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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1668959> since 2018-05-24T17:49:19Z

Published version:

DOI:10.1016/j.neuroscience.2018.03.023

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TLQP peptides in Amyotrophic Lateral Sclerosis: possible blood biomarkers with a neuroprotective role

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ABSTRACT

While the VGF-derived TLQP peptides have been shown to prevent neuronal apoptosis, and to act on synaptic strengthening, their involvement in Amyotrophic Lateral Sclerosis (ALS) remains unclarified. We studied human ALS patients' plasma (taken at early to late disease stages) and primary fibroblast cultures (patients vs controls), in parallel with SOD1-G93A transgenic mice (taken at pre-, early- and late symptomatic stages) and the mouse motor neuron cell line (NSC-34) treated with Sodium Arsenite (SA) to induce oxidative stress. TLQP peptides were measured by enzyme-linked immunosorbent assay, in parallel with gel chromatography characterization, while their localization were studied by immunohistochemistry. In controls, TLQP peptides, including forms compatible with TLQP-21 and -62, were revealed in plasma and spinal cord motor neurons, as well as in fibroblasts and NSC-34 cells. TLQP peptides were reduced in ALS patients' plasma starting in the early disease stage (14% of controls) and remaining so at the late stage (16% of controls). In mice, a comparable pattern of reduction was shown (vs wild type), in both plasma and spinal cord already in the pre-symptomatic phase (about 26% and 70%, respectively). Similarly, the levels of TLQP peptides were reduced in ALS fibroblasts (31% of controls) and in the NSC-34 treated with Sodium Arsenite (53% of decrease), however, the exogeneous TLQP-21 improved cell viability (SA treated cells with TLQP-21, vs SA treated cells only: about 83% vs. 75%). Hence, TLQP peptides, reduced upon oxidative stress, are suggested as blood biomarkers, while TLQP-21 exerts a neuroprotective activity.

Keywords:

TLQP-peptides, neurodegeneration, ALS, motor neurons, NSC-34 cells, human fibroblasts

LIST OF ABBREVIATIONS

ALS: Amyotrophic Lateral Sclerosis

ALSFRS-R: ALS Functional Rating Scale-Revised

C3AR: complement component 3a receptor 1

gC1q-R: receptor for the globular heads of c1q

DMEM: Dulbecco's modified Eagle's medium

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NSC-34: mouse motor neuron-like hybrid cell line

PBS: phosphate buffer saline

PIC: protease inhibitor cocktail

PFA: paraformaldehyde

SA: Sodium Arsenite

SOD1: superoxide dismutase 1

VACht: vesicular acetylcholine transporter

TARDBP: TAR DNA Binding Protein

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease characterized by neuronal degeneration in frontal cortex, brainstem and spinal cord. Virtually all muscles are gradually affected, with difficulties in speaking, swallowing and breathing, and death ensues 3 to 5 years after appearance of the first symptoms. Currently, no treatment is effective in stopping the progression of the disease, nor is any early diagnostic test available. While the aetiology of ALS is unknown, mutations of the superoxide dismutase 1 (SOD1) gene, or of the TARDBP (TAR DNA Binding Protein) gene have been hypothesized as common causes (Chiò et al., 2011; Zarei et al., 2015). In fact, oxidative stress, characterized by an altered equilibrium between the production of reactive oxygen species (free radicals) and antioxidant reactions, has been related to motor neuron degeneration in ALS (Bergeron, 1995; Robberecht, 2000). TLQP peptides are a family of peptides derived from the VGF (non acronymic) precursor protein, some of these originally identified in rat brain (Trani et al., 2002). They share a common N-terminal "TLQP" (Thr-Leu-Gln-Pro) amino acid sequence, are cleaved from the primary VGF product at the specific R-P-R (Arg-Pro-Arg) processing site found at rat/mouse VGF₅₅₃₋₅₅₅ (VGF₅₅₁₋₅₅₃ in human), and variably extend to the VGF precursor C-terminus (Brancia et al., 2010). In the brain, TLQP peptides appear to show a restricted localization compared to other VGF derived peptides, including a subpopulation of hypothalamic neurons projecting to a discrete area of median eminence (Brancia et al., 2010; Noli et al., 2014). Recently, a differential expression of several TLQP peptides was reported in the Syberian hamster brain. Namely, TLQP-21 (21 amino acid in length, rat VGF₅₅₆₋₅₇₆) was well represented in both hypothalamus and cortex while the longer form TLQP-62 (rat VGF₅₅₆₋₆₁₇ encompassing the VGF precursor's C-terminus) was abundant in cortex, and less expressed in hypothalamus (Noli et al., 2015). TLQP peptides were also found in hypothalamic-pituitary axis and plasma, differently expressed during the oestrous cycle phases (Noli et al., 2014) as well as in several peripheral locations including adrenal and stomach, changing in condition of stress and upon fasting, respectively (D'Amato et al., 2008; Brancia et al., 2010). Additional molecular forms compatible with predicted TLQP-30 and TLQP-42 peptides were revealed in certain endocrine organs (Cocco et al., 2007, Brancia et al., 2010) but have not been further studied so far. In human plasma, TLQP peptides were upregulated upon hyperglycaemia, and down-regulated in obese subjects (D'Amato et al., 2015). As to bioactivity and possible role/s, TLQP-21 has been shown to be involved in the regulation of metabolic mechanisms (Bartolomucci et al., 2006; Jethwa et al., 2007; Lewis et al., 2017),

reproduction (Aguilar et al., 2013; Noli et al., 2014), chronic stress (Razzoli et al., 2012) and inflammatory pain (Rizzi et al., 2008). The same peptide prevented apoptosis of rat cerebellar granules upon serum and potassium deprivation, with modulation of kinase phosphorylation (Severini et al., 2008). Also, it protected human umbilical vein endothelial cells against high-glucose-induced apoptosis, by enhancing glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate dehydrogenase, hence reducing reactive oxygen species (Zhang et al., 2013). Two receptor molecules have been identified for TLQP-21, namely the complement component 3a receptor (C3a-R: Hannedouche et al., 2013; Cero et al., 2014; 2016;) and the receptor for the globular heads of c1q (gC1q-R: Chen et al., 2013) and involved, with TLQP-21, in modulating lipolysis (Cero et al., 2016) and neuropathic pain (Chen et al., 2013), respectively. While the precise mechanisms involved are not entirely known, there is strong evidence that the TLQP-21 may act increasing intracellular calcium in Chinese hamster ovary cells (Cassina et al., 2013), microglia (Chen et al., 2013) and cerebellum (Severini et al., 2008). The longer form TLQP-62 has been widely investigated in hippocampus where it enhances synaptogenesis (Behnke et al., 2017), regulates memory formation, and induces both synaptic potentiation (Bozdagi et al., 2008; Lin et al., 2015) and neurogenesis (Takker-Varia et al., 2014). It can also cause dorsal horn cell hyper-excitability and behavioral hypersensitivity in rats (Moss et al., 2008). No specific receptor has been identified so far for TLQP-62. In ALS, despite the reported evidence that VGF expression is modulated in the animal model and humans (Pasinetti et al., 2006; Zhao et al., 2008), limited information is available regarding TLQP peptides. We have previously reported the involvement of the VGF C-terminal peptides in ALS, modulated in the SOD1 mutant mice and patient's plasma, but exclusively at the final disease phase (Brancia et al., 2016). Afterwards, we aimed at specifically investigating the role of the TLQP peptides in ALS, by studying their expression and changes (using ELISA and immunohistochemistry) in transgenic mice (SOD1-G93A) and the mouse motor neuron-like hybrid cell line (NSC-34), as experimental models. In parallel, we also investigated, by ELISA, ALS patients' plasma and primary fibroblast cultures, the latter being considered a good cellular model used in human ALS research (Sabatelli et al., 2015; Yang et al., 2015) and also, contain VGF (Brancia et al., 2016). Moreover, in the NSC-34 cells, the neuroprotective role of the TLQP-21 was addressed in parallel with the presence of its two known receptors (gC1q-R and C3a-R), examined by both western blot and immunocytochemistry.

