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

Posted Date: January 5th, 2021

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

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

Signaling), anti-mouse SQSTM1/p62 (1:1000-WB, Abcam), anti-rabbit NF-kB and phospho-NF-kB (1:1000-WB, Cell Signaling), anti-rabbit STAT3 (1:1000-WB, Cell Signaling), anti-rabbit phospho-STAT3 (1:2000-WB, Cell Signaling), anti-rabbit α-SMA (1:1000-WB, 1:500-IF, GeneTex), anti-rabbit N-cadherin (1:1000-WB, GeneTex), anti-mouse glial fibrillary acidic protein (GFAP) (1:500-IF, 1:1000-WB, Cell Signaling), anti-rabbit β-III Tubulin (1:500-IF, Cell Signaling), anti-mouse MyoG (1:200-WB, Hybridoma Bank, USA), anti-mouse platelet-derived growth factor receptor β (PDGFR-β) (1:250-WB, 1:500-IF, Santa Cruz), anti-mouse GAPDH (1:10000-WB, Calbiochem). Secondary immunoblot antibodies for WB were: anti-rabbit (1:2500) and anti-mouse (1:5000) IgG peroxidase-conjugated from Bio-Rad Laboratories (Hercules, CA, USA). Secondary fluorescent antibodies for IF were: Alexa-Flour 488-Donkey anti-rabbit (1:200), Cy3-Donkey anti-mouse (1:200) from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). DAPI was used to stain nuclei (1:1000, Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence and confocal analysis

FUS mice and age-matched controls were euthanized by CO_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 4ºC 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 $MgCl_2$, 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 hemizygousity 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'-TGCTGCTGTTGTACTGGTTCT-3'. Homozygous FUS mice were genotyped by qPCR using the following primers: Fwr5'-GCCAGAACACAGGCTATGGAA-3' and Rev5'-GTAAGACGATTGGGAGCTCTG-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–responses, 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 PDGFRβin 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-κB; 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

1. Apolloni S, Amadio S, Fabbrizio P, Morello G, Spampinato AG, Latagliata EC, Salvatori I, Proietti D, Ferri A, Madaro L, Puglisi-Allegra S, Cavallaro S, Volonté C (2019) Histaminergic transmission slows

progression of amyotrophic lateral sclerosis. J Cachexia Sarcopenia Muscle 10:872–893. doi: 10.1002/jcsm.12422

2. Beers DR, Appel SH (2019) Immune dysregulation in amyotrophic lateral sclerosis: mechanisms and emerging therapies. Lancet Neurol 18:211–220. doi: 10.1016/S1474-4422(18)30394-6
3. Boyapally R, Pulivendala G, Bale S, Godugu C (2019) Niclosamide alleviates pulmonary fibrosis in vitro and in vivo by attenuation of epithelial-to-mesenchymal transition, matrix proteins & Wnt/β-catenin signaling: A drug repurposing study. Life Sci 220:8–20. doi: 10.1016/j.lfs.2018.12.061
4. Bradbury EJ, Burnside ER (2019) Moving beyond the glial scar for spinal cord repair. Nat Commun 10:3879. doi: 10.1038/s41467-019-11707-7
5. Cabrita I, Benedetto R, Schreiber R, Kunzelmann K (2019) Niclosamide repurposed for the treatment of inflammatory airway disease. JCI Insight 4. doi: 10.1172/jci.insight.128414
6. Chakraborty D, Šumová B, Mallano T, Chen C-W, Distler A, Bergmann C, Ludolph I, Horch RE, Gelse K, Ramming A, Distler O, Schett G, Šenolt L, Distler JHW (2017) Activation of STAT3 integrates common profibrotic pathways to promote fibroblast activation and tissue fibrosis. Nat Commun 8:1130. doi: 10.1038/s41467-017-01236-6
7. Chen W, Mook RA, Premont RT, Wang J (2018) Niclosamide: Beyond an antihelminthic drug. Cell Signal 41:89–96. doi: 10.1016/j.cellsig.2017.04.001
8. D'Ambrosi N, Apolloni S (2020) Fibrotic Scar in Neurodegenerative Diseases. Front Immunol 11:1394. doi: 10.3389/fimmu.2020.01394
9. Dmytriyeva O, Pankratova S, Owczarek S, Sonn K, Soroka V, Ridley CM, Marsolais A, Lopez-Hoyos M, Ambartsumian N, Lukanidin E, Bock E, Berezin V, Kiryushko D (2012) The metastasis-promoting S100A4 protein confers neuroprotection in brain injury. Nat Commun 3:1197. doi: 10.1038/ncomms2202
10. Fabbrizio P, Apolloni S, Bianchi A, Salvatori I, Valle C, Lanzuolo C, Bendotti C, Nardo G, Volonté C (2020) P2X7 activation enhances skeletal muscle metabolism and regeneration in SOD1G93A mouse model of amyotrophic lateral sclerosis. Brain Pathol 30:272–282. doi: 10.1111/bpa.12774
11. Fei F, Qu J, Li C, Wang X, Li Y, Zhang S (2017) Role of metastasis-induced protein S100A4 in human non-tumor pathophysiologies. Cell Biosci 7:64. doi: 10.1186/s13578-017-0191-1
12. Fernández-Klett F, Priller J (2014) The fibrotic scar in neurological disorders. Brain Pathol 24:404–413. doi: 10.1111/bpa.12162
13. Gonzalez D, Contreras O, Rebolledo DL, Espinoza JP, van Zundert B, Brandan E (2017) ALS skeletal muscle shows enhanced TGF-β signaling, fibrosis and induction of fibro/adipogenic progenitor markers. PLoS One 12:e0177649. doi: 10.1371/journal.pone.0177649
14. Hamdoun S, Jung P, Efferth T (2017) Drug Repurposing of the Anthelmintic Niclosamide to Treat Multidrug-Resistant Leukemia. Front Pharmacol 8:110. doi: 10.3389/fphar.2017.00110
15. Hou J, Ma T, Cao H, Chen Y, Wang C, Chen X, Xiang Z, Han X (2018) TNF-α-induced NF-κB activation promotes myofibroblast differentiation of LR-MSCs and exacerbates bleomycin-induced pulmonary fibrosis. J Cell Physiol 233:2409–2419. doi: 10.1002/jcp.26112

