Niclosamide Targets Inflammatory and Profibrotic Pathways in Amyotrophic Lateral Sclerosis

Martina Milani  
University of Rome Tor Vergata: Universita degli Studi di Roma Tor Vergata

Eleonora Mammarella  
University of Rome Tor Vergata: Universita degli Studi di Roma Tor Vergata

Simona Rossi  
Consiglio Nazionale delle Ricerche

Serena Lattante  
Università Cattolica del Sacro Cuore: Universita Cattolica del Sacro Cuore

Mario Sabatelli  
Policlinico Universitario Agostino Gemelli

Mauro Cozzolino  
Consiglio Nazionale delle Ricerche

Nadia D’Ambrosi  
University of Rome Tor Vergata: Universita degli Studi di Roma Tor Vergata

Savina Apolloni (✉️ savina.apolloni@uniroma2.it)  
University of Rome Tor Vergata: Universita degli Studi di Roma Tor Vergata  https://orcid.org/0000-0002-5782-1665

Research

**Keywords:** ALS, S100A4, fibroblasts, FUS, α-SMA, neurodegeneration, inflammation

**DOI:** https://doi.org/10.21203/rs.3.rs-138253/v1

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**Abstract**

**Background**

An increasing number of studies evidence that amyotrophic lateral sclerosis (ALS) is characterized by extensive alterations in different cell types and in different regions besides the CNS. We previously reported the up-regulation in ALS models of a gene called fibroblast-specific protein (FSP)-1 or S100A4, generally recognized as a pro-inflammatory and profibrotic factor. Since inflammation and fibrosis are often mutual-sustaining events that contribute to establish a hostile environment for organ functioning, the comprehension of the elements responsible for these interconnected pathways is crucial to disclose novel aspects involved in ALS pathology.

**Methods**

Here we employed fibroblasts derived from ALS patients harboring the C9orf72 hexanucleotide repeat expansion and sporadic ALS patients with no mutations in known ALS-associated genes and we downregulated S100A4 using siRNA or the S100A4 transcriptional inhibitor niclosamide. Mice overexpressing human FUS were adopted to assess the effects of niclosamide in vivo on ALS pathology.

**Results**

We demonstrated that S100A4 underlies impaired autophagy and a profibrotic phenotype, which characterize ALS fibroblasts. Indeed, its inhibition reduces inflammatory, autophagic and profibrotic pathways in ALS fibroblasts, and to interfere with different markers known as pathogenic in the disease, such as mTOR, SQSTM1/p62, STAT3, α-SMA and NF-κB. Importantly, niclosamide in vivo treatment of ALS-FUS mice reduces the expression of S100A4, α-SMA and PDGFRβ in the spinal cord, as well as gliosis in central and peripheral nervous tissues, together with axonal impairment and displays beneficial effects on muscle atrophy, by promoting muscle regeneration and reducing fibrosis.

**Conclusion**

Our findings show that S100A4 has a role in ALS-related mechanisms, and that drugs such as niclosamide that are able to target inflammatory and fibrotic pathways could represent promising pharmacological tools for ALS.

**Background**

Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disease characterized by progressive loss of motor neurons in the brain and the spinal cord. It is the third most common neurodegenerative disease, with an onset occurring approximately at 60 years old and patients surviving on average three years from diagnosis. Most cases of ALS are sporadic (sALS), while 60% of familial ALS can be attributed to pathogenic variants in four genes: SOD1, TARDBP, FUS and C9orf72 [32].
An increasing number of studies supports the concept that ALS is not a disease restricted to motor neuron pathology, but a disorder characterized by an extensive involvement of the CNS, with documented causal roles exerted also by glial cells [2]. Moreover, tissue alterations in non-nervous districts, including skeletal muscles, adipose tissue and even dermis have been extensively documented [35, 47, 60]. Fibroblasts from ALS patients show indeed numerous abnormalities concerning mitochondria metabolism [19, 33] and the stability of RNA transcripts related to oxidative phosphorylation, protein synthesis and inflammation [53]. These peripheral cells therefore share common pathogenic pathways with different CNS resident cells and are therefore useful to recapitulate and study major pathologic hallmarks of the disease [43].