EXPERIMENTAL PROCEDURES

Human subjects

Subjects of Sardinian descent were studied, including ALS patients (females: n=20, males: n=24, age range: 25–85 yrs), and age-matched controls (unaffected by either neurological conditions, or diabetes; females: n=20, males: n=26). In patients, ALS related mutations were studied as follows: exon 6 of the TARDBP gene, and all five coding exons of the SOD1 gene were screened by polymerase chain reaction and sequenced using the Big-Dye Terminator v3.1 kit (Applied Biosystems Inc) and an ABI Prism 3130 Genetic Analyzer. A repeat-primed polymerase chain reaction assay was used to screen for the GGGGCC hexanucleotide expansion in the first intron of C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011). ALS patients studied showed either of: TARDBP-A382T mutation (n=16), SOD1-G93A mutation (n=3); expansion in the C9ORF72 gene (n=5), or no identifiable ALS-related mutation (n=20). The patients' motor and functional (which incorporates additional assessments of dyspnea, orthopnea, and the need for ventilatory support) performance was assessed at the time of blood sampling, by at least two experienced neurologists, according to the ALS Functional Rating Scale Revised (ALSFRS-R: Cedarbaum et al., 1999). Patients' data (summarised in Appendix A: Table 1A), including: age, gender, genetic mutation, ALSFRS-R score and co-morbidity at the time of blood sampling, as well as their clinical condition one year later (whether alive, or not, with or without tracheostomy). On the latter basis, patients were assigned to either of group I, "early disease stage" (n=25): patients who were alive and free of tracheostomy one year after blood sampling; or group II, "late disease stage" (n=19): patients who were deceased, or had undergone tracheostomy. The present study was approved by the Ethical Committee at the Cagliari AOU ("Azienda Ospedaliero Universitaria di Cagliari"), protocol n. 450/09/C.E. All patients provided their written informed consent to be part of the study according to the Italian legislation.

Human samples

Blood samples were collected with ethylenediaminetetraacetic acid (EDTA, 1.5 mg/ml), rapidly centrifuged (14,000 rpm, 5 min), hence plasma was aliquotted and stored frozen (at -80°C). Fibroblast primary cultures were set up using skin biopsies (taken under local anaesthesia) obtained from "late disease stage" ALS patients (n=4: two showed a heterozygous missense TARDBP-A382T mutation, two showed no identifiable ALS-

related mutation), and age-matched controls (n=3). Cultures were grown as previously reported (Orrù et al., 2016), using high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (20% vol/vol) and penicillin/streptomycin (10 ml/L of: 10,000 U penicillin + 10 mg/ml streptomycin in 150 mmol/L NaCl). Oxidative stress was induced adding sodium Arsenite (SA) to the culture medium (0.5 mmol/L, for 60 min). For ELISA, cultures were expanded, and four culture plates per patient (or control), and per treatment (SA, or no treatment), were separately extracted in phosphate buffer saline (PBS: 0.01 mol/L PO₄, pH 7.2, 0.15 mol/L NaCl) containing a protease inhibitor cocktail (PIC, Sigma-Aldrich P8340, 10 ml/g tissue). Remaining extracts (from controls' explants) were pooled and used for gel chromatography.

For immunocytochemistry, cells were grown on coverlips, and at least three coverlips (per patient/control, and per treatment) were fixed in paraformaldehyde (PFA: 40 g/L in PBS, 15 min), permeabilized with cold methanol (5 min), hence Triton X-100 (20 ml/L in PBS, 20 min), and rinsed with PBS. Coverslips were immunostained for TLQP peptides, and with an HuR antibody (Santa Cruz, Antibody Registry: AB627770, raised in mouse, 1:500) to label stress granules, while nuclei were counterstained with bisBenzimide (Hoechst 33342, 0.5 ug/ml in PBS).

SOD1-G93A mice

Transgenic B6SJL-TgN(SOD1-G93A)1Gur mice were used, over-expressing human SOD1 containing the Gly₉₃ to Ala mutation (Jackson Laboratory, Bar Harbor, ME; stock number 002726; housed at the University of Turin animal house facilities). All experimental procedures on live animals were carried out in accordance with the European Communities Council Directive 86/609/EEC (November 24, 1986), and the Italian Ministry of Health and University of Turin institutional guidelines on animal welfare (law 116/92 on Care and Protection of living animals undergoing experimental or other scientific procedures; authorization number 17/2010-B, June 30, 2010). The Ethical Committee at the University of Turin approved the study. All possible efforts were made to minimize the number of animals used and their suffering. Animal genotyping and behavioral testing used to assess disease progression (neurological test, rotarod and paw grip endurance tests) were previously described in detail (Boido et al., 2014). Transgenic animals were grouped according to age (days postnatal: P) and stage of motor dysfunctions (Brancia et al., 2016), as follows: (i) pre-symptomatic (around P45); (ii) early-symptomatic (around P90; two repeated deficits for two consecutive times); (iii) late-symptomatic (around P120; $\geq 20\%$ weight loss and inability to perform tests). Age-

matched wild type mice were used as controls: groups (iv) through (vi), respectively. Male mice were used in all cases, in view of our previous finding of TLQP modulations in female rodents along the oestrous cycle (Noli et al., 2014).