16. Hou S, Tian T, Qi D, Sun K, Yuan Q, Wang Z, Qin Z, Wu Z, Chen Z, Zhang J (2018) S100A4 promotes lung tumor development through β-catenin pathway-mediated autophagy inhibition. Cell Death Dis 9:277. doi: 10.1038/s41419-018-0319-1
17. Kadri H, Lambourne OA, Mehellou Y (2018) Niclosamide, a Drug with Many (Re)purposes. ChemMedChem 13:1088–1091. doi: 10.1002/cmdc.201800100
18. Kasembeli MM, Bharadwaj U, Robinson P, Tweardy DJ (2018) Contribution of STAT3 to Inflammatory and Fibrotic Diseases and Prospects for its Targeting for Treatment. Int J Mol Sci 19. doi: 10.3390/ijms19082299
19. Konrad C, Kawamata H, Bredvik KG, Arreguin AJ, Cajamarca SA, Hupf JC, Ravits JM, Miller TM, Maragakis NJ, Hales CM, Glass JD, Gross S, Mitsumoto H, Manfredi G (2017) Fibroblast bioenergetics to classify amyotrophic lateral sclerosis patients. Mol Neurodegener 12. doi: 10.1186/s13024-017-0217-5
20. Lattante S, Marangi G, Doronzio PN, Conte A, Bisogni G, Zollino M, Sabatelli M (2020) High-Throughput Genetic Testing in ALS: The Challenging Path of Variant Classification Considering the ACMG Guidelines. Genes (Basel) 11. doi: 10.3390/genes11101123
21. Lemke A, Penzenstadler C, Ferguson J, Lidinsky D, Hopf R, Bradl M, Redl H, Wolbank S, Hausner T (2017) A novel experimental rat model of peripheral nerve scarring that reliably mimics post-surgical complications and recurring adhesions. Dis Model Mech 10:1015–1025. doi: 10.1242/dmm.028852
22. Li Z, Li Y, Liu S, Qin Z (2020) Extracellular S100A4 as a key player in fibrotic diseases. J Cell Mol Med 24:5973–5983. doi: 10.1111/jcmm.15259
23. Liang L, Huang M, Xiao Y, Zen S, Lao M, Zou Y, Shi M, Yang X, Xu H (2015) Inhibitory effects of niclosamide on inflammation and migration of fibroblast-like synoviocytes from patients with rheumatoid arthritis. Inflamm Res 64:225–233. doi: 10.1007/s00011-015-0801-5
24. Ling S-C, Dastidar SG, Tokunaga S, Ho WY, Lim K, Ilieva H, Parone PA, Tyan S-H, Tse TM, Chang J-C, Platoshyn O, Bui NB, Bui A, Vetto A, Sun S, McAlonis-Downes M, Han JS, Swing D, Kapeli K, Yeo GW, Tessarollo L, Marsala M, Shaw CE, Tucker-Kellogg G, La Spada AR, Lagier-Tourenne C, Da Cruz S, Cleveland DW (2019) Overriding FUS autoregulation in mice triggers gain-of-toxic dysfunctions in RNA metabolism and autophagy-lysosome axis. Elife 8. doi: 10.7554/eLife.40811
25. Liu T, Zou X-Z, Huang N, Ge X-Y, Yao M-Z, Liu H, Zhang Z, Hu C-P (2019) Down-regulation of miR-204 attenuates endothelial-mesenchymal transition by enhancing autophagy in hypoxia-induced pulmonary hypertension. Eur J Pharmacol 863:172673. doi: 10.1016/j.ejphar.2019.172673
26. Liu Y, Luo X, Shan H, Fu Y, Gu Q, Zheng X, Dai Q, Xia F, Zheng Z, Liu P, Yin X-M, Hong L, Li M (2019) Niclosamide Triggers Non-Canonical LC3 Lipidation. Cells 8. doi: 10.3390/cells8030248
27. Ma J-H, Qi J, Lin S-Q, Zhang C-Y, Liu F-Y, Xie W-D, Li X (2019) STAT3 Targets ERR-α to Promote Epithelial-Mesenchymal Transition, Migration, and Invasion in Triple-Negative Breast Cancer Cells. Mol Cancer Res 17:2184–2195. doi: 10.1158/1541-7786.MCR-18-1194
28. Madaro L, Passafaro M, Sala D, Etxaniz U, Lugarini F, Proietti D, Alfonsi MV, Nicoletti C, Gatto S, De Bardi M, Rojas-García R, Giordani L, Marinelli S, Pagliarini V, Sette C, Sacco A, Puri PL (2018)

Denervation-activated STAT3-IL-6 signalling in fibro-adipogenic progenitors promotes myofibres atrophy and fibrosis. Nat Cell Biol 20:917–927. doi: 10.1038/s41556-018-0151-y

