

## Experimental Communications

### Cite

Gainutdinov T, Debska-Vielhaber G, Gizatullina Z, Vielhaber S, Orynbayeva Z, Gellerich FN (2022) Impaired Ca<sup>2+</sup> signalling as an indicator of disturbed mitochondrial function in fibroblasts from patients with sporadic and familial ALS. MitoFit Preprints 2022.15.  
<https://doi.org/10.26124/mitofit:2022-0015>

### Author contributions

ZG, FNG, SV conceived the idea; TG, GDV, ZG performed the experiments; ZS prepared the draft of the manuscript; all authors contributed to data analysis and writing.

### Conflicts of interest

The authors declare they have no conflict of interest.

**Received** 2022-04-16

**Accepted** 2022-04-16

**Online** 2022-04-21







### Data availability

The original data are available from the authors upon request.

### Keywords

ALS,  
CoQ<sub>10</sub>,  
cellular Ca<sup>2+</sup> homeostasis,  
histamine,  
mitochondria,  
Trolox

# Impaired Ca<sup>2+</sup> signalling as an indicator of disturbed mitochondrial function in fibroblasts from patients with sporadic and familial ALS

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## Abstract

**Amyotrophic lateral sclerosis (ALS) is a progressive, devastating, neurodegenerative disorder affecting upper and lower motor neurons. Common mechanisms of ALS pathogenesis are believed to be the disturbance of calcium homeostasis in the cell and dysfunction of mitochondria. Both factors mutually influence each other. As a result, chronic mitochondrial energy stress impairs fine cellular signaling and transport processes, leading to degeneration of motor neurons. In the current study we comparatively evaluated the cytosolic Ca<sup>2+</sup> in healthy and ALS fibroblasts. We found that the mitochondrial calcium capacity in fibroblasts obtained from patients with sporadic (sALS) and familial (fALS) ALS differs between two subtypes and from that in healthy individuals. The changes of**

**[Ca<sup>2+</sup>]<sub>cyt</sub> dynamics in ALS fibroblasts could be almost completely rescued by treatment with antioxidants (Trolox and CoQ<sub>10</sub>). These data confirm an important role of oxidative stress as a causative factor of mitochondrial dysfunction in ALS.**

## 1. Introduction

The calcium toxicity is one of the key factors of degeneration of motor neurons in Amyotrophic Lateral Sclerosis (ALS) (Grosskreutz et al 2010). Sustained neuronal hyperactivation leads to elevated cytosolic Ca<sup>2+</sup> and its abnormal intracellular redistribution causing depletion of an endoplasmic reticulum (ER) and overload of mitochondria (Marambaud et al 2009). Among the first line Ca<sup>2+</sup> toxicity consequences are mitochondrial injury, impaired autophagy, proteins misfolding, and oxidative stress (Rotunno und Bosco 2013; Edens et al 2016; Obrador et al 2021, Prell et al 2013). The mitochondrial Ca<sup>2+</sup> overload-associated energy deficit, along different intracellular systems, affects the activity of key Ca<sup>2+</sup> transporting mechanisms: plasma membrane Ca<sup>2+</sup>-ATPase (PMCA) (Brini et al 2011), endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) (Walker, Atkin 2011; Lautenschlaeger et al 2012), Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (Castaldo et al 2009), and mitochondrial Ca<sup>2+</sup>-uniporter (Kawamata et al 2010). The crosstalk between these systems is tightly controlled in healthy cells to maintain the physiological level of cytosolic Ca<sup>2+</sup>, but is misaligned in the ALS pathology (Kawamata et al 2010).

Most of the known ALS incidents are sporadic (sALS), and only 5-10 % are inherited familial (fALS), although, 10 % of sALS patients are found to have a genetic component (Renton et al 2014; Volk et al 2018). The available data show that the bioenergetic characteristics of neuronal mitochondria from sALS and fALS patients, including membrane depolarization, impaired oxidative phosphorylation and protein transport, and elevated oxidative stress, are quite similar (Singh et al 2021; Kaus, Sareen 2015). However, with regards to the non-neuronal tissues, i.e., fibroblasts, data are diverse and often contradictory (Konrad 2017; Volk et al 2018; Walczak et al 2019; Debska-Vielhaber et al 2021).