For ELISA and gel chromatography, animals (n=7 per group) were anaesthetized (3% isoflurane vaporized in O₂/N₂O 50:50), hence blood was drawn by cardiac puncture, collected in EDTA containing tubes (1.78 mg/ml), and centrifuged (11,000 rpm, 5 min). Plasma was aliquotted and stored frozen (-80°C). Upon blood sampling, animals were euthanized by cervical dislocation, and spinal cords were dissected. Tissues were coarsely minced with a scalpel, collected in tubes with ice-cold PBS containing PIC (10 ml/g tissue), treated with an Ultra-Turrax tissue homogenizer (Ika-Werke, Staufen, Germany, 3 min), hence tubes were heated in a vigorously boiling water bath (10 min), and centrifuged (3,000 rpm, 15 min). Supernatants were stored frozen until use (-20°C). For immunohistochemistry, mice (n=3 per group) were anaesthetized as above, and perfused transcardially with PFA (10 min). The whole spinal cord was removed and post-fixed in PFA (at 4°C, 2h). Cryosections of cervical and lumbar spinal cord (8 µm thickness) were immunostained (in single and double immunofluorescence) for TLQP peptides, and with an antibody to vesicular acetylcholine transporter (VACht, BIOMOL Research lab, Antibody Registry: AB2052813, raised in goat, 1:400) to label cholinergic motor neurons.

NSC-34 cells and oxidative stress

Cells were grown in high-glucose DMEM, supplemented with fetal bovine serum (10% vol/vol) and penicillin/streptomycin (as for fibroblasts, see above). For testing, cells were plated at a 50,000/ml density in 24-well plates (24h), hence underwent oxidative stress by addition of SA (0.5 mmol/L in culture medium, 60 min at 37°C) and measured the levels of TLQP-21, NERP-1, NAPP- and APGH-peptides, as well as VGF N-terminus and C- terminus. The effect of TLQP-21, NERP-1 (synthetic, custom produced for us by CPC Scientific, Sunnyvale, CA) was assessed by addition to the culture media, in the presence / absence of SA, at a range of concentrations (0.1 to 10 nmol/ml). Cell proliferation and viability was assessed used a colorimetric method based on the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent (MTT test, Sigma-Aldrich), according to the manufacturer's protocol. Absorbance was measured at 570nm (EnVision plate analyzer, Perkin Elmer, Milan, Italy). For ELISA and gel chromatography, cells preparations were extracted with PBS containing PIC (10 ml/g tissue), as described for fibroblasts. For immunocytochemistry, preparations grown on glass coverslips were fixed

with PFA (10 min), permeabilized with cold methanol (5 min), then with Triton X-100 (10 ml/L in PBS, 20 min), and rinsed in PBS. Coverslips were immunostained for TLQP peptides, and for the TLQP-21 receptors: gC1q-R (Abcam, Antibody Registry: AB10675815, raised in rabbit, 1:600), and C3a-R (Abcam, Antibody Registry: AB2687440, raised in rabbit, 1:200–1000). A HuR antibody (Santa Cruz, Antibody Registry: AB627770, raised in mouse, 1:500), to label stress granules, and a calnexin antibody (Sigma-Aldrich, Antibody Registry: AB2069152, raised in mouse, 1:300), to label endoplasmic reticulum (ER), were used in double-immunostaining with the TLQP antiserum. Cell nuclei were counterstained with bisBenzimide (Hoechst 33342, 0.5 ug/ml).

TLQP peptide/s antiserum

The guinea-pig primary antiserum to TLQP peptides, specific for their common N-terminal portion, previously described in detail (Brancia et al., 2005) was extensively used in different organs and tissues (Cocco et al., 2007, D'Amato et al., 2008; Brancia C et al., 2010; Noli et al., 2014; D'Amato et al., 2015; Noli et al., 2017). Briefly, a synthetic peptide corresponding to rat VGF_{556–564}, with the addition of a C-terminal cysteine residue, was conjugated *via* its C-terminus to keyhole limpet hemocyanin (KLH), and used for immunizations. See ELISA and immunohistochemistry sections (below), for specificity controls relevant to its use in each method.

ELISA

Competitive ELISA was carried out as previously described in detail (Cocco et al., 2007), and the characterization of the TLQP assay are summarized in Table 1, while calibration curve is shown in Fig.1. Briefly, multiwell plates (Nunc Thermoscientific, Milan, Italy) were coated with the relevant synthetic peptide, hence treated with PBS containing normal donkey serum (90 ml/L), aprotinin (20 nmol/L), and EDTA (1 g/L) for 2 hours. Primary incubations (3h) were carried out in duplicate, using TLQP (1:5k), NERP-1 (1:160k; Cocco et al., 2007), NAPP (1:100k; D'Amato et al., 2015) as well as N-terminus and C-terminus (both 1:12k; Cocco et al., 2010) antisera followed by biotinylated secondary antibodies (Jackson, West Grove, PA, Antibody registry: AB2340451; 1:10K, 1h), streptavidin-peroxidase conjugate (Biospa, Milan, Italy, 30 min), and tetramethylbenzidine substrate (TMB X-traKem-En-Tec, Taastrup, Denmark, 100 ml/well). Reaction was stopped with HCl (1 mol/L) and optical density was measured at 450nm using a multilabel plate reader (Chameleon: Hidex, Turku, Finland). Recovery of synthetic

peptide (same used for immunization, plate coating and measurement standard) added to plasma, or to tissue samples at extraction was >85%.

Gel chromatography

Plasma samples (human: 2 ml, or a pool from control mice: 1 ml), as well as extracts of spinal cord (pooled from control mice: 1.6 ml), NSC-34 cells (2 ml) and fibroblasts (1.2 ml) were individually loaded onto a Sephadex G-50S column (Sigma-Aldrich, 2 cm² x 1m). The column was equilibrated with ammonium bicarbonate solution (50 mmol/L in H₂O) and eluted with the same buffer (about 0.3 ml/min, using a membrane pump running at 5 impulses/min, -4°C). The column was calibrated using a kit (MVG70, Sigma-Aldrich) containing the following molecular weight markers: bovine albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa). Collected fractions (3 ml) were reduced in volume using a Vacufuge Concentrator (Eppendorf, Milan, Italy) and assessed by ELISA. Experiments were carried out in duplicate, or in triplicate, depending on sample availability. Overall recovery of loaded immunoreactive material/s resulted in an 81–102% range.

Detection of TLQP peptides in tissues and cells

TLQP antiserum (1:600 to 1:800) were diluted with PBS containing normal donkey serum (30 ml/L, from a pool of >5 animals) and NaN₃ (0.5 g/L), with the addition of normal mouse serum (30 ml/L: mouse tissue sections only). Primary incubations were carried out at room temperature, either overnight (16h: mouse spinal cord sections), or for 4 h (fibroblast and NSC-34 cell cultures). For double-labelling, the relevant primary antibodies were mixed and similarly incubated (see specific part). Sites of primary immune reaction were revealed using secondary IgG preparations absorbed against serum proteins from multiple species (Jackson ImmunoResearch Laboratories, West Grove, PA), at a 2 to 10 mg/L concentration (in PBS, 1h at room temperature). As appropriate, either of the following were used (1:200-300): (a) Cyanin 3.18 conjugated donkey anti-guinea pig IgG (Antibody registry:AB2340460), (b) Cyanin 3.18, or Alexa488 conjugated anti-mouse IgG (Antibody registry: AB2340460 and AB2341099, respectively), (c) a mixture of “a” and “b” above, (d) Cy3 conjugated anti-rabbit IgG (Antibody registry: AB 2307443) (e) Alexa488 conjugated donkey anti-sheep IgG (Antibody registry: AB 2340754). Routine controls included: substitution of each antibody layer, in turn, with PBS; the use of pre-immune, or non-immune sera; the use of inappropriate secondary antibodies. Pre-absorption of the TLQP antiserum with the

relevant (unconjugated) peptide (up to 100 mmol/L) resulted in virtually complete prevention of the corresponding labelling. Slides were coverslipped, and culture coverslips were mounted on slides, using Glycerol-PBS (1:2) containing NaN₃ (0.2 g/L). Preparations were observed and photographed using a BX51 fluorescence microscope (Olympus, Milan, Italy) equipped with a Fuji S3 Pro digital camera (Fujifilm, Milan, Italy).

