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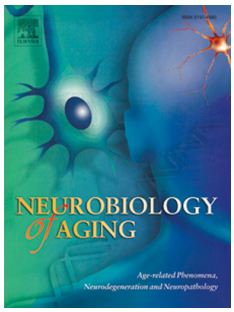

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Transforming Growth Factor beta 1 signaling is altered in the spinal cord and muscle of amyotrophic lateral sclerosis mice and patients.

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Abstract

Gender differences characterize amyotrophic lateral sclerosis (ALS). Since ALS patients have increased circulating levels of transforming growth factor beta 1 (TGFB1), here we analysed gender and disease progression related modification of TGFB1 and its related signaling molecules in spinal cord and skeletal muscle of ALS mice and in muscle biopsies from sporadic ALS patients. At pre-symptomatic (PS) stage *Tgfb1* expression is reduced in mouse spinal cord, but is increased selectively in male skeletal muscle. At symptomatic (S) stage *Tgfb1* is induced both in mouse spinal cord and muscle, as well as in muscle of ALS patients. *Tgfbr2* levels are induced only in mouse spinal cord. *Smad2* and *Smad4* are decreased in mouse spinal cord and muscle, but SMAD2 protein levels are augmented selectively in male mouse muscle. *Smad3* mRNA and SMAD3 protein are increased in mouse muscle. Genes controlled by TGFB1 in muscle (*Pax7*, *Collagen1a1* and *Fibronectin)* are reduced both in male and female ALS mice at S stage. Thus, TGFB1 modulation may serve as a novel therapeutic target for ALS.

Keywords

ALS; muscle; spinal cord; TGFB1; SMAD; Pax7; Col1a1; fibronectin

1. Introduction

ALS is an adult-onset progressive neurodegenerative disease affecting motoneurons in the brain and spinal cord, leading to paralysis. ALS is clinically heterogeneous in age and site of disease onset, as well as in the rate of progression. ALS is mainly sporadic (sALS), with familial forms (fALS) representing 10%–15% of ALS cases. Point mutations in human Cu/Zn superoxide dismutase 1 (*SOD1*) gene have been found in about 20% of fALS cases, while alterations of wild type (wt) SOD1 properties have been reported in some sALS patients (Bendotti and Carri, 2004; Pasinelli and Brown, 2006). sALS and fALS are clinically indistinguishable, and thus animal and cellular models expressing a mutated form of SOD1 with glycine 93 substitution with alanine (G93A) are widely used to study disease mechanisms (Pasinelli and Brown, 2006). The general incidence of ALS ranges from 2 to 5 per 100,000 person-years, with a prevalence of 7–9 per 100,000 persons. Interestingly, men have a higher risk of developing ALS than women. The male/female ratio of ALS incidence is 2.5 in younger groups (when androgen levels are high in males), it declines to 1.4 in older groups (Manjaly et al., 2010), and it becomes 1:1 at ages above 60 (when androgen levels decrease in males) (Groeneveld et al., 2004; Haverkamp et al., 1995; Logroscino et al., 2010). Moreover, women with ALS display an older average age of onset (Rudnicki, 1999). Notably, gender differences have been detected also in transgenic mice expressing G93A-SOD1. In these mice disease progression is significantly more aggressive in males than females (Heiman-Patterson et al., 2005; Veldink et al., 2003). Male hormonal steroids might have a role in this gender-related difference in ALS. Indeed, anabolic androgenic steroid abuse has been suggested to be responsible for the increased ALS prevalence in Italian soccer and American football players (Abel, 2007; Beghi et al., 2010; Belli and Vanacore, 2005; Chio et al., 2005; Piazza et al., 2004). In addition, reduced serum levels of androgens protected G93A-SOD1 mice from the toxicity of the mutant protein, whereas excessive androgen stimulation resulted in deterioration of motor function and reduced life span (Aggarwal et al., 2014).

There is a large body of evidence in support of the idea that ALS is not purely a motoneuron disease. Inflammation of nervous tissue and muscle degeneration contribute to motoneuron death in a non-cell autonomous fashion (Boillee et al., 2006). Toxicity to motoneurons might also derive from their target muscular cells. Indeed, while in G93A-SOD1 mice the reduction of the mutant protein in skeletal muscles has no effect on disease progression (Miller et al., 2006), selective expression of G93A-SOD1 in skeletal muscle results in progressive muscle atrophy (Corti et al., 2009; Dobrowolny et al., 2008; Levine et al.). Furthermore, muscle dysfunction and neuromuscular junction degeneration occur long before disease onset and motoneuron death (Fischer et al., 2004; Frey et al., 2000; Kennel et al., 1996). Several disease-associated alterations of the regenerative potential of skeletal muscles occur in the muscle of G93A-SOD1 mice; in particular, we and other authors previously reported modifications in the gene expression of TGFB1 (Galbiati et al., 2014; Galbiati et al., 2012; Si et al., 2015). TGFB1 is a pleiotropic growth factor with detrimental roles on muscular functions and regeneration (Kollias and McDermott, 2008; McLennan and Koishi, 2002). Nonetheless, TGFB1 enhances survival, development and maintenance of neurons (Bottner et al., 2000). TGFB1 functions are mediated by type 1 and type 2 transmembrane receptors forming a serine/threonine kinase complex. TGFB1 binds to a type 2 homodimer receptor that recruits and phosphorylates type 1 receptor to phosphorylate small mother against decapentaplegic 2 (SMAD2) and SMAD3. Phosphorylated SMAD2 and SMAD3 bind SMAD4, translocate to the nucleus, and act as transcription factors (reviewed by (Shi and Massague, 2003)).

In ALS patients, TGFB1 levels are increased both in serum and cerebrospinal fluid, and they positively correlate with disease duration (Houi et al., 2002; Ilzecka et al., 2002; Peters et al., 2017). Notably, a reduced activity of the transcription factor ZNF (Zinc Finger Protein) 512B, which is a regulator of TGFβ signaling pathway, has been linked to an increased susceptibility to ALS (Iida et al., 2011). In lumbar ventral horn motoneurons of G93A-SOD1 mice, TGFBR2 levels are increased (Phatnani et al., 2013). Moreover, an aberrant nucleocytoplasmic transport with an accumulation of

cytoplasmic pSMAD2/3 immunoreactivity has been reported in ALS (Nakamura et al., 2008; Zhang et al., 2006). However, few data are available on TGFB1 signaling in ALS muscle (Si et al., 2015).