In fALS with mutations in different ubiquitously expressed genes, including SOD1 or TARDBP, it was observed the accumulation of misfolded SOD1 protein aggregates (Bidhendi 2016) and the translocation of TDP43 in mitochondria (Ludolph et al 2015; Wang et al 2016; Wang et al 2017). However, it is still unclear whether the sporadic and familial ALS share common mechanisms or have distinct mechanisms of pathogenesis.

The focus of this study is to differentiate the nuances of cytosolic Ca<sup>2+</sup> homeostasis in fibroblasts from sporadic and familial ALS and explore the effect of antioxidants on crosstalk between cytosolic Ca<sup>2+</sup> and mitochondria driven damage.

## 2. Methods

### 2.1. Subjects.

In our study, we used fibroblasts from skin biopsies of three sALS and four fALS patients, and three healthy volunteers. The demographic and clinical data of the cohort are shown in the [Table 1](#). All ALS patients were diagnosed in accordance to the revised El Escorial criteria (Brooks et al 2000). Written informed consent was obtained from all participants prior to taking the skin biopsy. The study was approved by the Ethical Committee of the University of Magdeburg (No. 101-02/06-09; No. EK45022009).

**Table 1. Clinical characteristics of healthy and ALS patients involved in the study.**

Patient Groups	Healthy (n = 3)	sALS (n = 3)	fALS (n = 4)
<b>Age at onset (year)</b>	58.0 ± 8.8	64.7 ± 4.0	51.0 ± 7.9
Mean (SD)	59.5	65.8	51.5
Median Range	46-67	59-68	42-59
<b>Disease duration (months)</b>		17.7 ± 12.5	41.8 ± 32.1
Mean (SD)	-	12	36.5
Median Range		9-32	7-65
<b>ALSFRS-R*</b>		37.3 ± 4.9	33.3 ± 7.8
Mean (SD)	-	35	39
Median Range		34-43	9-83
<b>Gender</b>			
M:F ratio	1:2	2:1	1:1

\*ALSFRS-R, the ALS Functional Rating Scale (Revised), an established rating mechanism to monitor disease progression in patients with ALS (Cedarbaum et al 1999).

## 2.2. Cell Culture.

Primary fibroblasts from ALS and healthy participants were derived from 5x5 mm punch skin biopsies. Cells were grown in DMEM medium supplemented with 10 % FCS, 2 mM glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, 4 µg/mL Ciprolaxin and 10 µg/mL Tylosin, in a 5 % CO<sub>2</sub> atmosphere at 37 °C (Kunz et al 1995). All cultures were used between passages 6 and 10.

In the experiments with antioxidants, the ALS cells in the growth medium were exposed to 300 µM Trolox for 5 days or to 5 µM CoQ<sub>10</sub> for 3 weeks. Every second day the growth medium was changed, and fresh solutions of antioxidants were added.

## 2.3. Measurement of cytoplasmic Ca<sup>2+</sup>.

Cytoplasmic free Ca<sup>2+</sup> in fibroblasts was measured using a Carry Eclipse fluorescence multichannel spectrophotometer (Varian, Darmstadt, Germany). The cells were loaded with 2 µM Fura-2AM for 30 min at 37 °C in the dark (Grynkiewicz et al 1985). After incubation, cells were washed twice with HBSS (without Ca<sup>2+</sup>, Mg<sup>2+</sup>) and resuspended in the same buffer. For each experiment, 10<sup>6</sup> cells/ml were added in a quartz cuvette and placed in thermostatically controlled (37 °C) holder of spectrophotometer with continuous stirring. Cells were first exposed to 2 mM Ca<sup>2+</sup> and then sequentially challenged by the following stressors: Histamine 100 µM and FCCP 10 µM. The excitation and emission lights were 340/380 nm and 510 nm, respectively. At the end of each experiment, the cells were disrupted by addition of 0.5 % Triton X-100 to assess the maximal Ca<sup>2+</sup> signal. The 340/380 nm fluorescence excitation ratio (R) and the 380 nm fluorescence emission (F<sub>s</sub>) were both obtained at a saturating Ca<sup>2+</sup> concentration and at <1 nM Ca<sup>2+</sup>. The latter was reached in the presence of 1 mM EGTA in the buffer. The [Ca<sup>2+</sup>]<sub>cyt</sub> was calculated using the following equation (Grynkiewicz et al 1985):