Western blot of TLQP-21 receptors

NSC-43 cell preparations were lysed in 2% sodium dodecyl sulfate (20g/L, min), and a sample (10 ul) was set aside to assess protein concentration (BCA assay, Thermo Scientific). Loading buffer (75 mmol/L tris-hydrogen chloride buffer, pH 6.8, containing 200 ml/L glycerol, 0.4g/L% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue) was added to the extract, hence tubes were heated in a vigorously boiling water bath (3 min). Samples (containing about 15 µg proteins each) were loaded onto 4–20% gradient SDS (sodium dodecyl sulphate)-polyacrylamide gels and blotted onto polyvinylidene fluoride membranes (Hybond-P, Amersham). Membranes were blocked with 5% non-fat dry milk (50 g/L, 1h, in PBS) and incubated overnight (16h, at 4°C) with either of the TLQP-21 receptor antibodies (see above: NSC-34 cell cultures). On the following day, membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (Invitrogen, Life Technologies, 1:5000, 1h), hence revealed using a chemiluminescent substrate (Euroclone, SpA, Pero, Milan). A rabbit anti-actin antibody (Sigma-Aldrich, Antibody Registry: AB476697, 1:1000) was used to confirm an equal protein loading. Runs were carried out in triplicate in different days.

Statistical analyses

Statistical analyses were carried out using the StatistiXL software. For each experimental set, the normality of data distributions was preliminary checked using the Goodness-of-fit test. Resulting *p* values were >0.05 in all cases, hence the following parametric tests were applied. In case of unequal variance, the Welch's t-test (*t_w*-test) was carried out, otherwise the two-tailed Student's t-test with pooled variances was applied. Linear regression analysis was used to estimate possible correlations between plasma TLQP peptides, versus ALSFRS-R rating or the patients' age. In all cases, *p*-values <0.05 were deemed significant.

RESULTS

TLQP peptides in human

In plasma, levels of TLQP peptide immunoreactivity were roughly 80–90 pmol/ml in control subjects. A distinct, reduction in plasma TLQP peptides was seen in ALS patients (Fig. 2A), already in the early stage (mean \pm ES, controls: 87.2 ± 4.3 ; patients: 75.1 ± 4.0 , t_w test: $p < 0.05$, DF=65.4,) as also in the late stage (patients: 73.5 ± 3.8 , t_w test: $p < 0.05$, DF: 57.3). Data are expressed as percentage of the control samples (100%). In gel chromatography, the following profile of TLQP immunoreactivity was revealed (Fig. 2B): (i) a major peak at a ~ 7 –8 kDa elution position compatible with TLQP-62 (c) (ii) a broad peak in the ~ 6.5 –4 kDa region probably corresponding with the TLQP -42 and -30 forms (d–e), and a lower peak at ~ 2 –3 kDa compatible with TLQP-21 (f). Two larger forms were also found close to the void volume, at about 66 and 14–15 kDa elution positions may compatible to the VGF precursor (peak “a”) and NAPP-129 (peak “b”), respectively, both including the internal TLQP sequence. The sequences of the above TLQP peptides are summarized in Fig. 3. No correlation was found between patients’ plasma TLQP peptide levels and their corresponding ALSFRS-R score, age, or sex (Fig. 2C–E). In fibroblasts (Fig. 2F–I) a significant reduction in TLQP peptide/s immunoreactivity (pmol/ug; controls: 0.35 ± 0.04 , ALS: 0.24 ± 0.02 , t-test: $p < 0.05$, DF=19) was shown in culture extracts from ALS patients compared to controls (Fig. 2F). TLQP peptide/s immunoreactivity was revealed in a region of cytoplasm close to the nucleus, *bona fide* the Golgi area, as previously shown (Brancia et al., 2016) and in agreement with the role of VGF as precursor of secretory products (Brancia et al., 2005). In both ALS patients and controls (Fig. 2G,H; respectively) cytoplasmic stress granules appeared after SA treatment as previously reported (Orrù et al., 2016). Such granules were not labelled by the TLQP antibodies (Fig. 2I). The molecular forms seen upon gel chromatography were broadly comparable to those found in plasma (data not shown).

TLQP peptides in mice

In the cervical spinal cord of wild type mice of all ages studied, TLQP peptides were well represented in large and medium size perikarya in laminae VIII and IX, the majority surrounded by the VAcHT staining, hence identified as motor neurons (Fig. 4A, white arrows) while their levels (through ELISA) were in a range of 100–250 pmol/g. Instead, in the mutant mice, the TLQP immunoreactivity was significantly decreased, through both

IHC (Fig. 4A) and ELISA in the pre-symptomatic stage (158.7 ± 15.1 and 46.6 ± 20.4 , wild type vs. SOD1, t-test: $p < 0.005$, DF=9), remaining down regulated up to the late-stage (WT: 186.8 ± 10.6 , SOD1: 153.5 ± 19.9 , t-test: $p < 0.05$, DF=10) (Fig. 4B). Lumbar and cervical spinal cords were analysed in parallel showing similar immunostaining profiles in both IHC and ELISA. The molecular forms recognised by the TLQP antiserum were comparable to those observed in human samples, including peaks compatible with TLQP-62,-42,-30,-21 (peaks c, d, e, f) and the two peaks, “a” and “b”, may compatible with the VGF precursor and NAPP-129, respectively (Fig. 4C). In all wild type mice, plasma concentrations of TLQP peptides ranged between 170 and 200 pmol/ml, while they were significantly decreased in SOD1 mutant mice at the pre-symptomatic stage (WT: 170.5 ± 17.8 , SOD1: 125.7 ± 8.8 , t-test: $p < 0.05$, DF=10), remaining reduced in the late stage (WT: 171.5 ± 9.8 , SOD1: 132.3 ± 12.1 , t-test: $p < 0.05$, DF=10) (Fig. 4D). Gel chromatography applied to the mouse plasma revealed similar forms observed in human and mouse spinal cord hence it was not shown.