In this study, we analyzed the activation of TGFB1 signaling in both spinal cord and muscle of SOD1-G93A mice. We performed the analyses at pre-symptomatic (PS) and symptomatic (S) stage, taking into account gender-related effects. We also evaluated the expression of *TGFB1* and its signaling pathway in the muscle of a cohort of ALS patients. The results demonstrate that in the spinal cord *Tgfb1* expression is reduced at pre-symptomatic (PS) stage without gender differences, indicating a possible lack of its neuroprotective effect on motoneurons. In the skeletal muscle at PS stage, *Tgfb1* expression was induced selectively in males, while at the S stage it is increased in both ALS mouse- and human-derived specimens. TGFB1 signaling pathway is dysregulated mainly at the muscular level and this can negatively reflect on muscle functionality.

2. Material and methods

2.1 Animals

Mice were maintained according to the institutional guidelines, that are in compliance with national (D.L. 26/2014) and European laws and policies (2010/63/UE). All the experimental procedures were approved by the Italian Ministry of Health. All the animals were kept under controlled temperature and humidity conditions, with dark/light cycles of 12 h. Food and water were supplied ad libitum.

2.1.1 ALS model.

BL6JLTg(SOD1)2Gur/J (Stock number 002297, The Jackson Laboratory) male mice or BL6JL-Tg (SOD1-G93A)2Gur/J (Stock number 002726, The Jackson Laboratory) male mice were crossed with wild type female mice purchased (Stock number 100012, Charles River) or obtained in house by crossing C57Bl/6J female and SJL male mice. All the experiments were performed in mice coming from the F1 generation of the cross described above. Non-transgenic (NTg) littermates were used as controls. The genotyping of the litters was conducted by PCR on tail biopsy DNA as

previously described (Crippa et al., 2013; Galbiati et al., 2012). To evaluate disease stages, starting from the 8th week of age and twice a week, mice were tested for the deficits by rotarod, and hanging wire by the same operator as previously described (Aggarwal et al., 2014). Body weight loss was also monitored. Disease onset was set as the time at which the mouse permanently starts to lose body weight. Four mice per gender per genotype were anesthetized with isoflurane and sacrificed at 8 or 16 weeks of age, corresponding to presymptomatic (PS) or symptomatic (S) stage of disease. Spinal cord and quadriceps muscles were rapidly collected after the sacrifice, snap frozen on dry ice, and conserved at -80°C until extraction.

2.1.2 SBMA model.

Generation and genotyping of mice containing the exon 1 of the human androgen receptor gene with 113 CAG repeats (AR113Q mice) has been previously described (Rusmini et al., 2015; Yu et al., 2006a; Yu et al., 2006b). Mice genetic background was C57Bl/6J. Females carrying one copy of AR113Q in the X chromosome were crossed with C57Bl/6J mice to maintain the colony. Genotypes were verified by PCR on tail DNA. Symptomatic mice of 24 weeks of age were anesthetized with isoflurane and sacrificed; quadriceps muscles collected, snap frozen, and maintained at –80°C.

2.1.3 Denervation model.

Three-months-old NTg male mice were anaesthetized with ketamine and xylazine. An incision was made through the skin and the upper region of the left gluteal muscle to expose the sciatic nerve, which was then cut 1–2 mm distal to the sciatic notch. The proximal portion of the nerve was sutured to prevent errant re-innervation of the gastrocnemius muscle. Right sciatic nerve was exposed and utilized as sham internal control in each animal. Mice were sacrificed 7 days later, and gastrocnemius muscles collected, snap frozen and conserved at -80°C.

2.2 Human samples

All evaluations involving anonymized controls and patients and experiments involving muscle tissue samples were performed in accordance with relevant guidelines and regulations, and were

approved by the University of Padova Ethics Committee for Clinical Experimentation. Written informed consent to study procedures was obtained from each patient, or his legal guardians. Confidentiality was guaranteed by assigning a study code to each patient. Bioptic samples were conserved in a genetic biobank supported by Telethon, Italy, fully complying with highest quality standards, according to rigorous ethical principles complying with Italian laws and International Recommendations. All patients were clinically affected with the definite ALS diagnosis, according to the revised El Escorial criteria (Brooks et al., 2000) and were followed at Neuromuscular Clinic of the University of Padova. Supplementary table 1 reports demographic and clinical data of twenty three ALS patients enrolled (ten females and thirteen males). Control bioptic samples were obtained from healthy aged-matched subjects (nine females and five males), who had undergone hip arthroprosthesis. Muscle biopsies were obtained using an open biopsy procedure and 100–200 mg of muscle tissue was collected. All biopsies were immediately frozen in liquid nitrogen for histopathology and biochemical analyses and stored at −80°C until analyzed (Borgia et al., 2017). Total RNA was isolated from frozen muscle biopsies using TRIzol Reagent (Sigma-Aldrich), according to the manufacturer's instructions.

2.3 Cell cultures

The myoblast C2C12 cell line was originally obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line was routinely maintained in high glucose (4500 mg/L) Dulbecco's modified Eagle's medium (Biochrom KG, Berlin, Germany) supplemented with 4 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (Thermo Fisher Scientific, Monza, Italy) at 37°C with 5% CO_2. The plasmids pcDNA3-wtSOD1, pcDNA3-G93ASOD1 (Galbiati et al., 2012), were transiently transfected into C2C12 using Lipofectamine 2000^{TM} (Thermo Fisher Scientific) following Manufacturers' instructions. Briefly, 60,000 cells/ml were plated in 12-well dishes, and transfected with 1,6 µg of DNA, and 4 µL of Lipofectamine/well. Controls were mock transfected. Cells were harvested for RNA isolation at 48 h after transfection.