29. Maguire G (2018) Neurodegenerative diseases are a function of matrix breakdown: how to rebuild extracellular matrix and intracellular matrix. Neural Regen Res 13:1185–1186. doi: 10.4103/1673-5374.235026
30. Mirra A, Rossi S, Scaricamazza S, Di Salvio M, Salvatori I, Valle C, Rusmini P, Poletti A, Cestra G, Carrì MT, Cozzolino M (2017) Functional interaction between FUS and SMN underlies SMA-like splicing changes in wild-type hFUS mice. Sci Rep 7:2033. doi: 10.1038/s41598-017-02195-0
31. Natarajan R, Singal V, Benes R, Gao J, Chan H, Chen H, Yu Y, Zhou J, Wu P (2014) STAT3 modulation to enhance motor neuron differentiation in human neural stem cells. PLoS One 9:e100405. doi: 10.1371/journal.pone.0100405
32. Nguyen HP, Van Broeckhoven C, van der Zee J (2018) ALS Genes in the Genomic Era and their Implications for FTD. Trends Genet 34:404–423. doi: 10.1016/j.tig.2018.03.001
33. Onesto E, Colombrita C, Gumina V, Borghi MO, Dusi S, Doretti A, Fagiolari G, Invernizzi F, Moggio M, Tiranti V, Silani V, Ratti A (2016) Gene-specific mitochondria dysfunctions in human TARDBP and C9ORF72 fibroblasts. Acta Neuropathol Commun 4:47. doi: 10.1186/s40478-016-0316-5
34. Öztürk Akcora B, Vassilios Gabriël A, Ortiz-Perez A, Bansal R (2020) Pharmacological inhibition of STAT3 pathway ameliorates acute liver injury in vivo via inactivation of inflammatory macrophages and hepatic stellate cells. FASEB Bioadv 2:77–89. doi: 10.1096/fba.2019-00070
35. Paré B, Gros-Louis F (2017) Potential skin involvement in ALS: revisiting Charcot's observation - a review of skin abnormalities in ALS. Rev Neurosci 28:551–572. doi: 10.1515/revneuro-2017-0004
36. Peters S, Zitzelsperger E, Kuespert S, Iberl S, Heydn R, Johannesen S, Petri S, Aigner L, Thal DR, Hermann A, Weishaupt JH, Bruun T-H, Bogdahn U (2017) The TGF-β System As a Potential Pathogenic Player in Disease Modulation of Amyotrophic Lateral Sclerosis. Front Neurol 8:669. doi: 10.3389/fneur.2017.00669
37. Piera-Velazquez S, Jimenez SA (2019) Endothelial to Mesenchymal Transition: Role in Physiology and in the Pathogenesis of Human Diseases. Physiol Rev 99:1281–1324. doi: 10.1152/physrev.00021.2018
38. Pikor NB, Cupovic J, Onder L, Gommerman JL, Ludewig B (2017) Stromal Cell Niches in the Inflamed Central Nervous System. J Immunol 198:1775–1781. doi: 10.4049/jimmunol.1601566
39. Pölönen P, Jawahar Deen A, Leinonen HM, Jyrkkänen H-K, Kuosmanen S, Mononen M, Jain A, Tuomainen T, Pasonen-Seppänen S, Hartikainen JM, Mannermaa A, Nykter M, Tavi P, Johansen T, Heinäniemi M, Levonen A-L (2019) Nrf2 and SQSTM1/p62 jointly contribute to mesenchymal transition and invasion in glioblastoma. Oncogene 38:7473–7490. doi: 10.1038/s41388-019-0956-6
40. Qiu H, Lee S, Shang Y, Wang W-Y, Au KF, Kamiya S, Barmada SJ, Finkbeiner S, Lui H, Carlton CE, Tang AA, Oldham MC, Wang H, Shorter J, Filiano AJ, Roberson ED, Tourtellotte WG, Chen B, Tsai L-H, Huang EJ (2014) ALS-associated mutation FUS-R521C causes DNA damage and RNA splicing defects. J Clin Invest 124:981–999. doi: 10.1172/JCI72723

41. Ren X, Duan L, He Q, Zhang Z, Zhou Y, Wu D, Pan J, Pei D, Ding K (2010) Identification of Niclosamide as a New Small-Molecule Inhibitor of the STAT3 Signaling Pathway. ACS Med Chem Lett 1:454–459. doi: 10.1021/ml100146z
42. Renton AE, Majounie E, Waite A, Simón-Sánchez J, Rollinson S, Gibbs JR, Schymick JC, Laaksovirta H, van Swieten JC, Myllykangas L, Kalimo H, Paetau A, Abramzon Y, Remes AM, Kaganovich A, Scholz SW, Duckworth J, Ding J, Harmer DW, Hernandez DG, Johnson JO, Mok K, Ryten M, Trabzuni D, Guerreiro RJ, Orrell RW, Neal J, Murray A, Pearson J, Jansen IE, Sondervan D, Seelaar H, Blake D, Young K, Halliwell N, Callister JB, Toulson G, Richardson A, Gerhard A, Snowden J, Mann D, Neary D, Nalls MA, Peuralinna T, Jansson L, Isoviita V-M, Kaivorinne A-L, Hölttä-Vuori M, Ikonen E, Sulkava R, Benatar M, Wuu J, Chiò A, Restagno G, Borghero G, Sabatelli M, ITALSGEN Consortium, Heckerman D, Rogaeva E, Zinman L, Rothstein JD, Sendtner M, Drepper C, Eichler EE, Alkan C, Abdullaev Z, Pack SD, Dutra A, Pak E, Hardy J, Singleton A, Williams NM, Heutink P, Pickering-Brown S, Morris HR, Tienari PJ, Traynor BJ (2011) A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron 72:257–268. doi: 10.1016/j.neuron.2011.09.010
43. Riancho J, Arozamena S, López de Munaín A (2020) Dermic-derived fibroblasts for the study of amyotrophic lateral sclerosis. Neural Regen Res 15:2043–2044. doi: 10.4103/1673-5374.282257
44. Rossaert E, Pollari E, Jaspers T, Van Helleputte L, Jarpe M, Van Damme P, De Bock K, Moisse M, Van Den Bosch L (2019) Restoration of histone acetylation ameliorates disease and metabolic abnormalities in a FUS mouse model. Acta Neuropathol Commun 7:107. doi: 10.1186/s40478-019-0750-2
45. Ruma IMW, Kinoshita R, Tomonobu N, Inoue Y, Kondo E, Yamauchi A, Sato H, Sumardika IW, Chen Y, Yamamoto K-I, Murata H, Toyooka S, Nishibori M, Sakaguchi M (2018) Embigin Promotes Prostate Cancer Progression by S100A4-Dependent and-Independent Mechanisms. Cancers (Basel) 10. doi: 10.3390/cancers10070239
46. Sack U, Walther W, Scudiero D, Selby M, Kobelt D, Lemm M, Fichtner I, Schlag PM, Shoemaker RH, Stein U (2011) Novel effect of antihelminthic Niclosamide on S100A4-mediated metastatic progression in colon cancer. J Natl Cancer Inst 103:1018–1036. doi: 10.1093/jnci/djr190
47. Scaricamazza S, Salvatori I, Giacovazzo G, Loeffler JP, Renè F, Rosina M, Quessada C, Proietti D, Heil C, Rossi S, Battistini S, Giannini F, Volpi N, Steyn FJ, Ngo ST, Ferraro E, Madaro L, Coccurello R, Valle C, Ferri A (2020) Skeletal-Muscle Metabolic Reprogramming in ALS-SOD1G93A Mice Predates Disease Onset and Is A Promising Therapeutic Target. iScience 23:101087. doi: 10.1016/j.isci.2020.101087
48. Sekulovski N, Whorton AE, Tanaka T, Hirota Y, Shi M, MacLean JA, de Mola JRL, Groesch K, Diaz-Sylvester P, Wilson T, Hayashi K (2020) Niclosamide suppresses macrophage-induced inflammation in endometriosis†. Biol Reprod 102:1011–1019. doi: 10.1093/biolre/ioaa010
49. Serrano A, Apolloni S, Rossi S, Lattante S, Sabatelli M, Peric M, Andjus P, Michetti F, Carrì MT, Cozzolino M, D'Ambrosi N (2019) The S100A4 Transcriptional Inhibitor Niclosamide Reduces Pro-Inflammatory and Migratory Phenotypes of Microglia: Implications for Amyotrophic Lateral Sclerosis. Cells 8. doi: 10.3390/cells8101261