TLQP peptides and NSC-34 cells

TLQP immunoreactivity was found in the growth cones and axons of NSC-34 cells, as well as in their cytoplasm in a para-nuclear location suggestive of its abundance in the *bona fide* Golgi area (Fig. 5A left panel). No co-localization was found with markers of the ER (data not shown). Upon treatment with SA, to induce oxidative stress, cells mostly lost their axons and growth cones changing to a round shape while TLQP peptides maintained their cytoplasmic localization (Fig 5A, middle panel). No TLQP-immunoreactivity was detected in stress granules (Fig 5A, right panel). A significant decrease of TLQP peptides content (pmol/ug total protein; Fig. 5B) was found in SA treated cells (naïve: 0.199 ± 0.04 , treated: 0.094 ± 0.01 , t_w test: $p < 0.05$, DF=9). Addition of synthetic TLQP-21 (1nmol/ml) to culture media of SA treated cells significantly increased cell viability, compared to the SA treatment only (82.5% vs. 74.7%, $p < 0.05$; Fig. 5C). Several VGF derived peptides were measured in naive and SA stressed cells. Further to TLQP peptides, these included NERP-1, NAPP- and APGH-peptides, as well as VGF N-terminus and C- terminus related peptides. Only TLQP and NERP-1 peptide/s showed a significant change (reduction) in stressed cell cultures, hence were tested. However, addition of synthetic NERP-1 to the cell culture medium did not result in any detectable change in cell viability (data not shown). When we used the antibodies against the two TLQP receptors, the gC1q-R antibody showed a labelling in the nucleus with a feeble immunostaining into the cytoplasm (Fig. 5D), as expected (Soltys et al., 2000) while a

weak staining only was revealed for C3a-R. The presence of gC1q-R (predicted molecular weight: 33kD) was confirmed by western blot analysis (Fig. 5E). NSC-34 extracts revealed approximately the same MW forms observed in human and mouse samples, hence they were not shown.

DISCUSSION

We demonstrate here a downregulation of TLQP peptides in both stressed NSC-34 cells and untreated fibroblast cultures from ALS patients, as well as in motor neurons of SOD-1 mice before the onset of significant muscle weakness. In plasma, TLQP peptides were also reduced from the early clinical stages in ALS patients, and so were in the earliest stage studied in SOD-1 mice (pre-symptomatic stage). Hence, plasma TLQP peptides may have a value as possible biomarkers in the screening or diagnosis of suspected ALS patients.

TLQP peptides as blood biomarkers

The identification of potential biomarkers sensitive to the progression of disease is one of the present goals of ALS research. In SOD-1 mice, the reduction of TLQP peptide/s we observed in both plasma and motor neurons at the earliest, pre-symptomatic stage suggests that plasma changes may not only parallel, but also reflect early changes occurring in motor neurons. Hence, peptides of the overall TLQP family may show promise as indicators for early diagnosis of ALS. In fact, measurement of most neurotrophic factors, including BDNF (Tremolizzo et al., 2016), failed to selectively detect ALS patients and changes at an early phase (Turner et al., 2009). In a previous study, we revealed changes in peptides derived from a different part of VGF, namely the region encompassing the C-terminal end of the VGF precursor (Brancia et al., 2016). VGF C-terminus peptides also showed significant changes in ALS patients, but only at the advanced clinical stage (Brancia et al., 2016). Hence, it is conceivable that other VGF derived peptides, including those related to the VGF C-terminus, may decrease upon an extensive neuronal damage, while TLQP peptides are reduced at an earlier stage of initial cellular damage or breakdown. While further studies will be required, with the precise identification of the molecular forms involved, one might suggest that TLQP-21 and other related peptides, i.e TLQP-62, could be most prominently involved in the overall changes found in the present paper. The TLQP-62 peptide, deserves a special mention, because, since it extends from the TLQP sequence to the full C-terminus of the VGF precursor, it is also recognised and measured by VGF C-terminus assay (Brancia et al, 2016). Moreover, since the Sardinian population has a high predominance of TARDBP mutation (Chiò et al., 2011), as reflected in our cohort of patients, we were not able to study any correlation between TLQP levels and specific ALS mutation/s. While future studies will be done by us to investigate if the reduction of TLQP peptides is

peculiar for ALS, schizophrenia induced by phencyclidine has not produced any TLQP changes in the rat blood (Noli et al., 2017).

TLQP peptides are reduced in ALS tissues

TLQP peptides were reduced in spinal cord motor neurons of SOD1-G93A mice, in SA-stressed NSC-34 cells, as well as in fibroblast cultures from ALS patients. Interestingly, the latter patients showed a TARDBP mutation, which has also been proposed to induce cell death through oxidative stress (Duan et al., 2010; Braun et al., 2011; Zhan and Tibbets, 2015). Altogether, TLQP peptide/s changes we observed probably occurred in connection with oxydative stress and ensuing pathophysiological mechanisms. This way, the TLQP alterations occurring in the spinal cord of pre-symptomatic SOD1 mice may be relevant part of, or respond to the early modifications triggering the waterfall of events that cause motor neuron degeneration. It is worth noting that TLQP peptides were localized in the *bona fide* Golgi area as well as in growth cones and axons (Chevalier-Larsen and Holzbaur, 2006). Golgi fragmentation has been shown to be associated with ALS hallmarks, and to occur at an early, preclinical stage in both ALS patients (Maruyama et al., 2010), and SOD1 mice (Vlug et al., 2005; Van Dis et al., 2014). In the same mice, defects in retrograde transport, from the muscle cells to the cell body of motor neurons, have been suggested to be one of the earliest visible alterations (Ligon et al., 2005).

TLQP-21 protects neuronal cells from oxidative stress

We here reported that the TLQP peptides, localized in the cytoplasm and growth cones/axons of the NSC-34 cells, decreased in response to oxidative stress, while the TLQP-21, when added in the medium, is able to protect the cells from the death. The other VGF peptides tested in the stressed NSC-34 cells were not reduced, or, if they were, did not protect the cells from the death (as in the case of the NERP-1). Since the presence of both TLQP peptides and gCq1-R within the NSC-34 cells, we could speculate that the neuroprotection could be due to mechanisms linked to their relationship. Actions of TLQP-21 via the gC1q-R receptor has been shown to be implicated in hypersensitivity in the spinal cord dorsal horn (Chen et al., 2013). The gC1q-R is an ubiquitous protein of 33 kDa initially identified and characterized as a receptor for the globular heads of the complement activation component C1q (Ghebrehiwet et al., 1994) and involved in the inflammatory response (Peerschke and Ghebrehiwet, 2007). In spinal cord motor neurons of SOD1 mice, it was found to be

detectable before the appearance of the clinical symptoms (Heurich et al., 2011) with a major expression at the late stage (Lee et al., 2013). TLQP-21 has been previously reported, as mentioned, to prevent apoptosis induced by oxidative stress in both human (Zhang et al., 2013) and rat cells (Severini et al., 2008). All together these pieces of evidences highly suggest an involvement of TLQP-21-gC1q-R complex in the increased cell viability that we reported here, none the less, studies are warranted to investigate TLQP-21 activity on motor neurons in a variety of conditions. The gC1q-R is also expressed in fibroblasts (Bordin and Costa, 1998), where it might exert a protective action against oxidative stress (McGee and Baines, 2011). Hence, fibroblast cultures may be a further means to address the mechanisms implicated in TLQP-21 bioactivity, and its possible value in ALS.