Literature data and our previous work reported an evident up-regulation of a gene called fibroblast-specific protein (FSP)-1 or S100A4, in different models of ALS disease. S100A4 mRNA was found strongly increased in the lumbar spinal cord from pre-symptomatic and end-stage SOD1-G93A rats [49], in astrocytes from pre-symptomatic G37R mice [52] and is among the limited number of mRNAs displaying significant changes in their stability in both C9orf72 and sALS fibroblasts [53]. Accordingly, we found that S100A4 protein is overexpressed mainly by astrocytes and microglia from SOD1-G93A rats and by fibroblasts from ALS patients carrying SOD1 mutations [49]. The functions of S100A4 can be diverse and tissue-dependent but it is generally recognized as a pro-inflammatory and profibrotic gene, even though in the CNS its role seems more controversial, as in acute models of neurodegeneration it has been associated to trophic effects [9]. In contrast with this beneficial role, we previously demonstrated that in activated primary microglia cells the decrease of S100A4 obtained with its transcriptional inhibitor niclosamide is associated to a strong reduction of pro-inflammatory pathways [49]. Under this aspect, S100A4 is known to promote the release of cytokines at inflammatory sites and the remodeling of extracellular matrix components (ECM), and is a recognized inhibitor of autophagy, sustaining by this way inflammation and concomitant fibrotic events. Due to its properties, the protein has been implicated in the fibrosis of many organs, such as kidney, liver, lung and heart [22]. In neurodegenerative conditions, including ALS, an interplay between fibrosis and inflammation in different organs and tissues is an emerging concept that relies on data showing alterations of the ECM components and remodeling enzymes, increase in fibrotic markers as TGF-β, as well as in profibrotic genes [8, 12, 29]. Hence, the comprehension of the elements responsible for the inflammatory and fibrotic pathways appears to be crucial to dissect novel aspects contributing to the pathology of ALS.

Niclosamide is an FDA-approved anti-helminthic drug, with considerable safety [14, 50, 55]. In the last years, niclosamide has been repurposed for different diseases and preclinical validation proved that it has promising efficacy against solid cancers, rheumatoid arthritis and fibrotic conditions, due to potent anti-inflammatory and anti-fibrotic properties [5, 23, 50]. Niclosamide effects reside on its ability to target several signaling pathways, including S100A4, mammalian target of rapamycin (mTOR), signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB).
[17, 41, 48, 57], which, interestingly, have been found to be dysregulated in ALS [28, 49, 56], suggesting its potential use to interfere with these altered mechanisms in the pathology.

In this work, we have analyzed the role of S100A4 in the cellular pathways linked to human ALS-fibroblasts activation, such as mTOR, sequestosome 1 (SQSTM1/p62), NF-κB, α-smooth muscle actin (α-SMA) and N-cadherin. Moreover, we have tested niclosamide in vitro in ALS fibroblasts and in vivo in a transgenic mouse model of ALS overexpressing human FUS (hFUS), recapitulating pathological features of the disease, in order to understand its potential efficacy in ameliorating ALS pathology.

**Methods**

**Patients**

The study was approved by the ethics committee of the Università Cattolica del Sacro Cuore (Rome, Italy) on 30 July 2012, Prot nr. P740/CE/2012. A written informed consent was signed by all of the subjects. The diagnosis of ALS was made according to revise El Escorial/Airlie House Criteria. The presence of familiarity was deeply investigated. Patients with one or more affected relatives were diagnosed as familial ALS (fALS), while patients with no family history were classified as sporadic (sALS). Genetic analysis was performed on patients using massive parallel sequencing of genes associated to ALS, as previously described [20], and Repeat-Primed PCR was used to screen all patients for the C9orf72 expansion [42]. Three patients harboring the C9orf72 hexanucleotide repeat expansion (C9orf72) (2 fALS and 1 sALS), one patient harboring the p.R51C FUS pathogenic variant (fALS), two patients carrying the p.Q303H and the p.A382T variants in TARDBP (both sALS) were included in the study as well as three sporadic patients with no variants and five healthy controls.

**Fibroblast primary cultures**

All experiments were carried out in accordance to the approved guidelines of the ethics committee of the Catholic University, A written informed consent was obtained from patients and from healthy donors. Skin biopsies were performed using a 4-mm punch on the distal leg of the patients at NEMO Clinical Centre (Rome). Primary human dermal fibroblasts were isolated, as previously described [49]. Skin samples were dissected, transferred to a cell culture flask and cultured in BIO-AMF-2 complete medium (Biological Industries) in a 37 °C incubator. After the fibroblasts reached confluence, they were expanded up to 4th passage. Fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS, Euroclone) and 1% penicillin/streptomycin (Sigma) at 37 °C, 5% CO2.