$$[\text{Ca}^{2+}]_{\text{cyt}} = K_D \cdot F_0/F_s \cdot (R - R_{\text{min}})/(R_{\text{max}} - R),$$

where F<sub>0</sub> and R<sub>min</sub> are the Fura-2AM signal at 380 nm and 384/380 nm fluorescence excitation ratio, respectively, in a medium lacking Ca<sup>2+</sup>. The F<sub>s</sub> and R<sub>max</sub> are the corresponding values obtained at saturating Ca<sup>2+</sup> concentration. The K<sub>D</sub> for the Ca<sup>2+</sup>-Fura-2AM complex was 224 nM.

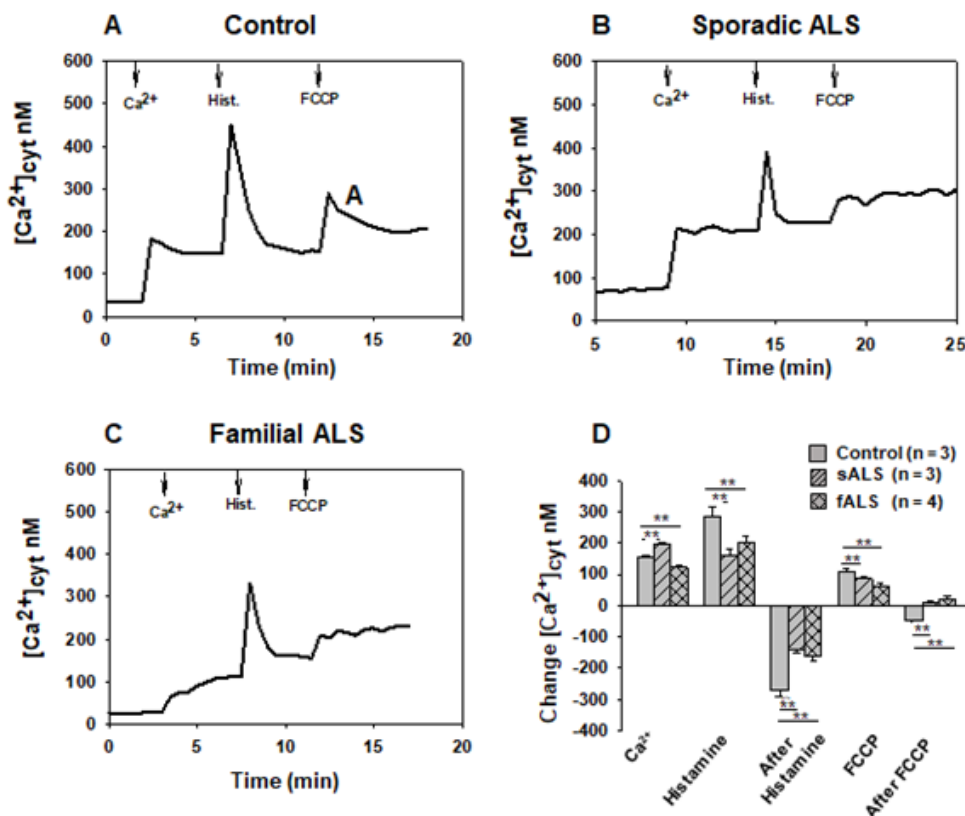
## 2.4. Statistical analysis.

All experimental runs were performed at least in triplicates. Data were analyzed using the paired, two-sample Student's t test assuming unequal variances. Data are presented as mean  $\pm$  S.D. Linear relationships between measured variables were assessed via Pearson product moment correlation (CP) using the Sigma Plot 11. Statistical significance was accepted at  $p \leq 0.05$ .

