†	x	X†	X			
Infectious markers	x									
Biological activity										
Autophagy on lymphoblasts		x					x			x
Autophagy on fibroblasts		x					x			
Autophagy on muscle cells							x‡			
Stress granule response on lymphoblasts		x					x			x
Stress granule response on fibroblasts		x					x			
Aggregates accumulation on lymphoblasts		x					x			
Aggregates accumulation on fibroblasts		x					x			
RNA-seq on PBMCs/fibroblasts		x					x			
Plasma extracellular vesicles and neurodegenerationbiomarkers		x					x			x
CSF extracellular vesicles and neurodegenerationbiomarkers		x					x			
Neuroinflammationmarkers (IL18, IL-18BP, MCP1 and IL17 in plasma and CSF).		X								
PBMCs/fibroblasts differentiation to iPS cells and MNs							x			

Continued

Table 4 Continued

Examinations	Pre-treatment		Treatment				Treatment end	Follow-up		Study end
	Screening (VS)	Baseline (W0)	W1	W2, W6, W10, W14, W16	W4, W8, W12	W18, W24	W30	W36	W42	W54
Quality of life assessment										
ALSAQ-40		x			x§		x			x
Study treatment dispensation and compliance										
Study treatment dispensation		x			x	x				
Study treatment compliance				x	x	x	x			
Concomitant medications	x	x	x	x	x	x	x	x	x	x

*Only at week 4 and 12.
†Pregnancy test has to be performed every month for fertile women (week: 4-8-12-16-20-24-28-30).
‡Muscle biopsy is optional for patients.
§Only at week 8 .

in the decline of ALSFRS-R, and that patients with ALS have on average an ALSFRS-R decline of 0.89 points/month (SD:0.13).[36–38] Therefore, we can consider a mean ALSFRS-R decline in 30 weeks of 6.23, with 90% of patients presenting a decline of the ALSFRS-R total score ranging from 4.7 to 7.7.

As a result, for this study, during the 30 weeks of treatment, we will expect that the minority (10%) of patients with ALS will not have a decline at ALSFRS-R of at least 4 points from baseline, whereas a relevant number of those treated, that we quantify in a maximum of 60%, will not have the same decline. In other words, we expect that after 30 weeks of treatment the percentage of positive response in the control group will be equal to 10%, whereas in the experimental group it will be equal to 60%.

The null hypothesis is that the percentage of positive response (increase of ALSFRS-R <4 points at 30 weeks) between Colchicine and placebo groups will not differ after 30 weeks of treatment.

The alternative hypothesis is that Colchicine determines an increase of positive response compared to placebo of at least 50%, in absolute scale (60% vs 10%).

The study has been designed to reject the null hypothesis with an alpha error of 0.025 (to take into account a multiple comparison with a control arm) and a power of 0.80. For this purpose, a sample of 51 patients randomised in 3 arms would be needed. Considering an average drop out of 5%, a recruitment of 54 patients will be necessary.

Concomitant medications and concerns

All medications taken by the patients at the onset of study and all medication given in addition to the IMP during the study will be regarded as concomitant medications.

Colchicine is available in an oral formulation (tablet with strength of 1 mg) with well-known pharmacokinetics, toxicology and dynamics. It has a bioavailability of 45%, with onset at 18–24 hours, time to peak effect at 48–72 hours, and peak plasma concentration of 6.2–6.8 ng/mL. The drug is metabolised by P-gp and CYP3A4, and it is demethylated to two primary metabolites and one minor metabolite. Half-life is reported to be within 26 and 31 hours and excretion occurs through feces and urine (65%).

Inducers of CYP3A4 and P-gp may decrease Colchicine concentrations whereas inhibitors of CYP3A4 and P-gp may increase Colchicine concentrations. To avoid toxicity, strong CYP3A4 and P-gp inhibitors will be prohibited as well as bitter oranges intake. Concomitant use of strong inducers (eg, rifampin, rifabutin) will be not recommended.