2.4 Western Blotting Assay

To obtain total proteins from spinal cord or muscle, samples were homogenized in lysis buffer [phosphate-buffered saline (PBS), pH 7.4, supplemented with 1% Nonidet P-40, protease inhibitor cocktail (Roche Diagnostic Spa, Monza, Italy), phosphatase inhibitors (sodium vanadate 100 mM and sodium fluoride 100 mM), and EDTA 1 μM], with TissueLyser II and stainless steel glass beads (Qiagen, Milano, Italy). Crude extracts were centrifuged for 10 min at 5000 rpm at 4°C to remove particulate matter. Supernatant proteins concentration was determined by the bicinchoninic acid method (BCA assay, EuroClone, Pero Italy). Western immunoblot analysis was performed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis loading 15 μg of total proteins. Samples were then electrotransferred to nitrocellulose membranes 0.45 μm (Bio-Rad Laboratories, Hercules, CA, USA) using a semi-dry transfer apparatus (Trans-Blot® Turbo™ Transfer System, Bio-Rad Laboratories). Nitrocellulose membranes were treated overnight with a blocking solution containing 5% non-fat dry milk in TBS-T (Tris-buffered saline with 0.1 % Tween 20) and then incubated with the right primary antibodies overnight at 4 °C (anti-TGFβ1 R&D, dilution 1:1000; anti-Smad2/3 Merck Millipore 1:1000; anti-GAPDH Santa Cruz Biotechnology 1: 1000).

Immunoreactivity was detected using the following secondary peroxidase-conjugated antibodies: goat anti-rabbit (Santa Cruz Biotechnology dilution 1:5000) was used to identify anti-Smad2/3, and anti-GAPDH; goat anti-mouse (Santa Cruz Biotechnology dilution 1:5000) was used to identify the anti-TGFβ1 antibody.

The immunoreactive regions were then visualized using the enhanced chemiluminescence detection kit reagents (Clarity™ Western ECL substrate; Bio-Rad Laboratories). A ChemiDoc XRS System (Bio-Rad Laboratories) was used for the image acquisition. Optical intensity of samples assayed was detected and analyzed using the Image Lab software (Bio-Rad Laboratories).

2.5 RNA extraction and RT-qPCR

Total RNA from frozen spinal cords, muscles or C2C12 cells were extracted using the TRI Reagent in accordance to the manufacturer's protocol (Sigma-Aldrich). RNA was dissolved in RNase-free

water and quantified. Total RNA (1µg) was treated for 15min at room temperature with 1U of DNaseI (SigmaAldrich). Samples were reverse-transcribed using the HighCapacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Real-time PCR was performed using the CFX 96 Real-Time System (Bio-Rad Laboratories) in a 10 µl total volume, using the iTaq SYBR Green Supermix (Bio-Rad Laboratories), and with 500 nm primers. PCR cycling conditions were as follows: 94°C for 10 min, 40 cycles at 94°C for 15 s and 60°C for 1 min. Primers for selected genes were designed using the program Primer 3 and purchased from Eurofins Genomics (Ebersberg, Germany). Primer sequences are reported in Supplementary Table 2. Melting curve analysis was performed at the end of each PCR assay as a control for specificity. Data have been normalized to the amount of the housekeeping gene "ribosomal protein lateral stalk subunit P0 (*Rplp0*) or *RPLP0*, whose expression, to our experience, is stable across samples of spinal cord and muscle. Sample C_t values were used for target relative quantification through the $\Delta\Delta C_t$ calculation. As internal reference we have choosen the female 8 week-old NTg mouse group, since from preliminary experiments in muscle it was the group with the lowest expression of *Tgfb1* mRNA. The *N*-fold changes in gene expression was obtained transforming data by the equation $2^{-\Delta\Delta Ct}$.

2.6 Statistical Analysis

Statistical analysis was performed through two-tailed Student *t-test* for comparisons between two groups (Control vs. axotomized, NTg vs. SBMA), one way analysis of variance (ANOVA) followed by Tukey *post-hoc* test for group comparison (pcDNA3 vs. wt-SOD1 and G93A-SOD1), and two-way ANOVA for group comparisons followed by Bonferroni *post-hoc* test to determine specific group pair(s) statistical difference, using the PRISM software (GraphPad, San Diego, CA, USA). Details of statistical analyses have been reported in the Supplementary Data.

3. Results

3.1 TGFB1 *expression in ALS tissues.*

We have previously shown that in skeletal muscle of PS male G93A-SOD1 mice *Tgfb1* expression is upregulated compared to age-matched non-transgenic (NTg) male mice (Galbiati et al., 2012). In addition, we found that the androgenic/anabolic steroid nandrolone further enhances *Tgfb1* expression in these mice (Galbiati et al., 2012). Here we evaluated whether gender affects *Tgfb1* expression in the same ALS mouse model. The study has been performed on 8 (PS) and 16 (S) week-old G93A-SOD1 mice. As control, we used age-matched non-transgenic (NTg) mice and Tg mice expressing human wt SOD1 (wt-SOD1). We analysed *Tgfb1* mRNA transcript levels in the spinal cords and quadriceps muscles of both PS and S male and female mice; at each time point, four mice per gender per genotype were utilized. In samples derived from spinal cord at the PS stage, *Tgfb1* mRNA levels were reduced in both female and male G93A-SOD1 mice compared to age-matched NTg and wt-SOD1 mice (Figure 1A; *$p<0.05$, **$p<0.01$). The decreased *Tgfb1* mRNA expression was reverted at the S stage; in fact, *Tgfb1* mRNA levels increased in females and males when compared to the levels detected at PS stage of G93A-SOD1 mice (Figure 1A; **$p<0.01$, ***$p<0.001$). *Tgfb1* gene expression in NTg and in wt-SOD1 mice remained unchanged at all the examined conditions.

In the quadriceps muscles at the S stage, *Tgfb1* mRNA levels were found to be greatly increased in female and male G93A-SOD1 mice compared to G93A-SOD1 mice at PS stage and to age-matched NTg and wt-SOD1 mice (Figure 1B; *$p<0.05$, **$p<0.01$, ***$p<0.001$). At the PS stage *Tgfb1* mRNA levels were significantly upregulated only in male G93A-SOD1 animals (* $p<0.05$ vs. age-matched NTg male mice) (Figure 1B). Combined with our previous data, this observation corroborate the notion that at the muscular level male androgens might exert a detrimental role in ALS, since they exacerbate some of the alterations induced by G93A-SOD1 (Aggarwal et al., 2014; Galbiati et al., 2012).

Since data from wt-SOD1 mice did not differ from those obtained from NTg animals, we decided to use the latter model as a control for subsequent analyses.