Collectively, our study suggests that the TLQP family, including both TLQP-21 and TLQP-62, respond early to oxidative stress, and could be of value as a biological diagnostic index for ALS. The TLQP-21 peptide might be of some relevance to prevent or reduce motor neuron death. Further studies may be of interest, to address the possible relevance of TLQP peptides other than TLQP-21. TLQP-62 has so far better studied, and has been shown to have a role in neurogenesis (Takker-Varia et al., 2014). Interestingly, in ALS patients, where impaired glucose tolerance has been reported (Sun et al., 2015), certain metabolites modulated in plasma are indicative of alterations in both mitochondrial activity and carbohydrate/lipid metabolism associated with neuronal changes (Lawton et al., 2012). Since TLQP-21 is a metabolic peptide acting on energy and lipolysis mechanisms (Bartolomucci et al., 2006), and TLQP-62 is a hypoglycaemic agent (Petrocchi-Passeri et al., 2015), we could speculate that both peptides could be possibly involved in the energy mechanisms that contribute to motor neuron degeneration (Dupuis et al., 2004). In conclusion, although currently there are no applicable blood diagnostic tests and pharmacological treatments for ALS, research on these topics may likely include the TLQP family.

AUTHORS' CONTRIBUTION

CB, GLF: conceived and planned the study; CB, BN, RP and MB performed experiments with assistance by AB and RP; CB, BN and CC analysed data; MB, FM and AV contributed materials and analytical tools, and reviewed the manuscript; CB, CC and GLF wrote the paper. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

Sandro Orrù provided fibroblast explants, Giuseppe Borghero human plasma samples. Giacomo Diaz is thanked for help with statistical analyses, Barbara Manconi for quality control of synthetic peptide preparations by HPLC-mass spectrometry, and Valeria Sogos for uncheasing encouragement (all at the University of Cagliari, Cagliari, Italy). Angelo Poletti (University of Milan) provided the NSC-34 cell line. This work was supported by: the Autonomous Region of Sardinia (Sardinia PO FSE 2007-1013 funds, L.R. 7/2007); NeuroCare onlus; Interdisciplinary Human Movement and Rehab Research Laboratory (HuMoRe).

CONFLICTS OF INTEREST

Authors declare no conflict of interest that could prejudice the impartiality of this scientific work.

FIGURE LEGENDS

Fig. 1. Calibration curve. The standard curve was obtained using a range of concentrations of the “reference” peptide in solution (VGF_{556–564}) and a fixed amount of the same peptide onto the well, competing for the TLQP antibody, OD: optical density; pmol/ml: picomoles/milliliters.

Fig. 2. TLQP peptides in human. Plasma levels of TLQP peptides (A). The levels of TLQP peptides in the early and late stage of the ALS patients (n= 25 and 19, respectively) are both reduced, when compared with the controls (n=46); percentage of reduction: 14 and 16, early and late stage, respectively, t_{wtest} : $p < 0.05$. Data are expressed as percentage of the control samples (100%). Chromatographic analysis coupled with ELISA (B). The following molecular forms are recognised by the TLQP-antiserum: a. ~66kDa, probably the VGF precursor; b. ~15kDa compatible to NAPP-129, as well as: c. ~7–8kDa, d–e. ~6.5–4 kDa, f. ~2–3 kDa, compatible to TLQP -62, -42, -30 and -21, respectively. Arrows in the top indicates the molecular weight markers, pmol: picomoles. ALS Functional Rating Scale Revised (ALSFRS-R) vs. TLQP peptides levels (C). There is not a statistically significant linear relationship ($p \leq 0.427$, $R = 0.124$) between TLQP peptides levels and the ALSFRS-R values; pmol/ml: picomoles/milliliter. Patient’s ages vs. TLQP peptides levels (D). We observe a no statistically significant linear relationship ($p \leq 0.507$; $R = 0.103$) between TLQP peptides levels and patient’s ages. TLQP peptides levels in female and male ALS patients (E). A no significant difference is found between males and females ALS patients ($p > 0.05$). TLQP in fibroblasts (F–I). TLQP peptides are reduced (F) in naïve ALS patient derived cells (n=4) compared to the corresponding controls (n=3; ~31% of controls; t-test: $p < 0.05$) pmol/ug: picomoles/micrograms. TLQP peptides are present in specific cytoplasm structures, probably the Golgi area, in cells from controls as well as ALS patients (G and H, respectively; red-orange, Cy3), either before (H) or after the treatment with SA (I), that produces visible stress granules (revealed with anti HuR, green, Alexa-488). The nuclei are revealed in Blue (Hoechst 33342).

Fig. 3. TLQP sequences. The different sequences of the putative TLQP peptides are described.

Fig. 4. TLQP peptides in mice. TLQP peptides in cervical motor neurons (A). TLQP peptides immunoreactivity (Cy3, red) is localized in a large number of perikarya of the ventral horns in wild type mice in the age of the pre-symptomatic and late stage of transgenic mice. These cell bodies are identified as motor neurons using VACHT antibody (Alexa-488, green). Instead, in the SOD1-G93A-mice, the TLQP immunoreactivity is weak visible in a minor number of cells already in the pre-symptomatic stage and remains reduced also in the late stage (n=3 per genotype). Levels of TLQP peptides in cervical spinal cord (B). TLQP levels are significantly decreased in mutant mice (vs the corresponding wild type) already in the pre-symptomatic phase (percentage of decrease: about 70; $p<0.005$) as well as in the late stage (18%; $p<0.05$). Data are expressed as percentage of the control samples (100%), n=7 per genotype. Chromatography analysis coupled with ELISA in spinal cord (C). Different molecular forms are recognised by the TLQP antiserum: a.~66kDa, compatible to the VGF precursor; b.~15kDa compatible to NAPP-129, and the peaks: c.~7–8kDa, d.~5kDa, e.~3kDa, and f. ~2 kDa, compatible to TLQP -62, -42, -30 and -21, respectively. Arrows in the top indicates the molecular weight markers; pmol:picomoles. Levels of TLQP peptides in plasma (D). TLQP levels are significantly decreased in mutant mice vs wild type already in the pre-symptomatic phase (26%; $p<0.05$) as well as in the late stage (23%; $p<0.05$). Data are expressed as percentage of the control samples (100%), n=7 per genotype.