Colchicine contains lactose. Patients with rare hereditary problems of galactose intolerance, the Lapp lactase deficiency or glucose-galactose malabsorption should not take this medication. Among other drug or substances to be alerted:

► Ciclosporin: possible increased risk of nephrotoxicity and myotoxicity.
► Vitamins: the absorption of vitamin B_{12} may be impaired by chronic administration or high doses of Colchicine; if required vitamin supplementation may be increased.
► Statins: acute myopathy has been reported in patients given Colchicine with statins.
► Grapefruit Juice: it strongly inhibits the CYP3A4-mediated metabolism of Colchicine, therefore it is forbidden.

Data analysis plan
Separate analyses will be performed in:
1. All randomised subjects receiving at least 1 dose of study medication (*intention-to-treat* population);
2. All randomised subjects excluding protocol deviations (*Per protocol*, PP population).

Descriptive statistics
Descriptive statistics will be performed comparing the groups of Colchicine treatment and placebo.

Continuous variables will be described using mean and SD or median and IQR; categorical variables will be described as counts and percentages.

Colchicine efficacy

The comparison of positive responses (as defined above) among arms will be carried out by using the logistic regression model. Results will be presented as ORs and 95% CI.

Biological activity

Mean differences in plasma and CFS concentrations of different biomarkers from baseline to week 30 will be calculated and compared using t-test or Wilcoxon-Mann-Whitney test.

The mean cellular biomarkers change over time will be assessed using repeated measures analysis of variance (ANOVA), with treatment as between-subjects factor and time as within-subjects factor. Different models will be used, each with a different biomarker of activity as the dependent variable. Models will be adjusted for any unbalanced distribution of the main prognostic factors (eg, age) between the treatment arms.

Safety analysis

Safety analysis will be performed in all subjects receiving at least one dose of the experimental drug. All AEs and SAEs will be recorded according to ICH Guidelines and compared in the treatment arms at any follow-up visit and at study end.

Differences in tracheostomy-free survival (Kaplan-Meier method) between the treated groups and placebo group will be compared using the log-rank test. Cox's proportional hazard model would be used to adjust for any possible unbalanced prognostic factors. Statistical significance will be set at 0.05 level for a two-tailed test. Missing data will be handled using the last observation carried forward.

Adverse events

AEs, SAEs, adverse drug reaction (ADR), Unexpected ADR, will be defined accordingly to 'ICH guidance for Clinical Safety Data Management: Definitions and Standards for Expedited Reporting'.

All AE occurring between the first study-related procedure and the last study-related procedure will be reported. Those meeting the definition of SAE will be reported in an ad hoc SAE Form; they will be reported to coordinating centre within 24 hours. All AE will be recorded in the eCRF with a diagnosis (whenever possible), and together with investigator's opinion concerning the relationship of the AE to study treatment.

Stopping rules for administering the drug (single patient)

The following rules will apply regardless of the relationship with treatment:

► First occurrence of moderate AE: study treatment will be interrupted until AE has returned to baseline value or mild intensity, then resumed at the same dose level.
► If the same moderate AE reoccurs, study treatment will be interrupted until AE has returned to baseline or mild intensity, then resumed with a dose reduction (according with investigator decision).

► In case of severe AE, study treatment will be interrupted until AE has returned to baseline level or mild intensity, then resumed with a dose reduction.
► In case of life-threatening or disabling AE, study treatment will be definitely discontinued.

Stopping rules for safety reasons

To establish stopping rules, we will consider the following list of toxicities as acceptable: diarrhea, abdominal cramping and pain, nausea, vomiting, pharyngolaryngeal pain; mild/moderate blood cells alterations; elevated CPK, muscle pain, alopecia, delayed corneal wound healing, hypersensitivity, rashes, urticaria, dermatoses, dermatitis (mild/moderate severity), elevated AST/ALT/ALP, loss of appetite.