Next, we analyzed TGFB1 protein expression (Figure 1C-D). Western blot (WB) analysis of spinal cord lysates showed that at S stage TGFB1 levels were increased when compared to PS stage both in male and female mice, even if this difference was not statistically significant (Fig. 1C). In muscle at PS stage TGFB1 levels were increased specifically in male G93A-SOD1 mice as compared to NTg mice (Fig. 1D; *p<0.05). At the S stage, TGFB1 levels were increased in female and male G93A-SOD1 mice, as compared to NTg mice (Fig. 1D; *p<0.05; ***p<0.001). Obtained data have also been presented in Table 1 (changes during the disease course in female and male G93A-SOD1 mice) and 2 (changes of mRNA and protein levels in female and male G93A-SOD1 mice *vs.* NTg mice).

We next asked whether alteration in the expression of *TGFB1* also occurs in the muscle of female and male ALS patients. Demographic and clinical data of controls and ALS patients have been reported in supplementary table 1. As reported in Figure 1E, the expression of *TGFB1* mRNA was increased in female and male sALS patients (disease effect p=0.0003). Notably, a statistically significant gender effect (p=0.0196) was observed related to *TGFB1* mRNA levels. These observations indicate that gene expression of TGFB1 is upregulated in the skeletal muscle of ALS mice at late stage of disease as in sALS patients.

3.2 Altered TGFB1 signaling in ALS spinal cord and muscle.

Not only TGFB1, but also its signaling pathway is dysregulated in different neurodegenerative diseases (Phatnani et al., 2013). Thus, we evaluated possible variations of the mRNA levels of *Tgfbr2*, both in the spinal cord and quadriceps muscle of PS and S G93A-SOD1 mice. In spinal cord, *Tgfbr2* expression was not altered at PS stage, whereas it was increased in female and male G93A-SOD1 mice at S stage (Figure 2A; *p<0.05; **p<0.01). In quadriceps muscles, *Tgfbr2* expression was unchanged at both PS and S stages (Figure 2B).

Then, we evaluated the expression of the canonical intracellular mediators of TGFB1 signaling pathway, i.e. SMAD proteins. In spinal cord samples, *Smad2* expression did not change at PS stage,

whereas it decreased in female and male mice during disease progression (Figure 3A; *p<0.05, **p<0.01), while *Smad3* expression in G93A-SOD1 mice at S stage showed gender differences (Figure 3B; *p<0.05). In quadriceps muscles, *Smad2* mRNA levels were decreased in S stage female and male mice compared to those in PS stage (Figure 3C; *p<0.05; **p<0.01). *Smad3* mRNA levels were up-regulated in the muscle of S stage female and male mice, compared to those in PS stage (Figure 3D; *p<0.05, **p<0.01). By WB analysis of spinal cord lysates, we did not find any significative changes in the levels of SMAD2 and SMAD3 except for an increase in the SMAD3 levels in female G93A-SOD1 mice during the disease course (Fig. 3 E,F, and G; *p<0.05). In quadriceps muscles, we found that SMAD2 levels are upregulated selectively in males at PS ans S stage (Figure 3H, and J; *p<0.05; **p<0.01). SMAD3 levels were increased at PS and S stage both in male and female mice (Fig. 3H, and K; *p<0.05). We also analyzed the expression of *Smad4*, and we found that in spinal cord of G93A-SOD1 mice *Smad4* mRNA levels are reduced during disease progression in female, but not in male mice (Figure 4A; *p<0.05). In quadriceps, *Smad4* mRNA levels were reduced at the S stage compared to PS stage in G93A-SOD1 mice in both male and female mice (Figure 4B; **p<0.01; ***p<0.001).

We then evaluated possible alteration in the TGFB1 signalling pathway in the muscle of female and male ALS patients. As reported in Figure 5, the levels of *SMAD3* mRNA were diminished in female patients, while *TGFBR2*, *SMAD2*, and *SMAD4* did not change in ALS patients vs. controls (**p<0.01). A statistically significant gender effect was observed in controls for *SMAD2* and *SMAD3 mRNA* (**p<0.01; ***p<0.001).

These results indicate that the expression of TGFB1 effectors, i.e. TGFBR2, SMAD2, SMAD3, and SMAD4, is altered in the spinal cord, and specially in the muscle of ALS mice, and some of these alteration are recapitulated in ALS patients. In Table 1 and 2 we have summarized results grouped for changes during the disease course or between male and female in ALS animals *vs.* NTg mice.

3.3 TGFB1 related functions in ALS muscle

TGFB1 suppresses muscle differentiation by inhibiting the expression of muscle-specific genes (Galbiati et al., 2012; Massague et al., 1986; Olson et al., 1986). Moreover, TGFB1 hampers muscle regeneration in muscular diseases by inhibiting satellite cell proliferation and myofiber fusion (Burks et al., 2011; Cohn et al., 2007). During myogenesis satellite cells are mainly regulated by the transcription factor paired box protein 7 (PAX7), which in turn is known to be negatively modulated by TGFB1 (Braga et al., 2012). Thus, we analysed whether *Pax7* expression is altered in the muscle of G93A-SOD1 mice. The results reported in Figure 6A show that *Pax7* mRNA levels are reduced in female and male G93A-SOD1 mice, at S stage compared to PS stage (***$p<0.001$). To evaluate whether this effect could be generally ascribed to myofiber denervation, we measured *Pax7* expression in two other conditions characterized by motoneuron loss: i) muscles derived from acutely axotomized NTg mice; ii) muscles derived from a mouse model of SBMA (spinal bulbar muscular atrophy, or Kennedy's disease), which represents another type of motoneuron disease sharing progressive denervation with ALS (La Spada et al., 1991; Lieberman et al., 2014). Figure 6B indicates that denervation due to axotomy (7 days) leads to a decrease of *Pax7* expression (** $p<0.01$). *Pax7* expression was increased in the muscle of SBMA mouse model at S stage (24 weeks) compared to age-matched NTg mice (Figure 6C; *** $p<0.001$).