Fig. 5. TLQP peptides in NSC-34 cells. TLQP immunolocalization (A). TLQP peptides (Cy3, red-yellow,) are found within the cytoplasm, probably in the Golgi area as well as in the axons and growth cones (left panel). When treated with Sodium Arsenite (SA), the cells lose their growth cones changing to a round shape (nucleus revealed in Blue: Hoechst 33342) and TLQP peptides are present exclusively within the cytoplasm (middle panel) and not visible in the stress granules (revealed with anti HuR, ALEXA488, green; right panel). TLQP levels (B). A significant TLQP peptides decrease is seen (B) in the SA treated compared to the naïve cells (about 53%, $p<0.05$). Pmol/ug prot tot: picomoles/micrograms of total protein. MTT viability test (C). The NSC-34 viability increases when the cells are treated with SA together with TLQP-21, compared to the treatment with the SA only (about 83% vs. 75%, $p<0.05$). TLQP-21 alone is not able to produce any effect. The gC1q-R immunoreactivity (D, E). The gC1q-R antibody stains the nucleus as well as weakly the cytoplasm (D, Cy3 red-yellow), and also labelled a form of about 31–33kDa revealed through Western blot (E)

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Appendix A

Table 1A: Characteristics of patients

P#	Age	Gender	ALSFRS-R Score	Genetic mutation	Co-morbidity
1	67	M	25	TDP-43 A382T	
2	66	F	19	TDP-43 A382T	
3	81	F	30	nd	MDS
4*	39	F	22	TDP-43 A382T	
5	56	F	43	SOD1	
6*	66	F	14	TDP-43 A382T	
7	82	M	21	nd	
8	72	F	22	TDP-43 A382T	
9*	53	M	28	nd	
10*	72	M	11	C9ORF expansion	
11	48	M	26	TDP-43 A382T	
12*	64	M	27	nd	
13	56	M	41	C9ORF expansion	DVT
14	71	M	42	SOD1	
15*	55	M	21	TDP-43 A382T	
16	64	F	38	SOD1	
17	78	M	17	TDP-43 A382T	
18*	42	F	30	nd	
19	53	F	31	nd	
20	65	F	38	C9ORF72 expansion	
21	70	M	15	nd	
22*	69	M	6	TDP-43 A382T	
23	70	F	31	TDP-43 A382T	psychosis
24*	82	M	23	TDP-43 A382T	
25*	76	F	20	TDP-43 A382T	
26*	61	M	35	nd	
27	45	M	41	TDP-43 A382T	
28	53	F	25	nd	
29*	64	M	30	nd	
30	76	M	32	nd	
31*	64	M	17	nd	
32*	53	M	37	nd	
33	72	M	35	TDP-43 A382T	
34	58	M	33	ndr	
35	52	M	41	C9ORF72 expansion	
36	67	M	16	nd	K colon
37*	72	F	23	C9ORF72 expansion	
38*	74	F	32	nd	
39	67	F	21	TDP-43 A382T	Sjögren syndrome
40	74	F	21	nd	
41*	59	F	27	nd	
42*	64	F	16	nd	
43*	63	F	0	nd	
44	65	M	32	TDP-43 A382T	Parkinsonism

* = Late stage of ALS disease; nd: not determined; DVT:deep vein thrombosis; MDS: Myelodysplastic syndrome

Table 1. VGF assay characterization

Assay	Peptide	IC ₅₀	CV1 %	CV2%	CR%	
TLQP	rVGF ₅₅₆₋₅₆₄ ¹	8	2-3	7-3	100	IC ₅₀ : co nc ent rati
	rVGF ₅₅₅₋₅₆₄ (R-TLQPPASSR)*				3.5	
	rVGF ₅₅₇₋₅₆₄ (-LQPPASSR)				20	
	rVGF ₅₅₆₋₅₆₅ (TLQP11)				122	
	rVGF ₅₅₆₋₅₇₆ (TLQP21)				183	
	hVGF ₅₅₄₋₅₇₇ (TLQP24)				65	

10 on of peptide producing 50% inhibition of the maximum signal (picomoles/milliliters);
 CV1 and CV2: intra- and inter-assay variation, respectively; r: rat; h: human
 15 ¹“reference” peptide used for immunization, plate coating, and standard. *Arginine (R):
 residue added at the peptide N-terminus, to mimic its extended sequence within the
 VGF precursor. The cross-reactivity (CR) of each peptide is expressed compared to
 the “reference” peptide, a >100% reactivity is indicated for the authentic TLQP-21 that
 is more reactive compared to the reference one. CV1: mean values of six different
 known concentration of TLQP calibrators (500pmol/ml, 50pmol/ml, 5pmol/ml,
 0.5pmol/ml, 0.05pmol/ml, 0.005pmol/ml; 8 replicates). CV2: three known calibrators
 (50pmol/ml, 5pmol/ml, 0.5pmol/ml; 10 independent experiments, in duplicate).