50. Stewart RL, Carpenter BL, West DS, Knifley T, Liu L, Wang C, Weiss HL, Gal TS, Durbin EB, Arnold SM, O'Connor KL, Chen M (2016) S100A4 drives non-small cell lung cancer invasion, associates with poor prognosis, and is effectively targeted by the FDA-approved anti-helminthic agent niclosamide. Oncotarget 7:34630–34642. doi: 10.18632/oncotarget.8969
51. Šumová B, Cerezo LA, Szczuková L, Nekvindová L, Uher M, Hulejová H, Moravcová R, Grigorian M, Pavelka K, Vencovský J, Šenolt L, Závada J (2019) Circulating S100 proteins effectively discriminate SLE patients from healthy controls: a cross-sectional study. Rheumatol Int 39:469–478. doi: 10.1007/s00296-018-4190-2
52. Sun S, Sun Y, Ling S-C, Ferraiuolo L, McAlonis-Downes M, Zou Y, Drenner K, Wang Y, Ditsworth D, Tokunaga S, Kopelevich A, Kaspar BK, Lagier-Tourenne C, Cleveland DW (2015) Translational profiling identifies a cascade of damage initiated in motor neurons and spreading to glia in mutant SOD1-mediated ALS. Proc Natl Acad Sci U S A 112:E6993-7002. doi: 10.1073/pnas.1520639112
53. Tank EM, Figueroa-Romero C, Hinder LM, Bedi K, Archbold HC, Li X, Weskamp K, Safren N, Paez-Colasante X, Pacut C, Thumma S, Paulsen MT, Guo K, Hur J, Ljungman M, Feldman EL, Barmada SJ (2018) Abnormal RNA stability in amyotrophic lateral sclerosis. Nat Commun 9:2845. doi: 10.1038/s41467-018-05049-z
54. Tomcik M, Palumbo-Zerr K, Zerr P, Avouac J, Dees C, Sumova B, Distler A, Beyer C, Cerezo LA, Becvar R, Distler O, Grigorian M, Schett G, Senolt L, Distler JHW (2015) S100A4 amplifies TGF-β-induced fibroblast activation in systemic sclerosis. Ann Rheum Dis 74:1748–1755. doi: 10.1136/annrheumdis-2013-204516
55. Wieland A, Trageser D, Gogolok S, Reinartz R, Höfer H, Keller M, Leinhaas A, Schelle R, Normann S, Klaas L, Waha A, Koch P, Fimmers R, Pietsch T, Yachnis AT, Pincus DW, Steindler DA, Brüstle O, Simon M, Glas M, Scheffler B (2013) Anticancer effects of niclosamide in human glioblastoma. Clin Cancer Res 19:4124–4136. doi: 10.1158/1078-0432.CCR-12-2895
56. Wolozin B, Ivanov P (2019) Stress granules and neurodegeneration. Nat Rev Neurosci 20:649–666. doi: 10.1038/s41583-019-0222-5
57. Wu C-S, Li Y-R, Chen JJW, Chen Y-C, Chu C-L, Pan I-H, Wu Y-S, Lin C-C (2014) Antihelminthic niclosamide modulates dendritic cells activation and function. Cell Immunol 288:15–23. doi: 10.1016/j.cellimm.2013.12.006
58. Yahn SL, Li J, Goo I, Gao H, Brambilla R, Lee JK (2020) Fibrotic scar after experimental autoimmune encephalomyelitis inhibits oligodendrocyte differentiation. Neurobiol Dis 134:104674. doi: 10.1016/j.nbd.2019.104674
59. Ye T, Xiong Y, Yan Y, Xia Y, Song X, Liu L, Li D, Wang N, Zhang L, Zhu Y, Zeng J, Wei Y, Yu L (2014) The anthelmintic drug niclosamide induces apoptosis, impairs metastasis and reduces immunosuppressive cells in breast cancer model. PLoS One 9:e85887. doi: 10.1371/journal.pone.0085887
60. Zhang L, Tang L, Huang T, Fan D (2020) Life Course Adiposity and Amyotrophic Lateral Sclerosis: A Mendelian Randomization Study. Ann Neurol 87:434–441. doi: 10.1002/ana.25671

61. Zhou T, Zheng Y, Sun L, Badea SR, Jin Y, Liu Y, Rolfe AJ, Sun H, Wang X, Cheng Z, Huang Z, Zhao N, Sun X, Li J, Fan J, Lee C, Megraw TL, Wu W, Wang G, Ren Y (2019) Microvascular endothelial cells engulf myelin debris and promote macrophage recruitment and fibrosis after neural injury. Nat Neurosci 22:421–435. doi: 10.1038/s41593-018-0324-9