Since in different muscle disorders, TGFB1 promotes an aberrant differentiation of myogenic cells to myofibroblasts inducing fibrosis (Burks and Cohn, 2011; Li et al., 2004), and muscular fibrosis is a pathological feature of ALS (Qi et al., 2015), we analyzed in G93A-SOD1 mice the mRNA expression of two extracellular matrix proteins, whose increase in expression represents a hallmark of fibrosis: collagen 1 alpha 1 (*Col1a1*) and fibronectin (*F*n). We found that in the muscle of NTg mice the expression of *Col1a1* decreases with age. In G93A-SOD1 mice *Col1a1* expression was further diminished at S stage as compared to PS stage and to age-matched NTg mice (Figure 7A; * $p<0.05$; **$p<0.01$; *** $p<0.001$). The expression of *Fn* was reduced in the muscle of both female and male G93A-SOD1 mice at S stage compared to PS stage and to age matched NTg mice (Fig. 7B; ** $p<0.01$, *** $p<0.001$). To determine whether the effect on *Col1a1* and *Fn* gene expression

in G93A-SOD1 is due to the presence of the mutated form of SOD1 or to denervation, we measured their expression in axotomized mouse muscle. Figure 7C and 7D show that denervation due to acute axotomy cause a dramatic increase in *Col1a1* and *Fn* expression (*p<0.05; **p<0.01), suggesting that the reduction in the expression of these two genes in muscle of G93A-SOD1 is not due to muscle denervation, but, probably, to the presence of the G93A-SOD1. To validate this hypothesis, we analysed the expression of *Col1a1* and *Fn* in C2C12 myoblasts transiently transfected with G93A-SOD1 (Galbiati et al., 2012; Onesto et al., 2011). *Tgfb1* mRNA levels were increased in C2C12 expressing G93A-SOD1 as compared to control myoblasts (pcDNA3) (Figure 7E; *p<0.05), and *Col1a1* and *Fn* mRNA levels were decreased (Figure 7F, and G; *p<0.05). No effect was detected in cells transfected with the wt-SOD1.

4. Discussion

In the last few years, it has become clear that ALS is a non-cell autonomous disease of motoneurons, and that other cell types are involved and modulate ALS onset and progression. An emerging body of evidence supports the idea that muscle cells could primarily contribute to G93A-SOD1-mediated toxicity (Crippa et al., 2013; Dobrowolny et al., 2008; Galbiati et al., 2012; Onesto et al., 2011; Wong and Martin, 2010). Gender is a risk factor for developing ALS (Manjaly et al., 2010). Consistent with this idea, our results show that, although TGFB1 is highly expressed in muscle at the S stage both in female and male mice, the increase of TGFB1 takes place earlier in male than in female (at the PS stage). Since *Tgfb1* is an androgen responsive gene (Singh et al., 2009), it is possible that the increased risk of ALS in males is linked to the higher serum levels of male sex steroids. The hypothesis of TGFB1 involvement in ALS is corroborated by the fact that its gene expression is also increased in the muscle of ALS patients. This result is in agreement with the increase of *TGFB3* in ALS patient muscle reported by other authors (Pradat et al., 2012). An inconsistency appear to be present between human and mice at the S stage. This may merely reflect the fact that G93A-SOD1 mice have an identical genetic background and a severe and constant

disease progression, while sALS patients are characterized by highly variable genetic background and their disease progression may be more directly affected by gender differences, since sex hormone levels greatly change throughout life. High TGFB1 plasma levels have been detected in ALS patients, with a significant positive correlation with disease duration (Houi et al., 2002; Ilzecka et al., 2002; Peters et al., 2017). On the contrary, the expression levels of endogenous *Tgfβ1* mRNA at the end stage negatively correlated with the survival of G93A-SOD1 mice (Endo et al., 2015). All these evidence indicate that a prolonged increase of TGFB1 levels might promote a more rapid disease progression and that TGFB1 could be a potential biomarker of ALS progression in males and females (Peters et al., 2017).

TGFB1 has a protective role in neurons (Krieglstein et al., 2002), and reduced neuronal TGFB signaling is implicated in age-dependent neurodegeneration (Tesseur et al., 2006). Gene expression analysis in the spinal cord of G93A-SOD1 mice showed decreased *Tgfb1* levels at the PS stage, possibly indicating a lack of neuroprotective effects in the early stages of disease, which may in turn contribute to the death of motoneurons at later stages. In ALS mouse spinal cord, *Tgfb1* mRNA increases with disease progression, a phenomenon that can be explained by the development of reactive astrogliosis (Phatnani et al., 2013). Indeed, astrocyte-derived TGFB1 accelerates ALS progression, while the same parameter is counteracted by the administration of a TGFB inhibitor (Endo et al., 2015). Furthermore, the effects of TGFB1 are also mediated by the inhibition of microglial activation with the consequent reduction of neuroprotective properties of microglial cells (Endo et al., 2015).

We found that *Tgfbr2* mRNA levels are increased in the spinal cord, but not in the muscle, of G93A-SOD1 mice at S stage. These results are in agreement with previous studies showing a high TGFBR2 immunoreactivity in spinal cord motoneurons of mice expressing G93A-SOD1, which correlates with reactive astrogliosis and disease progression (Phatnani et al., 2013). Furthermore, the tissue specific modulation of the *Tgfbr2* expression could also mediate different roles of TGFB1 in muscle and spinal cord. Increased levels of *Tgfbr2* in the spinal cord has been reported also by

other authors both in human and mouse spinal cord samples ((D'Arrigo et al., 2010; Kirby et al., 2011)

Different *Tgfbr2* expression levels might also correlate with dysregulation(s) of the intracellular pathway mediating TGFB effects. We found that *Smad2* expression in the spinal cord of ALS mice decreases along disease course, yet protein levels were unchanged. It is unclear which factors differentially regulated RNA (transcription, stability) and protein (translation, posttranslational modification, clearance) levels. In muscle *Smad2* mRNA were reduced and *Smad3* mRNA were increased with disease progression. Nontheless, SMAD2 and SMAD3 were increased at S stage. An increased *SMAD3* expression in patient muscle was reported also by Pradat and collaborators (Pradat et al., 2012). Although SMAD2 and SMAD3 are both receptor-activated SMADs, SMAD2 is unable to bind DNA directly, whereas SMAD3 binds DNA. Furthermore, in some systems SMAD2 and SMAD3 are antagonists, suggesting that they trigger different biological response (Labbe et al., 1998). Additionally, cultured hepatic cells turning to fibrogenic lineage show decreased SMAD2 expression accompanied by activation of SMAD3 (Dooley et al., 2000). Thus, SMAD2 and SMAD3 mediate different cellular responses, and this is also supported by data showing that *Smad2* knockout is lethal at the embryonic stage, whereas *Smad3* knockout mice survive to adulthood. Furthermore, to be imported into the nucleus phosphorylated SMAD3 associates with importin-beta in the cytoplasm, whereas phosphorylated SMAD2 is autonomously imported into the nucleus (Kurisaki et al., 2001). Nakamura et al. proposed that alteration of SMAD nuclear translocation may impair the neuroprotective TGFB signaling, leading to neurodegeneration (Nakamura et al., 2008). SMAD4 is the protein that shuttles receptor regulated SMADs into the nucleus, even if SMAD4-independent effects of TGFB have been described (Hocevar et al., 1999). In line with the work of Saris et al., we found decreased *Smad4* expression both in spinal cord and muscle, suggesting a further site of dysregulation of TGFB intracellular signaling (Saris et al., 2013).