A procedure consisting of stopping the trial in case of excess of AE recording the treating group in case of 25% of the occurrence of the following: agranulocytosis, severe thrombocytopenia, aplastic anaemia; moderate/severe peripheral neuropathy, myopathy, rhabdomyolysis, renal damage, hepatic damage.

Based on the occurrence of AEs in each group (0.01 mg/kg/day or 0.005 mg/kg/day) or other clinically significant safety evaluations, it will be decided which dose of Colchicine has the best risk/benefit ratio to conclude for further studies.

ETHICS AND DISSEMINATION

The study will be carried out in accordance with the Declaration of Helsinki, as amended by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013.

All subjects will sign and personally date an approved Informed Consent (IC) Form after receiving detailed written and verbal information about the reason, the nature, the required procedures, the intended duration and the possible risks and benefits and any discomfort associated with the study. No study procedure will be performed before the written IC has been provided. This study will be conducted in accordance with the Declaration of Helsinki and the ICH E6 guideline (Good Clinical Practice). Both the Patient Information Sheet and the IC Form have been approved by the Ethics Committee with the study protocol. The results of the study will be presented during scientific symposia or published in scientific journals only after review and written approval by the involved parties in full respect of the privacy of the participating subjects.

An insurance company will provide insurance coverage for damages emerging from the trial.

Patient and public involvement

This research protocol was written without patient involvement. Patients were not invited to comment on the study design and were not consulted to develop patient-relevant outcomes or interpret the results. Patients were not expected to contribute to conducting this study and to the writing of this document for readability or accuracy.

Patients' association will be involved in disseminating the study design at patients' enrolment beginning, to allow patients to participate in it; patients' association will be involved in study results dissemination not only to participants but to the entire patient communities (eg, by website information)

Author affiliations

[1]Department of Neurosciences, St. Agostino Estense Hospital, Azienda Ospedaliero Universitaria di Modena, Modena, Italy

[2]Dipartimento di Scienze Farmacologiche e Biomolecolari, Centro di Eccellenza sulle Malattie Neurodegenerative, Università degli Studi di Milano, Milano, Italy

[3]Genomics and Post-Genomics Center, IRCCS Mondino Foundation, Pavia, Italy

[4]Laboratory of Translational Biomarkers, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy

[5]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy

[6]Department of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy

[7]Department of General Neurology, Rare Diseases Unit, IRCCS Mondino Foundation, Pavia, Italy

[8]"Rita Levi Montalcini" Departmentof Neurosciences, ALS Centre, University of Turin and Azienda Ospedaliero Universitaria Città della Salute e della Scienza, Turin, Italy

[9]Department of Diagnostic, Clinical and Public Health Medicine, University of Modena and Reggio Emilia, Modena, Italy

[10]Dipartimento ad attività integratedi Medicina Interna e Specialistica, Azienda Ospedaliero Universitaria "L. Vanvitelli", Napoli, Italy

[11]Department of Neurology, Institute of Experimental Neurology (INSPE), Division of Neuroscience, IRCCS San Raffaele Scientific Institute, Vita-Salute San Raffaele University, Milan, Italy

[12]Neuromuscular Omni Centre (NEMO), Fondazione Serena Onlus, Policlinico A. Gemelli IRCCS, Roma, Italy

[13]Istituto di Neurologia, Università Cattolica del Sacro Cuore, Roma, Italy

[14]UOC di Neurologia, Dipartimento di Scienze dell'invecchiamento, Neurologiche, ortopediche e della testa collo, Fondazione Policlinico Universitario A. Gemelli IRCCS, Roma, Italy

[15]Department of Neurology-Stroke Unitand Laboratory of Neuroscience, Istituto Auxologico Italiano IRCCS, Milan, Italy

[16]Department of Pathophysiology and Transplantation, "Dino Ferrari" Center, Università degli Studi di Milano, Milano, Italy