Figures

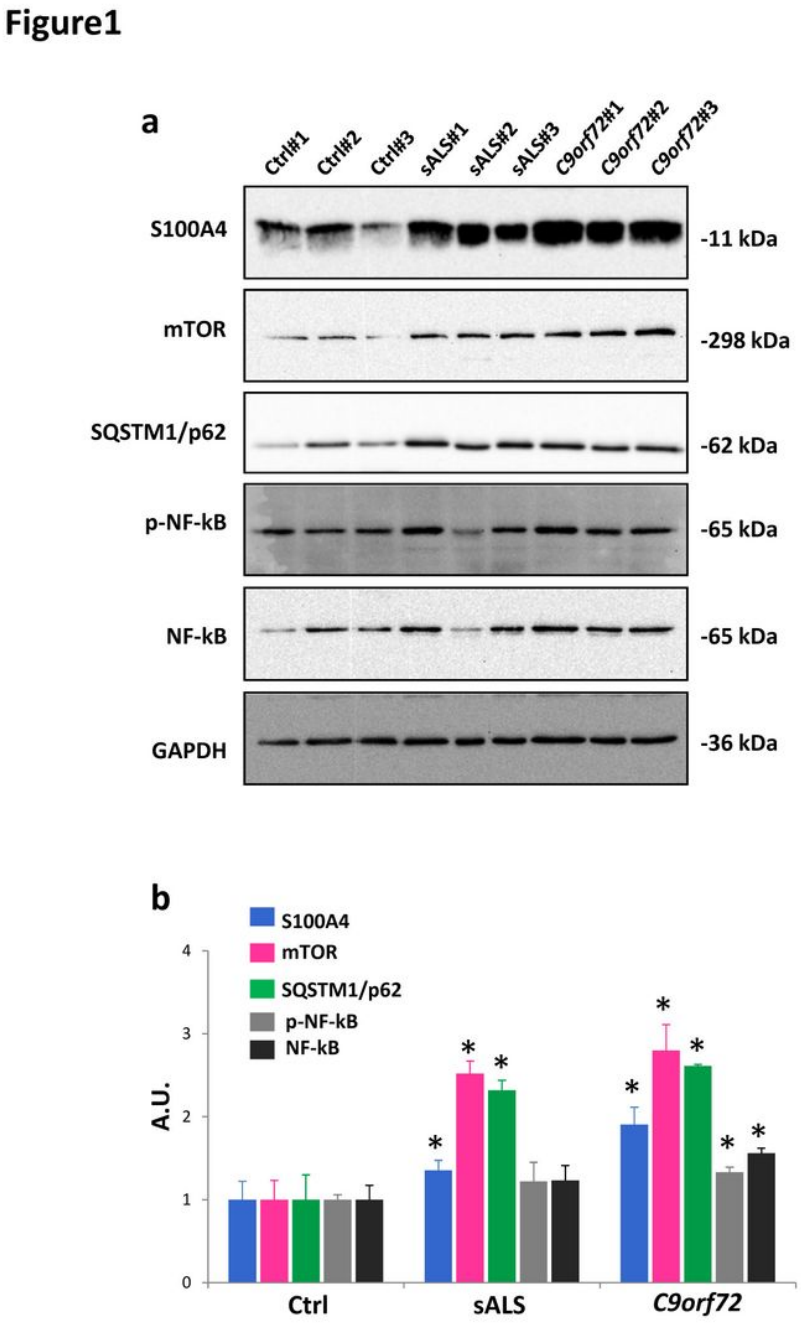

Figure 1

ALS-derived fibroblasts show increased activation-related pathways (a) Protein lysates of fibroblasts from controls, sporadic (sALS) and C9orf72 ALS patients (n=3/group) were analysed by Western blot using anti-S100A4, anti-mTOR, anti-SQSTM1/p62, anti-p-NF-κB and anti-NF-κB. GAPDH was used to normalize samples. (b) The expression levels were calculated by densitometric analyses. Data represent mean ± SEM. Statistical significance was calculated by ANOVA and values significantly different from controls are indicated with an asterisk when $p \leq 0.05$.