An excessive TGFB1 has been proposed to be a harmful factor in muscle (Burks and Cohn, 2011). TGFB profoundly influences the differentiation of myoblasts by inhibiting the induction of muscle-specific gene expression and myotube formation (Liu et al., 2001). TGFB1 targets one or more master regulators of myogenesis, such as PAX7. Indeed, impaired regenerative capacity concomitant with a progressive loss of satellite cells has been reported in *Pax7*-null mice (von Maltzahn et al., 2013). Notably, we found that *Pax7* expression is decreased in the muscle of ALS mice at S stage, suggesting that the myogenic potential and the ability for tissue repair of ALS muscle is impaired. This result is in agreement with a previous work on G93A-SOD1 mice (Calvo et al., 2012), and has been confirmed in patient biopsies showing a reduction in mRNA levels of markers of satellite cells and early myogenesis (Jensen et al., 2016). In agreement, denervation induced by axotomy diminished *Pax7* mRNA levels. On the contrary, *Pax7* gene expression was increased in muscle of SBMA mouse model. SBMA is another type of motoneuron pathology sharing some features with ALS (La Spada et al., 1991; Lieberman et al., 2014), and it is characterized by progressive death of motoneuron and denervation. This result indicates that the reduction of *Pax7* in the ALS muscle may be due to a specific action of G93A-SOD1 rather than to denervation alone, and could lead to an impairment of muscle regeneration. TGFB1 promotes an aberrant differentiation of myogenic cells to myofibroblasts and induces fibrosis (Burks and Cohn, 2011; Li et al., 2004), which is a pathological feature of ALS (Qi et al., 2015). In fact, FN and COL1 protein levels were found greatly increased in muscle of symptomatic ALS mice (Gonzalez et al., 2017). *Col1a1* gene expression was age-dependent in NTg mice, in line with the normal age-related rearrangement of skeletal muscle fibers and of the connective tissue network (Trappe, 2009). In contrast to increased protein levels, in ALS mice we found that *Col1a1* and *Fn* mRNA levels were much lower in symptomatic mice. This effect was not due to denervation, since the expression of these two extracellular matrix proteins is highly increased in axotomized muscle. On the other hand, *Col1a1* and *Fn* mRNA reduction could be due to the decline in the ability to move of mice during disease progression, since poor mobility downregulates total muscular collagen synthesis

(Ahtikoski et al., 2001). Furthermore, in C2C12 myoblasts the expression of G93A-SOD1 led to decreased levels of *Col1a1* and *Fn* mRNA, indicating that mutant SOD1 could be directly responsible for modulation of *Col1a1* and *Fn* gene expression. It is possible that the decrease in collagen expression is an attempt to prevent collagen accumulation that leads to fibrosis; a similar mechanism has been proposed in hepatic cells (Cao et al., 2002). TGFβ promotes *Col1a1* transcription through specific responsive sequences (Sato et al., 2004). Therefore it remains to understand why the levels of *Col1a1* mRNA and *Fn* are reduced in the presence of high levels of TGFB1. A possible explanation comes from a work demonstrating that high doses of TGFB potently suppress *Col1a1* transcription via the transcription factor CUX1 (Fragiadaki et al., 2011). However, also other Authors reported decreased muscle *Col1a1* and *FN* expression respectively in G86R-SOD1 mice or ALS patient (Gonzalez de Aguilar et al., 2008; Pradat et al., 2012).

5. Conclusions

The data here reported indicate that TGFB1 signaling pathway is altered in ALS spinal cord and muscle. In the spinal cord, the PS reduction of *Tgfb1* expression could reflect a lack of its neuroprotective effect. In muscle, the higher levels of TGFB1 could contribute to muscular atrophy since this correlates with an altered *Pax7* expression, and changes in extracellular matrix production. Androgen sensitivity of *Tgfb1* and its higher levels in males could also be a possible explanation of their higher risk of developing ALS. All together, these data increase the possibilities of new and complementary therapies to treat the disease or, at least, to delay muscle wasting.

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7. Authors' contributions

MG and AP designed and wrote the manuscript. MM performed the experiments; VC, RC and PR assisted in data analysis and interpretation; MEC, VF, GV, BT helped in data collection. EM and MPi critically revised the manuscript and the figures. MPe provided ALS mice tissues and revised manuscript. GS provided RNA from patient muscle biopsies. All authors have provided final approval of the manuscript.

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Figure Legends

Figure 1: TGFB1 levels in ALS tissues.

A,B) RT-qPCR analyses of *Tgfb1* mRNA performed on total RNA extracted from spinal cord (A) or quadriceps muscles (B) of female and male mice [NTg (non-transgenic), Tg expressing either the wild type human SOD1 transgene (wt-SOD1) or the G93A mutant form of human SOD1 (G93A-SOD1)], at 8 and 16 weeks of age. Data have been normalized to *Rplp0* mRNA, expressed relative to the levels determined in female NTg mice at 8 weeks of age, which are taken as internal reference, and expressed as fold changes. Each bar represents the mean ± s.e.m. of four independent replicates. *p<0.05; **p<0.01; ***p<0.001. C,D) Representative immunoblot and densitometric analysis of TGFB1 and GAPDH proteins extracted from spinal cords (C) or quadriceps muscles (D;) of female and male NTg, and G93A-SOD1mice, at 8 or 16 weeks of age. GAPDH was used to normalize protein loading. Each bar represents the mean ± s.e.m. of independent replicates (three for spinal cord and four for quadriceps). *p<0.05, **p<0.01, ***p<0.001. E) RT-qPCR analyses of *TGFB1* mRNA levels from muscle biopsies of male and female human controls and ALS patients. Data have been normalized to *RPLP0* mRNA, expressed relative to the levels determined in female controls, and expressed as fold changes. Disease effect p=0.0003; gender effect p=0.0196; *p<0.05; **p<0.01. Statistical analysis has been performed through two-way ANOVA for group comparisons followed by Bonferroni *post-hoc* test.