**Chemicals and antibodies**

Niclosamide and all reagents, unless otherwise specified, were purchased from Sigma-Aldrich. Immunofluorescences (IF) and immunoblots (WB) were performed with the following primary antibodies: anti-rabbit S100A4 (1:500-IF, 1:1000-WB, Millipore), anti-rabbit mTOR and phospho-mTOR (1:100-WB, Cell
Immunofluorescence and confocal analysis

FUS mice and age-matched controls were euthanized by CO\textsubscript{2} and decapitated. Spinal cords were immediately dissected and post-fixed in 4% PFA for 12 h, incubated in 30% sucrose in PBS solution for 24 h at 4 °C and then cut into 30 μm thick slices with a freezing cryostat. Lumbar spinal cord slices from at least three animals per group were blocked for 1 h in 10% NDS in PBS, 0.3% Triton X-100 and then incubated 3 days at 4 °C with primary antibodies diluted in 2% NDS in PBS, 0.3% Triton X-100 and then for 3 h at room temperature with appropriate secondary antibody, diluted in the same solution. After two rinses, 10 min each in PBS, nuclei were stained with 1 μg/ml DAPI (Sigma-Aldrich) for 10 minutes. Whole mount sciatic nerves were post-fixed in 4% PFA for 24 h, incubated with PBS at 4°C for 48h and blocked with blocking buffer of 10% NDS in PBS, 0.3% Triton X-100 for 6 h at RT. Nerves were then incubated 3 days at 4 °C with primary antibodies diluted in 2% NDS in PBS, 0.3% Triton X-100 and then for 3 h at room temperature with appropriate secondary antibody, diluted in the same solution. After two rinses, 10 min each in PBS, nuclei were stained with 1 μg/ml DAPI for 10 minutes. Images were visualized by Nikon Eclipse TE200 epifluorescence microscope (Nikon, Florence, Italy) connected to a CCD camera. Images were captured under constant exposure time, gain and offset. After creating a region of interest, background was subtracted, and the average pixel intensity was determined. All images quantifications were done using ImageJ software (NIH, Bethesda, USA).

Western blot

Cell were lysed on plates in 2xLaemmli buffer and the lysates were boiled at 100°C for 5 min. Spinal cords, sciatic nerves and gastrocnemius muscles of at least 3 animals per group were dissected [1] and lysed in homogenization buffer (50 mM Tris HCl pH 7.4, 250 mM NaCl, 1 mM EDTA, 5 mM MgCl\textsubscript{2}, 1% Triton X-100, 0.25% Na-deoxycholate, 0.1% SDS, protease inhibitor cocktail). After 2 × 10" sonication cycles, samples were incubated on ice and then centrifuged at 15000 × g for 20' at 4 °C. Supernatants were then quantified with Bradford protein assay (Bio-Rad) and resuspended in Laemmli Buffer before SDS-PAGE (Sigma-Aldrich). Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose.
membranes, followed by incubation with 5% skimmed milk for 1 h and with primary antibodies at 4°C overnight. HRP-conjugated secondary antibodies (1:2,500, Jackson ImmunoResearch) were applied at RT for 1 h. ECL solution (Roche) was used for chemiluminescent detection. GAPDH was used as a control for equal loading. Following densitometry-based quantification and analysis using ImageJ software, the relative density of each identified protein was calculated.

S100A4 Silencing

Primary fibroblasts were seeded in 12-well plate at a density of 50,000 cells per well approximately 24 h before transfection and at the confluence of about 50%, the cells were transfected with two types of siRNAs for S100A4 (50 nM) (Thermo Fischer). A scrambled siRNA (100 nM) (Thermo Fischer) was used as a negative control. Transfection was performed using Metafectene (Biontex, Germany) following the manufacturer’s instructions. After transfection for 48 or 72 h cells were harvested for further experiments.

FUS transgenic mice

Adult Tg (Prnp-FUS) WT3Cshw/J mice expressing hemagglutinin-tagged human wild-type FUS (hFUS) were obtained from Jackson Laboratories. Animals were housed in our indoor animal facility at constant temperature (22 ± 1 °C) and relative humidity (50%) with 12-h light cycle (light 7 am–7 pm). Mice were maintained in hemizygosity on the same C57BL/6 genetic background. Hemizygous FUS mice were backcrossed to obtain homozygous mice, used as experimental subjects. Food and water were freely available. When animals showed symptoms of paralysis, wet food was given daily into the cages for easy access to nutrition and hydration. Mice were genotyped by PCR analysis of tissue extracts from tail tips. Hemizygous FUS mice were identified using PCR primers: Fwr5′-AGGGCTATTCCCAGCAGAG-3′, Rev5′-TGCTGCTGTTGTACTGTTCT-3′. Homozygous FUS mice were genotyped by qPCR using the following primers: Fwr5′-GCCAGAACACAGGCTATGGAA-3′ and Rev5′-GTAAGACGATTGGGAGCCTCTG-5′.