Figure 1

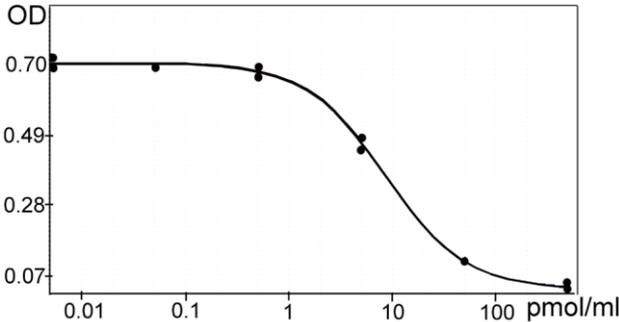


Figure 2

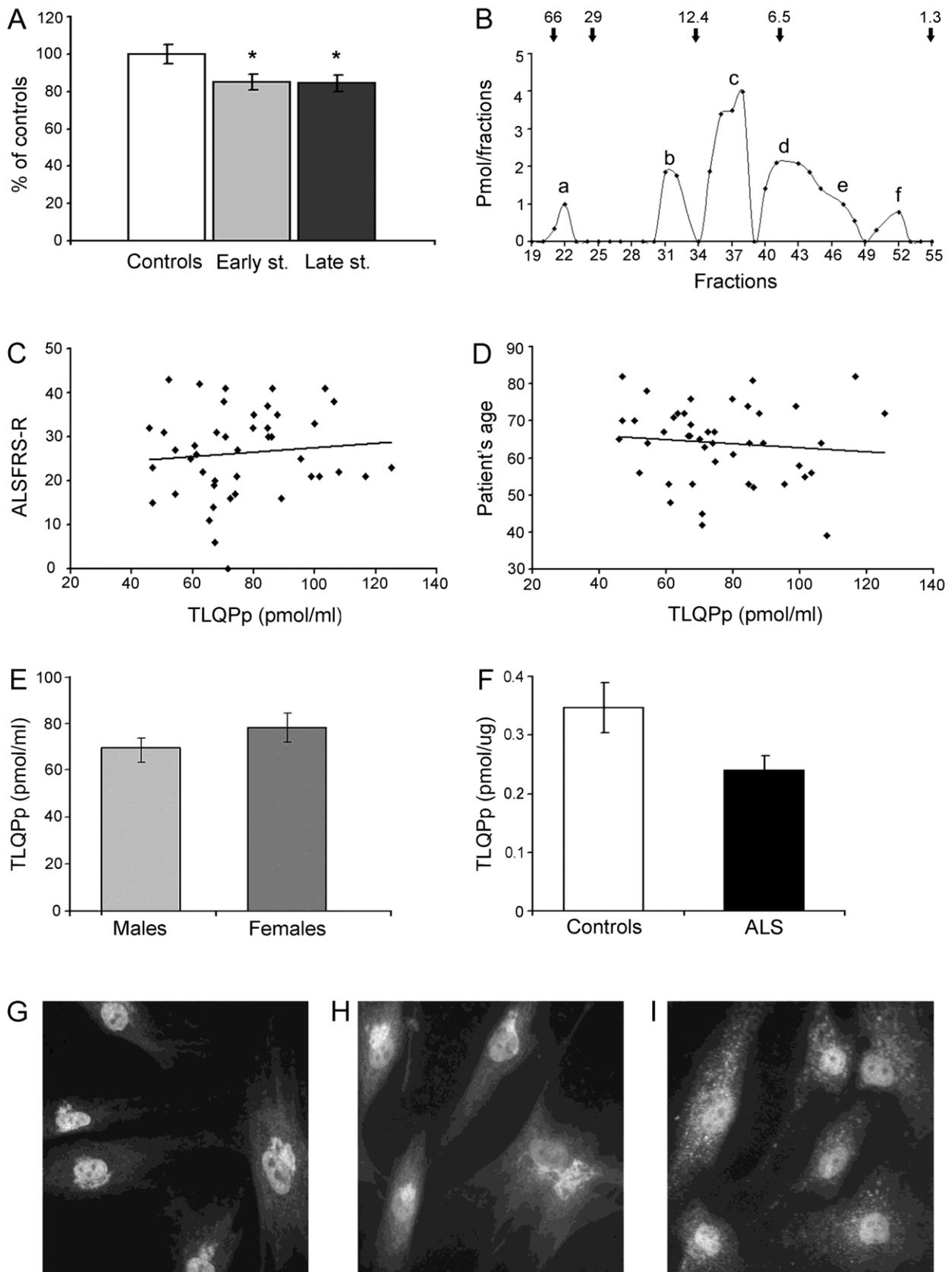


Figure 3



TLQP-62 seq 556-617	TLQPPASSRRRHFHHALPPARHHPDLEAQARRAQEEADAEERRLQEQEELENYIEHVLLHRP
TLQP-42 seq 556-598	TLQPPASSRRRHFHHALPPARHHPDLEAQARRAQEEADAEER
TLQP-30 seq 556-587	TLQPPASSRRRHFHHALPPARHHPDLEAQA
TLQP-21 seq 556-677	TLQPPASSRRRHFHHALPPAR

Figure 4

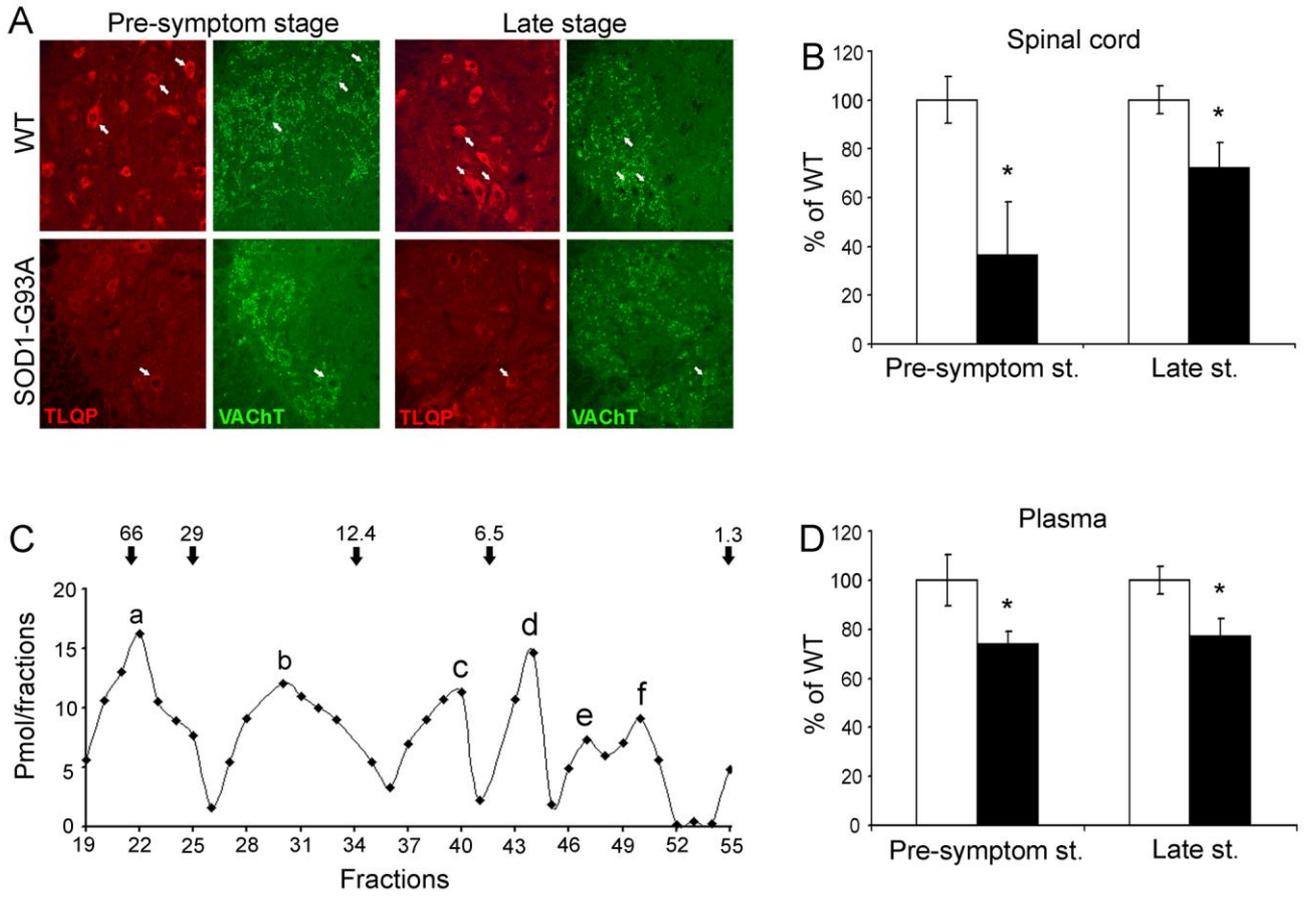


Figure 5