[17]Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari, Bari, Italy

[18]Department of Neurosciences, University of Padua, Padua, Italy

[19]Centre of Integrative Biology (CIBIO), University of Trento, Trento, Italy

[20]Centre for Neuroscience and Nanotechnology, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy

REFERENCES

1. Taylor JP, Brown RH, Cleveland DW. Decoding ALS: from genes to mechanism. *Nature* 2016;539:197–206.
2. Calvo A, Moglia C, Lunetta C, *et al*. Factors predicting survival in ALS: a multicenter Italian study. *J Neurol* 2017;264:54–63.
3. Miller RG, Mitchell JD, Moore DH. Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Cochrane Database Syst Rev* 2012:CD001447.
4. Writing GroupEdaravone (MCI-186) ALS 19 Study Group. Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol* 2017;16:505–12.
5. Mitsumoto H, Brooks BR, Silani V. Clinical trials in amyotrophic lateral sclerosis: why so many negative trials and how can trials be improved? *Lancet Neurol* 2014;13:1127–38.
6. van den Berg LH, Sorenson E, Gronseth G, *et al*. Revised Airlie House consensus guidelines for design and implementation of ALS clinical trials. *Neurology* 2019;92:e1610–23.
7. Thomas M, Alegre-Abarrategui J, Wade-Martins R. RNA dysfunction and aggrephagy at the centre of an amyotrophic lateral sclerosis/frontotemporal dementia disease continuum. *Brain* 2013;136:1345–60.
8. Iguchi Y, Eid L, Parent M, *et al*. Exosome secretion is a key pathway for clearance of pathological TDP-43. *Brain* 2016;139:3187–201.
9. Buchan JR, Kolaitis RM, Taylor JP, *et al*. Eukaryotic stress granules are cleared by autophagy and Cdc48/VCP function. *Cell* 2013;153:1461–74.
10. Chitiprolu M, Jagow C, Tremblay V, *et al*. A complex of C9ORF72 and p62 uses arginine methylation to eliminate stress granules by autophagy. *Nat Commun* 2018;9:2794.
11. Zhao W, Beers DR, Bell S, *et al*. TDP-43 activates microglia through NF-κB and NLRP3 inflammasome. *Exp Neurol* 2015;273:24–35.
12. Cadwell K. Crosstalk between autophagy and inflammatory signalling pathways: balancing defence and homeostasis. *Nat Rev Immunol* 2016;16:661–75.