Figure 2: *Tgfbr2* mRNA is upregulated in the spinal cord of ALS mice.

RT-qPCR analyses of *Tgfbr2* mRNA performed on total RNA extracted from spinal cord (A) and quadriceps muscle (B) of female and male mice [NTg (non-transgenic), and Tg mice expressing the G93A mutant form of human SOD1 (G93A-SOD1)], at 8 (presymptomatic stage) and 16 (symptomatic stage) weeks of age. Data have been normalized to *Rplp0* mRNA, expressed relative to the levels determined in female NTg mice at 8 weeks of age, which are taken as internal

reference and expressed as fold changes. Each bar represents the mean ± s.e.m. of four independent replicates. *p<0.05, **p<0.01. Statistical analysis has been performed through two-way ANOVA for group comparisons followed by Bonferroni *post-hoc* test.

Figure 3: SMAD levels in ALS tissues are dysregulated.

RT-qPCR analyses of the mRNA levels of *Smads*2 and 3 performed on total RNA extracted from spinal cord (A,B) or quadriceps muscles (C,D) of female and male mice [NTg (non-transgenic), Tg expressing the G93A mutant form of human SOD1 (G93A-SOD1)], at 8 (presymptomatic stage) or 16 (symptomatic stage) weeks of age. Data have been normalized to *Rplp0* mRNA, expressed relative to the levels determined in female NTg mice at 8 weeks of age, which are taken as internal reference, and expressed as fold changes. Each bar represents the mean ± s.e.m. of four independent replicates (*p<0.05, **p<0.01). E - K) Representative immunoblot and densitometric analyses of SMAD2 and 3. Proteins were extracted from spinal cords or quadriceps muscles of female and male NTg, and G93A-SOD1mice, at 8 or 16 weeks of age. GAPDH was used to normalize protein loading. Each bar represents the mean ± s.e.m. of independent replicates (three for spinal cord and four for quadriceps). Images have been cropped from different parts of the same gel, and full-length blots are included in the Supplementary Figure 1. *p<0.05; **p<0.01.

Statistical analysis has been performed through two-way ANOVA for group comparisons followed by Bonferroni *post-hoc* test.

Figure 4: *Smad4* mRNA levels are decreased in ALS muscle.

RT-qPCR analyses of *Smad4* mRNA performed on total RNA extracted from spinal cord (A) or quadriceps muscles (B) of female and male mice [NTg (non-transgenic), and Tg expressing the G93A mutant form of human SOD1 (G93A-SOD1)], at 8 (presymptomatic stage) or 16 (symptomatic stage) weeks of age. Data have been normalized to *Rplp0* mRNA, expressed relative

to the levels determined in female NTg mice at 8 weeks of age, which are taken as internal reference, and expressed as fold changes. Each bar represents the mean ± s.e.m. of four independent replicates. * p<0.05, **p<0.01, ***p<0.001. Statistical analysis has been performed through two-way ANOVA for group comparisons followed by Bonferroni *post-hoc* test.

Figure 5: TGFB1 signaling in muscle of ALS patients.

RT-qPCR analyses of the mRNA levels for: *TGFBR2* (A); *SMAD2* (B); *SMAD3* (C); *SMAD4* (D) in muscle biopsies of male and female human controls and ALS patients. Data have been normalized to *RPLP0* mRNA, expressed relative to the levels determined in female controls, and expressed as fold changes. Statistical analysis has been performed through two-way ANOVA for group comparisons followed by Bonferroni *post-hoc* test. Gender effect for *SMAD2* mRNA: ***p<0.001. Gender effect for Smad3: **p<0.01. Disease effect in female: **p<0.01.

Figure 6: *Pax7* mRNA expression in different models of denervated muscles.

RT-qPCR analyses of the *Pax7* mRNA levels performed on total RNA extracted from: (A) quadriceps muscles of female and male mice [NTg (non-transgenic), and transgenic expressing the G93A mutant form of human SOD1 (G93A-SOD1)], at 8 (presymptomatic stage) or 16 (symptomatic stage) weeks of age. Data have been normalized to of *Rplp0* mRNA, expressed relative to the levels determined in female NTg mice at 8 weeks of age, which are taken as internal reference, and expressed as fold changes. Each bar represents the mean ± s.e.m. of four independent replicates. **p<0.01,***p<0.001. (B) gastrocnemius muscles of left axotomized non-transgenic mice (Axo). Right gastrocnemius muscles of the same animals were used as controls (Ctrl). Data have been normalized to the amount of *Rplp0* mRNA, expressed relative to the levels determined in control muscles, which are taken as internal reference, and expressed as fold changes. **p<0.01. (C) quadriceps muscles of male non-transgenic (WT) mice, and of Knock In AR113Q (SBMA)

mice at 24 weeks of age (corresponding to symptomatic stage). Animals were age-matched. Data have been normalized to the amount of *Rplp0* mRNA, expressed relative to the levels determined in WT mice taken as internal reference, and expressed as fold changes. ***p<0.001. Statistical analysis has been performed through two-way ANOVA for group comparisons followed by Bonferroni *post-hoc* test.

Figure 7: Changes in *Col1a1* and *Fn* mRNA levels.