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the European Guidelines for the use of animals in research (2010/63/EU) and the requirements of Italian laws (D.L. 26/2014). The ethical procedure was approved by the Italian Ministry of Health (protocol number 406/2019 PR). All efforts were made to minimize animal suffering and the number of animals necessary to produce reliable results.

Niclosamide in vitro and in vivo treatment

The inhibitor of S100A4 niclosamide (2′,5-dichloro-4′-nitrosalicylanilide) was solubilized in dimethyl sulfoxide (DMSO) for in vitro experiments. Control cells were treated with the equal amount of solvent. For the in vivo experiments niclosamide (20 mg/kg/d, dissolved in Cremophor®) was administered daily from post-natal day 25 via intraperitoneal (i.p.) injections, when hFUS mice showed first signs of destabilized gait [30]. Control mice were treated with the appropriate volume of solvent solution. Survival was determined by the loss of righting reflex within 20 s after laying the mouse on its side [1].

Statistics
Data are reported as mean ± SEM. Two-tailed Student’s t test (for paired or unpaired samples as appropriate) or one-way ANOVA was used for statistical analysis. A p value less than 0.05 was accepted as a significant difference.

**Results**

**ALS fibroblasts show aberrant levels of S100A4, mTOR, SQSTM1/p62 and NF-κB**

In a previous work, we demonstrated that S100A4 was increased in fibroblasts from patients with different SOD1 pathogenic variants [49]. To investigate whether an augmented expression of S100A4 is a common trait of fibroblasts derived from patients with ALS, we have analyzed the protein expression in primary fibroblasts from sporadic ALS (sALS) patients without known variants in ALS-associated genes, and from patients carrying pathogenic **C9orf72** expansions, the most common cause of familial and sporadic ALS found to date. As shown, primary fibroblasts derived from both groups of patients display a strong increase in S100A4 protein levels, compared with those obtained from healthy subjects (Figure 1a, b). Furthermore, S100A4 is overexpressed also in a fibroblast line derived from a patient carrying the **FUS** p.R521C pathogenic variant (Additional file 1: Figure S1a) and from patients with the **TARDBP** p.Q303H and p.A382T mutations (Additional file 1: Figure S1b), demonstrating that S100A4 is upregulated in fibroblasts from fALS and sALS patients, carrying different pathogenic mutations.

Since the overexpression of S100A4 is correlated with autophagy impairment and inflammation, we also analyzed key markers related to these pathways in ALS fibroblasts. Cells from ALS patients show increased mTOR expression and an accumulation of SQSTM1/p62, compared to cells from healthy controls (Figure 1a). Moreover, although sALS fibroblasts do not show significant differences in both total and p-NF-κB levels compared to controls, fibroblasts carrying the **C9orf72** expansions display increased total and activated NF-κB (Figure 1a). These findings indicate that ALS-derived primary fibroblasts show features of autophagic and inflammatory pathway alterations, which may suggest an activated phenotype.

**S100A4 silencing inhibits activation markers in ALS fibroblasts**

In order to directly assess the contribution of S100A4 in supporting the autophagic and inflammatory dysregulated pathways shown by ALS fibroblasts, we silenced S100A4 expression in both sALS and **C9orf72** fibroblasts. We found that a 60% down-regulation of S100A4 is sufficient to strongly decrease the levels of mTOR and SQSTM1/p62 proteins in both sALS (Figure 2a) and **C9orf72** (Figure 2b) cells, as well as the expression of p-NF-κB in **C9orf72** fibroblasts (Figure 2b), with respect to controls.

The transformation of fibroblasts into activated cells as profibrotic myofibroblasts is characterized by the upregulation of several distinctive markers, including S100A4, α-SMA, N-cadherin, and by the activation of the STAT3 pathway. To explore whether the inhibition of S100A4 may affect the expression of these markers, we adopted the conditions of S100A4 silencing described before, and tested the levels of these proteins. As shown, S100A4 silencing leads to a decreased expression of STAT3, N-cadherin and α-SMA,
both in sALS (Figure 3a) and C9orf72-ALS (Figure 3b) fibroblasts, compared to controls. These findings thus suggest that S100A4 is directly involved in aberrant pathways related to autophagy and inflammation and contributes to the phenotypic transition of ALS fibroblasts toward a profibrotic and activated state.