Figure 2

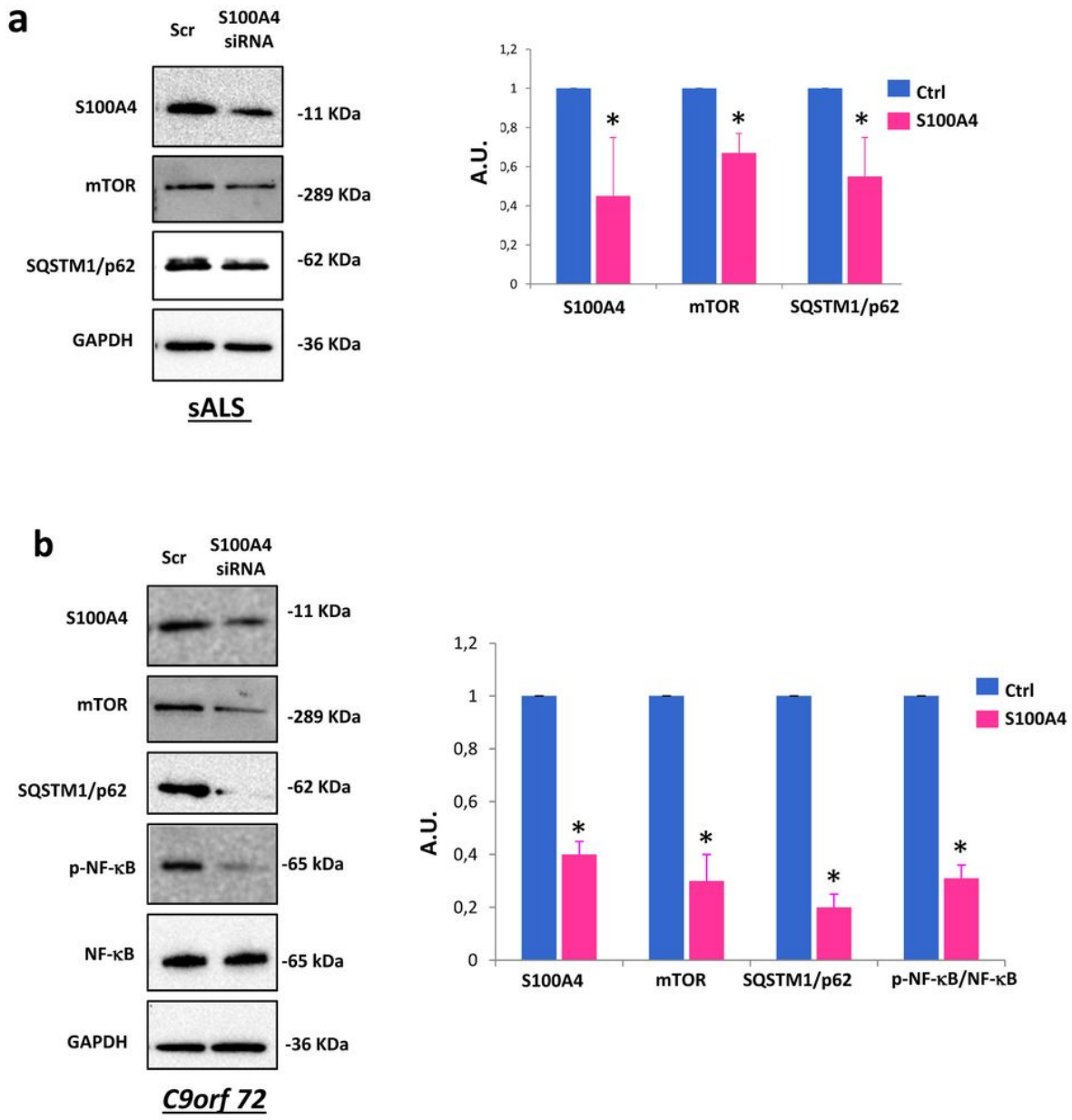

Figure 2

S100A4 silencing inhibits activation markers of ALS fibroblasts Skin fibroblasts from sporadic ALS (sALS) and C9orf72 ALS patients were treated with S100A4 siRNA and non-silencing siRNA (Scr) and then harvested after 72 h of transfection. (a) Protein lysates of sALS fibroblasts were subjected to western blot using anti-S100A4, anti-mTOR, anti-SQSTM1/p62. (b) Protein lysates of C9orf72 fibroblasts were subjected to western blot using anti-S100A4, anti-mTOR, anti-SQSTM1/p62, anti-NF-κB and anti-p-NF-κB. GAPDH was used to normalize samples. 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 and the significantly different values are indicated with an asterisk when $p \leq 0.05$.

Figure 3

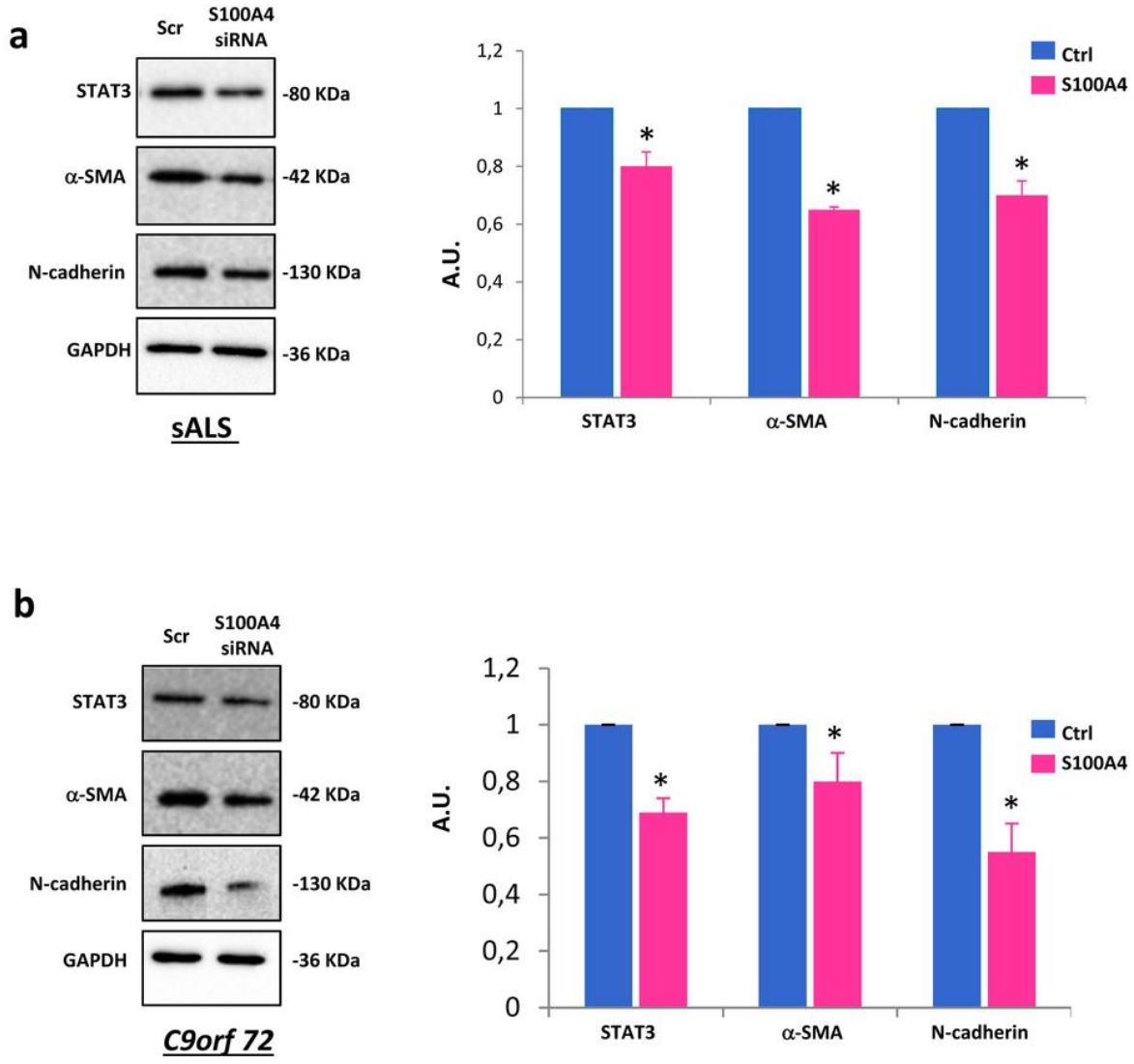

Figure 3

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 $p \leq 0.05$.