(A,B) RT-qPCR analyses of *Col1a1* and *Fn* mRNA levels performed on total RNA extracted from quadriceps muscles of female and male mice [NTg (non-transgenic), and transgenic expressing the G93A mutant form of human SOD1 (G93A-SOD1)], at 8 (presymptomatic stage) or 16 (symptomatic stage) weeks of age. Data have been normalized to the amount of *Rplp0* mRNA, expressed relative to the levels determined in female NTg mice at 8 weeks of age, which are taken as internal reference, and expressed as fold changes. Each bar represents the mean ± s.e.m. of four independent replicates. *p<0.05, **p<0.01, ***p<0.001. (C,D) RT-qPCR analyses of *Col1α1* and *Fn* mRNA levels performed on total RNA extracted from gastrocnemius muscles of left axotomized NTg mice (Axo). Right gastrocnemius muscles of the same animals were used as controls (Ctrl). Data have been normalized to the amount of *Rplp0* mRNA, expressed relative to the levels determined in control muscles, which are taken as internal reference, and expressed as fold changes. *p<0.05, **p<0.01. (E,F,G) RT-qPCR analyses of *Tgfb1*, *Col1a*1, and *Fn* mRNAs, performed on total RNA extracted from C2C12 cells transfected with wild type human SOD1 (wt-SOD1) or G93A mutant form of human SOD1 (G93A-SOD1). Data have been normalized to the amount of *Rplp0* mRNA, expressed relative to the levels determined in control cells (mock transfected, pcDNA3), which are taken as internal reference, and expressed as fold changes. Each bar represents the mean ± s.e.m. of four independent replicates. *p<0.05. Statistical analysis has been performed through two-way ANOVA for group comparisons followed by Bonferroni *post-hoc* test.

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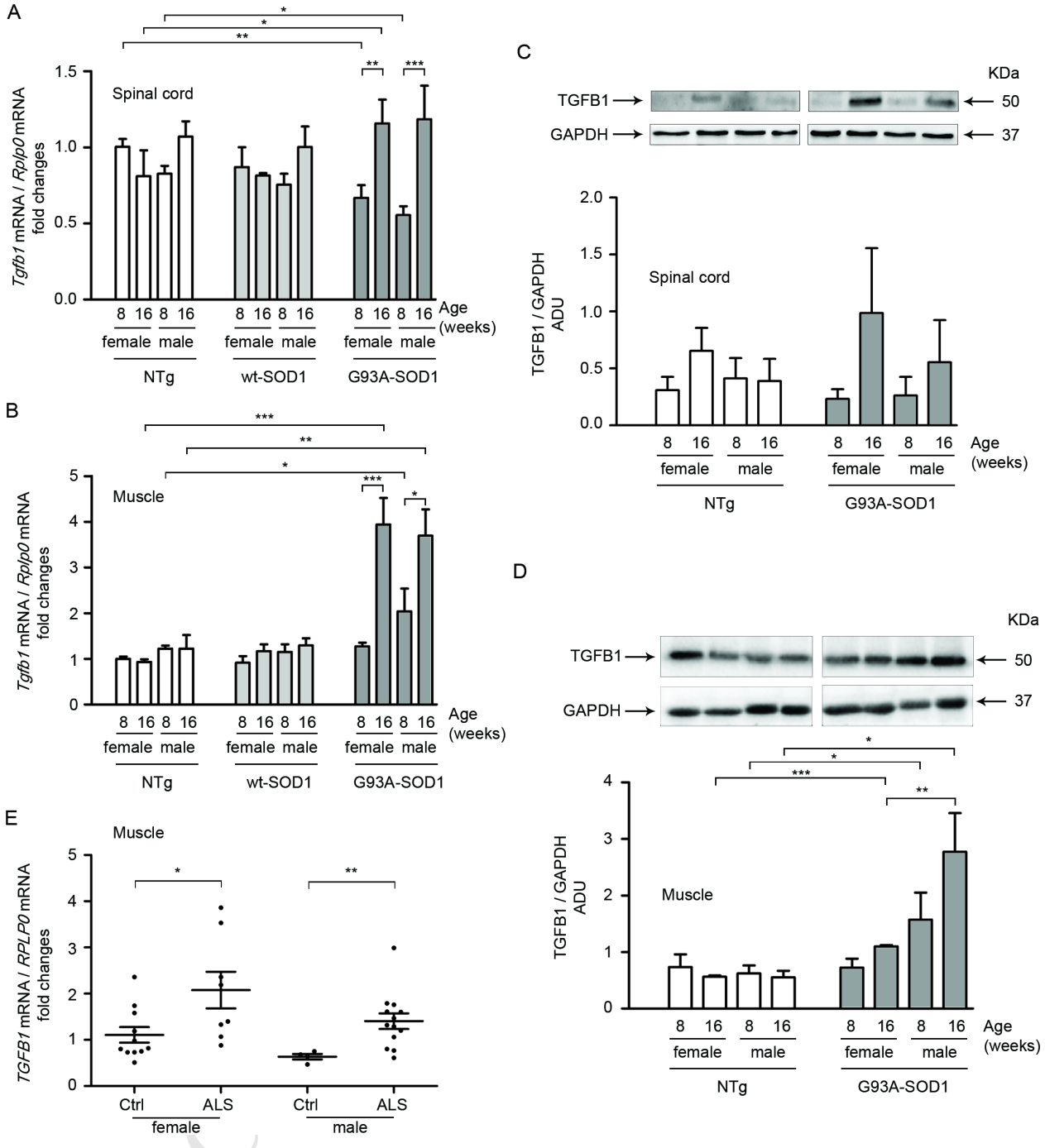
Milan, 28-02-2019

To the Editor of Neurobiology of Aging

On behalf of all the authors, I, the undersigned corresponding author of the manuscript entitled:
"**Transforming Growth Factor beta 1 signaling is altered in the spinal cord and muscle of amyotrophic lateral sclerosis mice and patients.**" by Meroni M, Crippa V, Cristofani R, Rusmini P, Cicardi M, Messi E, Piccolella M, Tedesco B, Ferrari V, Sorarù G, Pennuto M, Poletti A, Galbiati M
hereby declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.
I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of them.
I further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.
I confirm that the data contained in the manuscript being submitted have not been previously published, have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging.

Mariarita Galbiati


A
Spinal cord
Tgfb1 mRNA / Rplp0 mRNA fold changes
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0.5
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Age (weeks)
female male female male female male
NTg wt-SOD1 G93A-SOD1
B
Muscle
Tgfb1 mRNA / Rplp0 mRNA fold changes
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Age (weeks)
female male female male female male
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GAPDH
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Spinal cord
TGFB1 / GAPDH ADU
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Age (weeks)
female male female male
NTg G93A-SOD1
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KDa
TGFB1
GAPDH
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Muscle
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8 16 8 16 8 16 8 16
Age (weeks)
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NTg G93A-SOD1
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Muscle
TGFB1 mRNA / RPLP0 mRNA fold changes
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female male