**Niclosamide decreases S100A4, mTOR and profibrotic markers in ALS fibroblasts**

Previous studies reported that niclosamide, a pleiotropic drug recognized as a transcriptional inhibitor of S100A4, can induce canonical autophagy via feedback downregulation of mTOR [26] and can exert a potent inhibitory activity on STAT3 [7]. Thus, we tested the effects of niclosamide on sALS and C9orf72 fibroblasts as a way to evaluate its ability to reverse the aberrant pathways observed in ALS fibroblasts. Niclosamide treatment decreases S100A4, p-mTOR, p-STAT3 levels in both sALS (Figure 4a) and C9orf72 (Figure 4b) fibroblasts. Moreover, niclosamide inhibits α-SMA and N-cadherin protein expression (Figure 4a, b), a result in line with its well-recognized anti-fibrotic action [5]. Overall, these data show that niclosamide reverse several parameters linked to inflammation, impaired autophagy, fibrosis and activation of ALS fibroblasts.

**Niclosamide reduces ALS pathology in transgenic mice carrying hFUS mutation**

It is established that S100A4 is up-regulated in mutant SOD1 transgenic rat and mouse models of ALS during the disease course [49, 52] (Sun et al., 2015; Serrano et al., 2019). To understand whether the increase of S100A4 is a common trait in rodent models originating from different ALS genes, here we analyzed its protein expression in wild-type human FUS-overexpressing mice. The hFUS model recapitulates all key features of ALS such as motor neuron degeneration, muscle atrophy, physiological decline, cachexia, and neuroinflammation, and represents a model of the highly aggressive disease forms as those occurring particularly in FUS patients [24]. Notably, S100A4 is increased in the lumbar spinal cord (Additional file 1: Figure S2a and b) of diseased hFUS mice. This result indicates that the protein expression is commonly deregulated in different *in vivo* models of ALS, and prompted us to test the effects of S100A4 inhibition on disease phenotypes. To this aim, we treated hFUS mice with niclosamide at the dose of 20 mg/kg [46, 59], starting from the early symptom onset and analyzed the efficacy of the compound to restore several aberrant parameters occurring in these mice (Figure 5A). At the employed dose, niclosamide slightly but significantly increases the disease duration, compared to vehicle-treated mice (Figure 5b). Further, spinal cord pathology is improved, as indicated by the decrease in the levels of S100A4, as well as of GFAP and α-SMA in hFUS treated mice, compared to vehicle-treated mice (Figure 5c). As shown in Figure 5d, while spinal cord sections from Non-Tg mice show PDGFRβ-positive cells (indicating cells of mesenchymal origin) in the meninges and around blood vessels, in hFUS mice PDGFRβ staining is infiltrated into the white matter parenchyma, suggesting the presence of fibrotic regions. Interestingly, PDGFRβ-positive infiltrates in the white matter are reduced after niclosamide treatment (Fig. 5d). Next, since peripheral nerves are strongly affected in the hFUS model [24], we investigated the effects of niclosamide on sciatic nerves. As observed, the sciatic nerve of hFUS mice shows an axonal impairment as demonstrated by the decrease in β-III tubulin-positive fibers and the
concomitant upregulation of GFAP, in accordance with a Wallerian degeneration, evidencing a disorganization of Schwann cells compared with sciatic nerve from control littermate mice (Non-Tg) (Figure 5e, f). Niclosamide treatment partially restores the levels of β-III tubulin and GFAP and, in the niclosamide group both β-III tubulin and GFAP expression appear flatter and less frayed with respect to the vehicle group, suggesting that the treatment ameliorates axonal impairment in hFUS mice sciatic nerves (Figure 5e, f).