13. Crippa V, D'Agostino VG, Cristofani R, *et al*. Transcriptional induction of the heat shock protein B8 mediates the clearance of misfolded proteins responsible for motor neuron diseases. *Sci Rep* 2016;6:22827.
14. Carra S, Seguin SJ, Lambert H, *et al*. HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. *J Biol Chem* 2008;283:1437–44.
15. Crippa V, Sau D, Rusmini P, *et al*. The small heat shock protein B8 (HspB8) promotes autophagic removal of misfolded proteins involved in amyotrophic lateral sclerosis (ALS). *Hum Mol Genet* 2010;19:3440–56.
16. Rusmini P, Cristofani R, Galbiati M, *et al*. The Role of the Heat Shock Protein B8 (HSPB8) in Motoneuron Diseases. *Front Mol Neurosci* 2017;10:176.
17. Anagnostou G, Akbar MT, Paul P, *et al*. Vesicle associated membrane protein B (VAPB) is decreased in ALS spinal cord. *Neurobiol Aging* 2010;31:969–85.
18. Cristofani R, Crippa V, Rusmini P, *et al*. Inhibition of retrograde transport modulates misfolded protein accumulation and clearance in motoneuron diseases. *Autophagy* 2017;13:1280–303.
19. Crippa V, Cicardi ME, Ramesh N, *et al*. The chaperone HSPB8 reduces the accumulation of truncated TDP-43 species in cells and protects against TDP-43-mediated toxicity. *Hum Mol Genet* 2016;25:3908–24.
20. Cristofani R, Crippa V, Vezzoli G, *et al*. The small heat shock protein B8 (HSPB8) efficiently removes aggregating species of dipeptides produced in C9ORF72-related neurodegenerative diseases. *Cell Stress Chaperones* 2018;23:1–12.
21. Huang CC, Bose JK, Majumder P, *et al*. Metabolism and mis-metabolism of the neuropathological signature protein TDP-43. *J Cell Sci* 2014;127:3024–38.
22. Cicardi ME, Cristofani R, Rusmini P, *et al*. Tdp-25 Routing to Autophagy and Proteasome Ameliorates its Aggregation in Amyotrophic Lateral Sclerosis Target Cells. *Sci Rep* 2018;8:12390.
23. Carra S, Seguin SJ, Landry J. HspB8 and Bag3: a new chaperone complex targeting misfolded proteins to macroautophagy. *Autophagy* 2008;4:237–9.
24. Ganassi M, Mateju D, Bigi I, *et al*. A Surveillance Function of the HSPB8-BAG3-HSP70 Chaperone Complex Ensures Stress Granule Integrity and Dynamism. *Mol Cell* 2016;63:796–810.
25. Terkeltaub RA. Colchicine update: 2008. *Semin Arthritis Rheum* 2009;38:411–9.
26. Dalbeth N, Lauterio TJ, Wolfe HR. Mechanism of action of colchicine in the treatment of gout. *Clin Ther* 2014;36:1465–79.
27. Brooks BR, Miller RG, Swash M, *et al*. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2000;1:293–9.
28. Chiò A, Calvo A, Moglia C, *et al*. Phenotypic heterogeneity of amyotrophic lateral sclerosis: a population based study. *J Neurol Neurosurg Psychiatry* 2011;82:740–6.
29. Kimura F, Fujimura C, Ishida S, *et al*. Progression rate of ALSFRS-R at time of diagnosis predicts survival time in ALS. *Neurology* 2006;66:265–7.
30. Sabatelli M, Zollino M, Conte A, *et al*. Primary fibroblasts cultures reveal TDP-43 abnormalities in amyotrophic lateral sclerosis patients with and without SOD1 mutations. *Neurobiol Aging* 2015;36:2005. e5–2005.e13.
31. De Marco G, Lomartire A, Calvo A, *et al*. Monocytes of patients with amyotrophic lateral sclerosis linked to gene mutations display altered TDP-43 subcellular distribution. *Neuropathol Appl Neurobiol* 2017;43:133–53.
32. Pansarasa O, Bordoni M, Drufuca L, *et al*. Lymphoblastoid cell lines as a model to understand amyotrophic lateral sclerosis disease mechanisms. *Dis Model Mech* 2018;11:dmm031625.
33. Gagliardi S, Zucca S, Pandini C, *et al*. Long non-coding and coding RNAs characterization in Peripheral Blood Mononuclear Cells and Spinal Cord from Amyotrophic Lateral Sclerosis patients. *Sci Rep* 2018;8:2378.
34. Ching JK, Elizabeth SV, Ju JS, *et al*. mTOR dysfunction contributes to vacuolar pathology and weakness in valosin-containing protein associated inclusion body myopathy. *Hum Mol Genet* 2013;22:1167–79.
35. Sproviero D, La Salvia S, Giannini M, *et al*. Pathological proteins are transported by extracellular vesicles of sporadic amyotrophic lateral sclerosis patients. *Front Neurosci* 2018;12:487.
36. Kaufmann P, Levy G, Thompson JL, *et al*. The ALSFRSr predicts survival time in an ALS clinic population. *Neurology* 2005;64:38–43.
37. Castrillo-Viguera C, Grasso DL, Simpson E, *et al*. Clinical significance in the change of decline in ALSFRS-R. *Amyotroph Lateral Scler* 2010;11:178–80.
38. Kollewe K, Mauss U, Krampfl K, *et al*. ALSFRS-R score and its ratio: a useful predictor for ALS-progression. *J Neurol Sci* 2008;275:69–73.