Figure 4

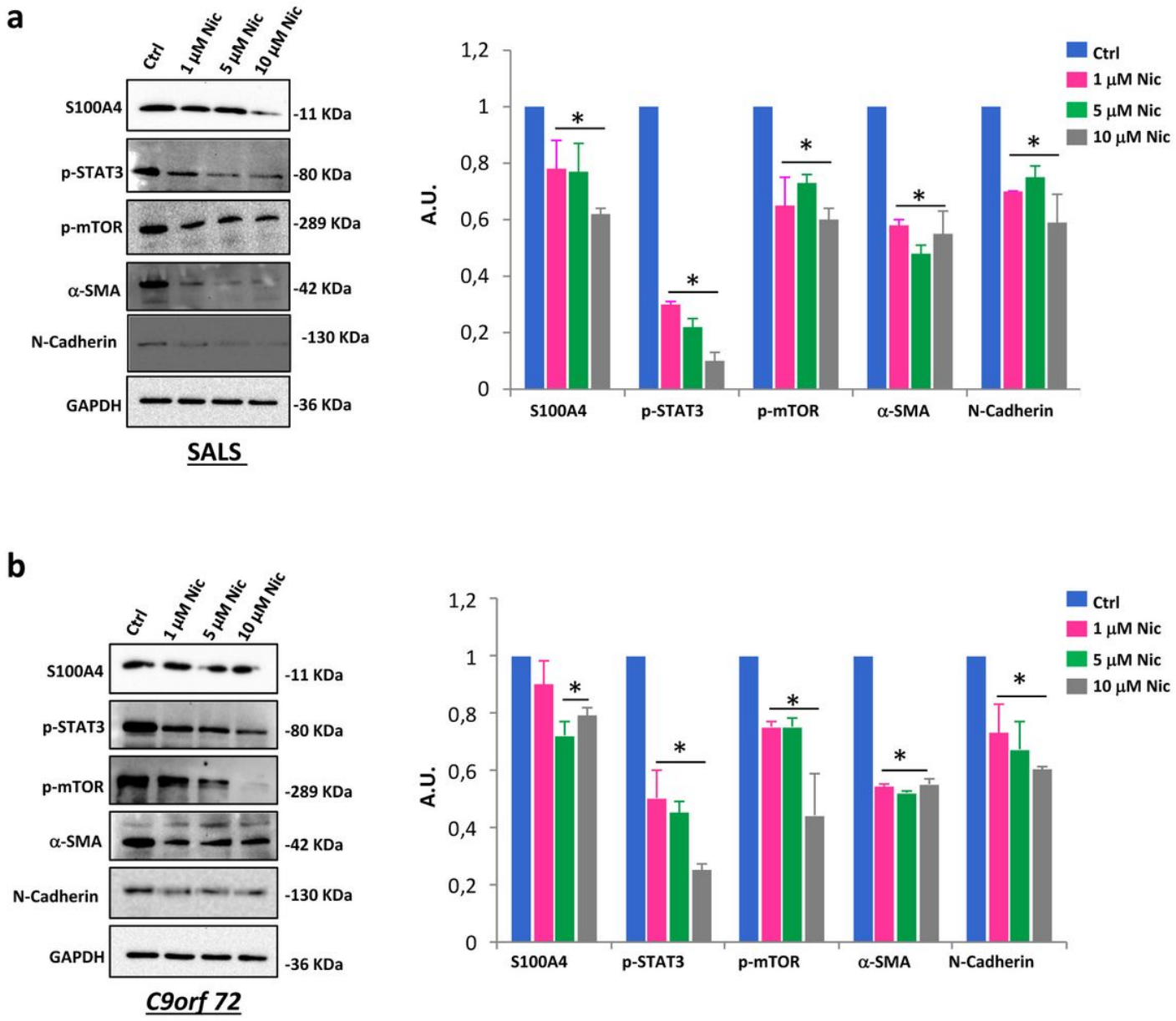

Figure 4

Niclosamide induces phenotypic changes in ALS fibroblasts Fibroblasts from sporadic ALS (sALS) (a) and C9orf72 ALS patients (b) were treated for 72 h with three different concentration of niclosamide

(Nic): 1 µM, 5 µM and 10 µM. Protein expression levels were analyzed by western blot with anti-S100A4, anti-p-STAT3, anti-p-mTOR and anti-α-SMA. GAPDH was used as a loading control. Data represent mean ± SEM of n=3 independent experiments. Statistical significance was calculated by ANOVA and significantly different values from controls are indicated with an asterisk when p≤0.05.