Finally, we explored the effects of niclosamide treatment on hFUS muscle pathology. At first, we found that hFUS mice show a strong increase in S100A4 protein in the gastrocnemius muscle compared to healthy mice and that niclosamide strongly inhibits its level (Figure 6a). We next assessed the expression of the key myogenic transcription factor MyoG, a marker of muscle differentiation [10] and we found that, compared to vehicle-treated mice, niclosamide administration increases MyoG expression (Figure 6a), suggesting an improved myogenic differentiation. Importantly, muscles from hFUS mice show increased levels of p-STAT3 and p-mTOR (Figure 6b), which suggest that pathways involved in skeletal muscle atrophy and fibrosis are activated in these animals. Further, profibrotic markers, such as PDGFR-β and α-SMA, are also upregulated compared to Non-Tg mice (Figure 6c). Remarkably, niclosamide decreases the expression of all aforementioned molecules (Figure 6b, c), confirming that they represent crucial targets of the drug also in vivo (Figure 6d).

Discussion

In this work, we provide evidence for the contribution of S100A4 in ALS pathogenesis and the potential repurposing of niclosamide for preclinical trials in the disease. Indeed, we have demonstrated here that S100A4 is upregulated in fibroblasts derived from different ALS patients as well as in the ALS model represented by hFUS mice. These data are consistent with our previous results, showing an increase of S100A4 in the SOD1 rat model in vivo and in mutant SOD1 fibroblasts in vitro [49] and suggest that an increased level of S100A4 is a common pathological trait of ALS, shared by different experimental models and disease-associated gene variants. Remarkably, in a recent paper S100A4 mRNA was identified together with other 333 transcripts, out of 22,977 annotated transcripts, among those whose stability is altered in C9orf72 ALS and sALS fibroblasts [53], sustaining our hypothesis that S100A4 dysregulation is a pathological hallmark of the disease. S100A4 belongs to the S100 superfamily, constituted by small proteins that are generally secreted by cells under stressful conditions, and that are undergoing extensive research as biomarkers in different fields, such as oncology, cardiology, fibrosis and inflammation as well as brain injury pathologies [11, 51]. The upregulation of S100A4 in fibroblasts from patients with sporadic and familial forms of the disease, together with the notion that the protein is released into biological fluids, make S100A4 an ideal candidate to be tested as a biomarker.

Recently, primary skin fibroblasts derived from patients have been extensively used as a model to study ALS because they share pathological alterations with neural cells, concerning energy metabolism, stress–response, autophagy, inflammation and RNA processing [43]. Under this aspect, they are useful tools to validate new pathogenic mechanisms and perform preliminary assessments of novel potential
treatments. Moreover, fibroblasts represent a cell type that can become resident in the nervous system during inflammation [38], as well as in skeletal muscle. Indeed, activated fibroblasts (deriving from endothelial cells, pericytes, immune cells) can be accounted as cellular players in the development of fibrosis and inflammation during several neurodegenerative conditions, including ALS [4, 8, 36, 58]. Thus, the identification of the molecules and pathways involved in the transition of fibroblasts from a quiescent to an activated phenotype, when their homeostasis is disturbed, might unveil pathogenic mechanisms that occur in nervous and peripheral tissues and that can contribute to the disease progression. Extensive studies have shown that the transformation into activated fibroblasts is an extremely complex process involving numerous signaling pathways and that depends on the physiological or pathological status of the cells and on their specific cellular contexts [37]. Among these, recent studies indicate that mTOR and the substrate of autophagy SQSTM1/p62 contribute to mesenchymal transition and that autophagy enhancers can attenuate fibroblast activation [25, 37, 39]. Moreover, the NF-κB pathway also plays an important role in inducing a myofibroblast-like phenotype, especially under inflammatory conditions, elicited for instance by TNF-α or IL-6 [15]. We have demonstrated here that high levels of S100A4 in ALS-fibroblasts correlate with signs of impaired autophagy and inflammation, as suggested by high expression of mTOR, SQSTM1/p62 and NF-κB. It is well known that an increase in S100A4 characterizes profibrotic activated fibroblasts, as those induced by TGFβ [54]. Therefore, the dysregulation of these markers points to an activated pro-inflammatory and fibrotic phenotype of fibroblasts derived from patients with ALS compared to cells from healthy donors.

Our results show that the molecular changes characterizing the activated state of ALS-fibroblasts are limited when the expression of S1004 is knocked-down, demonstrating that S100A4 is not only a marker of activation, but a necessary driver of the aberrant phenotypes of ALS-fibroblasts. Consistently, S100A4 is a well-known activator of the NF-κB axis [22] and its down-regulation promotes autophagy, while its overexpression inhibits starvation-induced autophagic pathways [16, 45]. Remarkably, the depletion of S100A4 decreases the levels of the typical fibrotic markers α-SMA and N-cadherin and of the pro-fibrotic factor STAT3. STAT3 contributes to fibrosis by inducing the production of ECM, generally sustaining the differentiation of organ resident cells via canonical and non-canonical pathways. Indeed, several lines of evidence report the fundamental role of STAT3 in fibroblast plasticity in different tissues [6, 18], where the inhibition of its signaling pathway attenuates fibrosis by decreasing several markers, among which α-SMA [34] and N-cadherin [27]. Since S100A4 is a known inducer of JAK/STAT pathway, it is possible that S100A4 knock-down can indirectly decrease the levels of the fibrotic molecules α-SMA and N-cadherin through the inhibition of STAT3. Nevertheless, we may not exclude a direct effect of S100A4 depletion on these markers, in particular on α-SMA, through the c-Myb and sphingosine-1-phosphate (S1P pathway) [22]. Independently of the molecular pathways, we have shown here that the specific inhibition of S100A4 can revert a pathological phenotype of ALS-fibroblasts, suggesting a role for the protein in sustaining harmful mechanisms in ALS.

To evaluate the effects of S100A4 down-regulation in ALS-fibroblasts by a pharmacological approach, we employed niclosamide, a well-known S100A4 transcriptional inhibitor, which is also recognized as a multi-target drug that promotes autophagy and inhibits STAT3 and NF-κB and acknowledged as a potent
blocker of fibrotic signaling in fibroblasts [3]. Our results demonstrate that the drug is able to reduce inflammatory/autophagic/fibrotic pathways in ALS fibroblasts, thereby interfering with different mechanisms characterized as pathogenic in ALS. Most interestingly, our in vivo results demonstrate that niclosamide relieves ALS-related pathological features in spinal cord, sciatic nerve and skeletal muscle of hFUS mice. Central and peripheral nerve pathology with inflammation and fibrosis is a major harmful mechanism contributing to degeneration [21, 61]. In ALS, neuronal regeneration and axonal growth may be limited by a hostile environment characterized by extensive gliosis and aberrant remodeling of ECM components [8]. Accordingly, gene ontology analysis of differently expressed genes in the spinal cord of hFUS mice show ECM matrix disorganization and increased expression of proteoglycans [40, 44]. Treatment with niclosamide in vivo clearly reduces the levels of S100A4, α-SMA and PDGFRβ in the spinal cord, as well as inflammation in central and peripheral nervous tissues, together with axonal impairment. These data are consistent with the in vitro results, demonstrating the anti-inflammatory and anti-fibrotic properties of niclosamide toward activated CNS glial cells, such as microglia and astrocytes [31, 49], and toward ALS-activated fibroblasts. Overall, these results show that niclosamide can control the excessive gliogenic/fibrotic environment and enhance neural repair in vivo in the hFUS model of ALS. Interestingly, skeletal muscles of hFUS mice display a strong increase in S100A4 expression, accompanied by augmented levels of α-SMA, PDGFRβ and STAT3, all proteins that have been widely demonstrated to be involved in muscle fibrosis and atrophy in both mutant SOD1 mouse models and in ALS patients [13, 28]. We have shown here that niclosamide displays positive effects also on muscle atrophy by promoting muscle regeneration and inhibiting muscle fibrosis, indicating that the targeting of these pathways can affect disease also in muscle tissue.

Our findings deserve further research to validate this new mechanism of action of niclosamide in preclinical experiments, performing dose-response treatments and one important goal will be to test the drug in additional ALS models since none of them completely recapitulates all aspects of the disease.

In conclusion, our findings show that S100A4 plays an important role in ALS-related mechanisms, and suggest that the use of a pleiotropic compound such as niclosamide, capable of affecting inflammatory, autophagic and profibrotic mechanisms in multiple districts of an ALS model, can meet the requirements of a possible treatment for ALS, that necessarily must be multifunctional and multitarget.

**Abbreviations**

ALS: Amyotrophic Lateral Sclerosis; ECM: Extracellular matrix; fALS: familial ALS; FSP-1: Fibroblast-specific protein-1; GFAP: Glial fibrillary acidic protein; hFUS: human FUS; mTOR: mammalian target of rapamycin; NF-κB: Nuclear factor-kB; Non-Tg: Non-transgenic; PDGFRβ: Platelet-derived growth factor receptor β; α-SMA: α-smooth muscle actin; SQSTM1/p62: Sequestosome 1; sALS: sporadic ALS; STAT3: Signal transducer and activator of transcription 3; TGF-β: Transforming growth factor β.