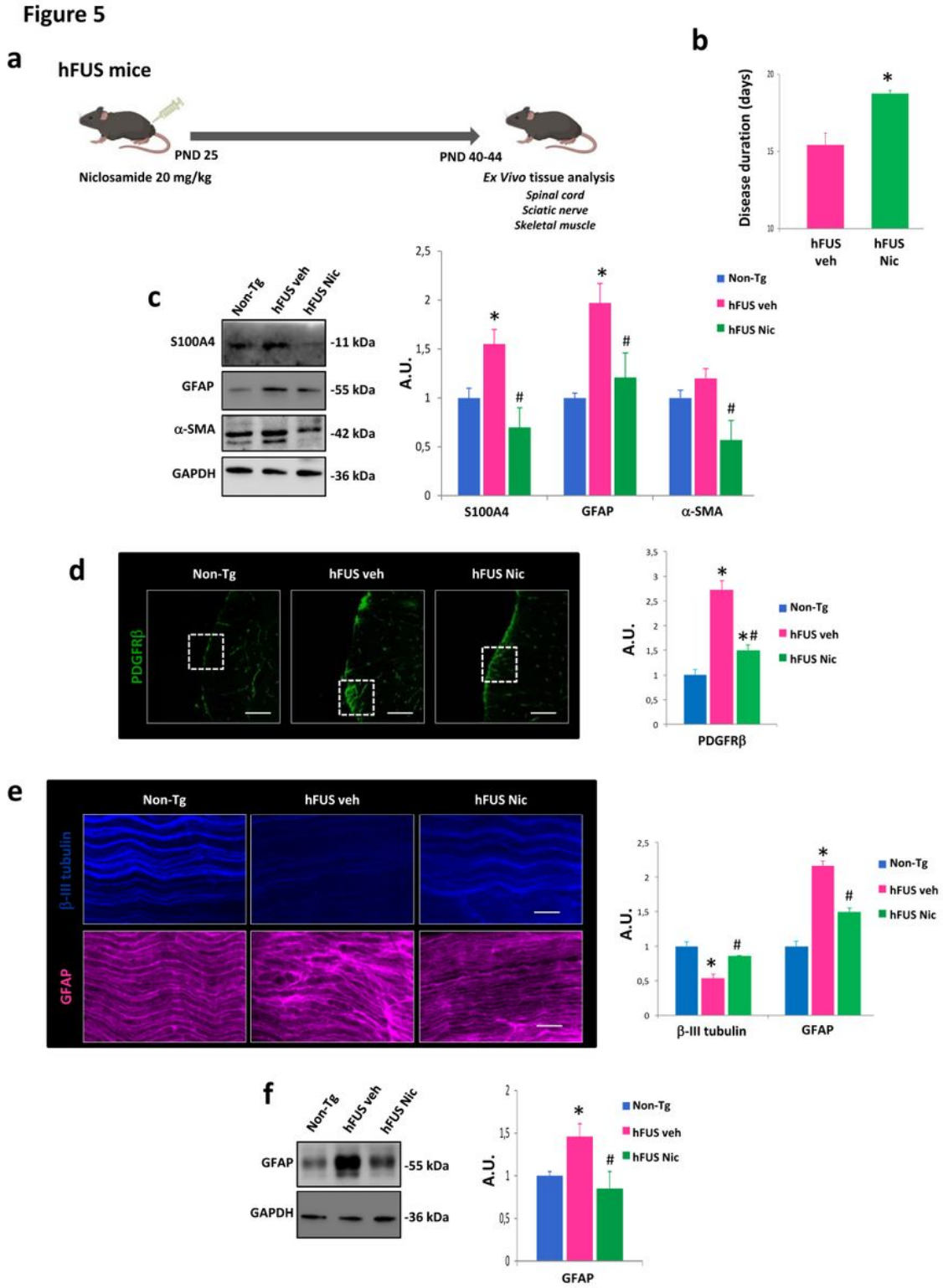

Figure 5

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 $p \leq 0.05$ 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 $p \leq 0.05$.

Figure 6

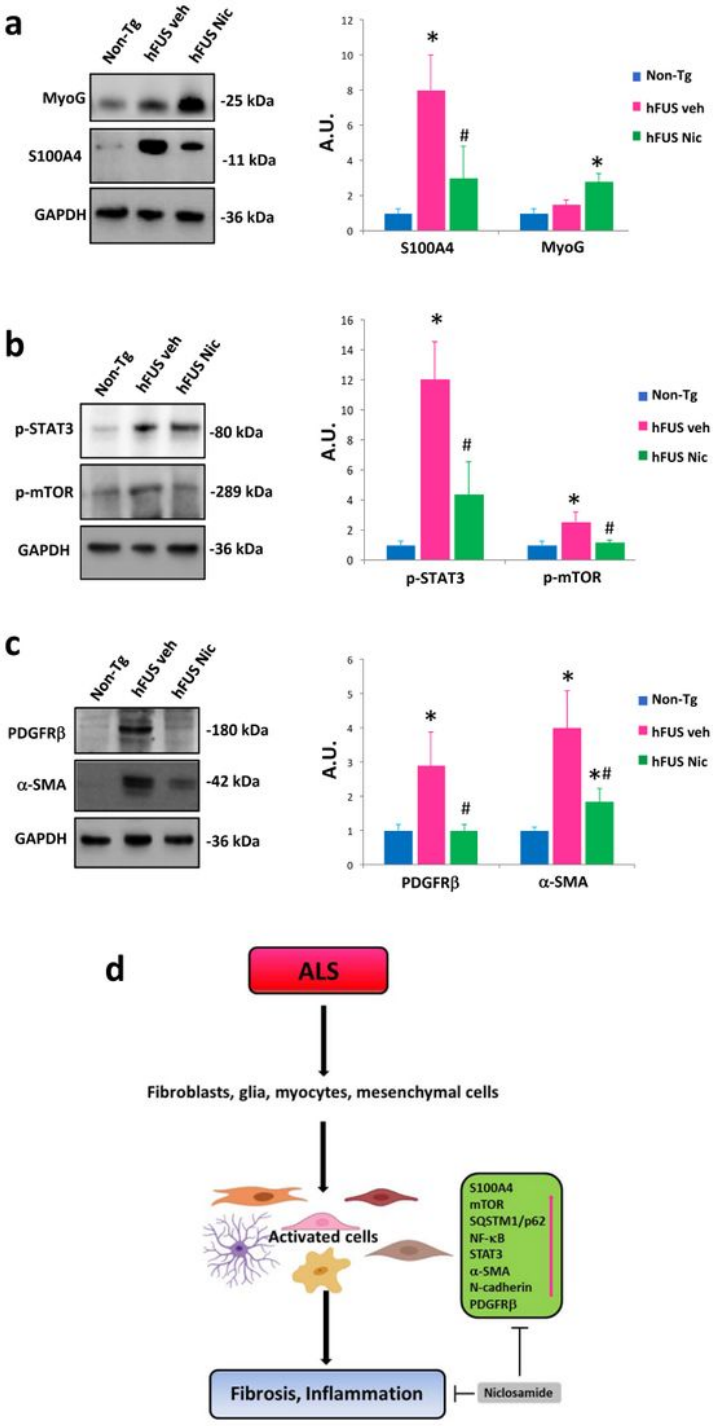

Figure 6

Niclosamide reduces profibrotic and inflammatory pathways in the skeletal muscles of hFUS symptomatic mice Protein lysates from gastrocnemius muscles of non-transgenic (Non-Tg) ~40 days, vehicle-treated (hFUS veh) and niclosamide-treated hFUS mice (hFUS nic) at end stage of disease were subjected to western blot with anti-MyoG and anti-S100A4 (a), with anti-p-STAT3 and anti-p-mTOR (b) and with anti-PDGFR-β and α-SMA (c). GAPDH served as loading control, n=4 mice/group. Alongside the

blots are reported the relative densitometric values. 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 $p \leq 0.05$. (d) During ALS, numerous molecular and signalling pathways trigger an activation of different cells (fibroblasts, glial cells, myocytes cells and generally mesenchymal cells) that, depending on the specific cellular context and on the pathological status of the cells, involves the increased expression of proteins including S100A4, mTOR, SQSTM1/p62, NF-κB, STAT3, α-SMA, N-cadherin and PDGFRβ. These processes lead to an inflammatory and fibrotic state. The identification of molecules (i.e. niclosamide) and regulatory pathways (i.e. S100A4) involved in this phenomenon could offer novel approaches for ALS disease.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Milanietal.SupplementaryMaterials.docx