**Declarations**
Ethics approval and consent to participate

The study was approved by the ethics committee of the Università Cattolica del Sacro Cuore (Roma, Italy) on 30 July 2012, Prot nr. P740/CE/2012. All individuals signed a written informed consent. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the European Guidelines for the use of animals in research (2010/63/EU) and the requirements of Italian laws (D.L. 26/2014). The ethical procedure was approved by the Italian Ministry of Health.

Consent for publication

Not applicable

Availability of data and material

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

Funding

ND and MC are funded by Arisla-SPLICEALS Project.

Authors' contributions

SA and NDA conceived and designed the study and drafted the manuscript. MM and EM performed the experiments. All authors contributed to the data analysis, interpretation. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Paola Fabbrizio for the assistance with muscles tissues experiments and Chiara Miele and Valeria Durante for technical help.

Supplementary information

Additional file 1

References


S100A4 is involved in profibrotic pathways. Fibroblasts from sporadic ALS (sALS) (a) and C9orf72 ALS patients (b) were transfected with S100A4 siRNA and non-silencing siRNA (Scr). Cells were lysed after 72
h of transfection and assayed by western blot with anti-STAT3, anti-α-SMA, anti-N-cadherin. The levels of GAPDH expression were used as loading control. Data represent mean ± SEM of n=3 independent experiments. Statistical significance was calculated by student’s t-test and compared with the non-silencing siRNA. Significantly different values are indicated with an asterisk when p≤0.05.

Figure 5

a) hFUS mice

PND 25
Niclosamide 20 mg/kg

PND 40-44
Ex vivo tissue analysis
Spinal cord, Sciatic nerve, Skeletal muscle

b) Disease duration (days)

hFUSveh
hFUS Nic

Non-Tg
hFUS veh
hFUS Nic

C) S100A4
GFAP
α-SMA
GAPDH

A.U.

-11 kDa
-55 kDa
-42 kDa
-36 kDa

* #

Non-Tg
hFUS veh
hFUS Nic

d) Non-Tg, hFUS veh, hFUS Nic

PDGFRβ

A.U.

* #

Non-Tg
hFUS veh
hFUS Nic

e) Non-Tg, hFUS veh, hFUS Nic

β-III tubulin, GFAP

A.U.

* #

Non-Tg
hFUS veh
hFUS Nic

f) GFAP
GAPDH

A.U.

-55 kDa
-36 kDa

* #

Non-Tg
hFUS veh
hFUS Nic

Figure 5
Niclosamide ameliorates pathology in hFUS symptomatic mice (a) Schematic illustration of niclosamide treatment in hFUS mice. Male mice were intraperitoneally injected daily with 20 mg/kg niclosamide from postnatal day (PND) 25 until death and spinal cord, sciatic nerves and skeletal muscles tissues were then analysed. (b) Niclosamide-treated hFUS mice (hFUS Nic) show a significant difference in the disease duration with respect to vehicle-treated hFUS mice (hFUS veh), n= 6 mice/group. Data are presented as means ± SEM and statistical difference was calculated by student t-test and indicated with an asterisk when p≤0.05 with respect to vehicle-treated hFUS mice. (c) Protein lysates from lumbar spinal cord of non-transgenic (Non-Tg) (~40 days), vehicle (hFUS veh) and niclosamide-treated hFUS mice (hFUS nic) at end stage of the disease were assayed by western blot with anti-GFAP, anti-S100A4 and anti-α-SMA. Data represent mean ± SEM of n=4 mice/group. (d) Representative fluorescence images of PDGFRβ (green) in the lumbar spinal cord of Non-Tg, hFUS veh and hFUS nic mice at end stage of the disease. Scale bars: 50 µm. (e) Representative fluorescence images of β-III tubulin (blue) and GFAP (purple) in the sciatic nerves of Non-Tg, hFUS veh and hFUS nic mice at end stage of the disease. Scale bars: 50 µm. Immunofluorescence intensities were calculated by densitometric analyses. Data represent mean ± SEM of four sections per animal (n=4 mice/group). (f) Protein lysates from sciatic nerves of Non-Tg, hFUS veh and hFUS Nic mice at end stage of the disease were assayed by western blot with anti-GFAP. GAPDH served as loading control. Relative densitometric values are reported on the right. Data represent mean ± SEM of n=4 mice/group. Statistical significance was calculated by ANOVA and values significantly different from Non-Tg or hFUS veh mice are indicated respectively with an asterisk (*) or a hash (#) when p≤0.05.