## 3. Results

### 3.1. Comparative assessment of cytosolic $\text{Ca}^{2+}$ in control vs ALS fibroblasts.

The cytosolic  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) was monitored as a function of signal changes of endogenously accumulated Fura-2AM before and upon subsequent addition of Histamine and FCCP in the experimental medium. To mimic a physiological interstitial environment, 2 mM  $\text{Ca}^{2+}$  was added to the medium before challenging the cells with the modulators. The Figure 1 illustrates the typical  $\text{Ca}^{2+}$  responses to the stressors of human control and ALS fibroblasts. The  $[\text{Ca}^{2+}]_{\text{cyt}}$  in sALS was by 28 % higher and in fALS by 20 % lower compared to the control cells (Figure 1A).



**Figure 1. Assessment of cytosolic  $[\text{Ca}^{2+}]_{\text{cyt}}$  in fibroblasts from healthy volunteers and ALS patients. (A). Healthy control. (B). ALS sporadic. (C). ALS familial (SOD1 mutation). (D). Quantitative summary of the changes of fluorescence signal upon addition of modulators. Additions: fibroblasts  $10^6$  cells/ml,  $\text{Ca}^{2+}$  2 mM, Histamine 100  $\mu\text{M}$ , FCCP 10  $\mu\text{M}$ . Data are means  $\pm$  S.D. (n = individuals). \*\*  $p < 0.01$ .**

Next, the cells were excited with Histamine, the  $\text{Ca}^{2+}$  mobilizing agonist that acts via H- and G-protein-coupled receptors (Kirischuk et al 1996; Hofstra et al 2003). This compound was used to modulate a scenario of an elevated cytosolic  $\text{Ca}^{2+}$  and evaluate the vulnerability and/or functional activity of  $\text{Ca}^{2+}$  signaling mechanisms of ALS affected cells. The 100  $\mu\text{M}$  Histamine increased the cytosolic  $\text{Ca}^{2+}$  signal, presumably by stimulating the ion influx from extracellular environment (Li et al 2012). The  $\text{Ca}^{2+}$  peaks were lower in ALS fibroblasts than in the control, i.e., by 45 % in sALS (Figure 1B) and by 30 % in fALS (Figure 1C). However, after 3-5 minutes of Histamine stimulation, the levels of cytosolic  $\text{Ca}^{2+}$  recovered back by -84 % in sALS and -78 % in fALS versus -98 % in the control (Figure 1D). This cytosolic signal decrease occurs due to a removal of free  $\text{Ca}^{2+}$  ions from cytosol by their redistribution between the intracellular compartments (mitochondria and ER), and extrusion of  $\text{Ca}^{2+}$  out of cell via the mechanism of PMCA.

To address the mitochondrial functionality, FCCP was added over the Histamine-stimulated cells. The uncoupling facilitated  $\text{Ca}^{2+}$  release from mitochondria. The value of FCCP-induced  $\text{Ca}^{2+}$  peak is proportional to the amount of free  $\text{Ca}^{2+}$  in the mitochondria and thus, corresponds to the mitochondrial calcium retention capacity. As seen in Figure 1D (FCCP columns), the  $\text{Ca}^{2+}$  signal in sALS cells was 68 % and in fALS was 57 % of that in the control cells. This data suggest that the calcium capacity of ALS mitochondria is much lower than in the control, and they are no longer capable of retaining the calcium ions in the matrix.

The “After FCCP” columns demonstrate the changes of  $[\text{Ca}^{2+}]_{\text{cyt}}$  after the mitochondria depolarization with FCCP. As seen in Figure 1A, in the control sample, the FCCP-induced increase of  $[\text{Ca}^{2+}]_{\text{cyt}}$  was transient because cells tend to eliminate the excess of free  $\text{Ca}^{2+}$ ; the level of basal  $[\text{Ca}^{2+}]_{\text{cyt}}$  quickly restored to 60 %, by virtue of available cellular ATP (Figure 1D, After FCCP). However, the elevated signals in both ALS samples remained sustained, indicating the compromised ability of ALS cells to maintain  $[\text{Ca}^{2+}]_{\text{cyt}}$ .

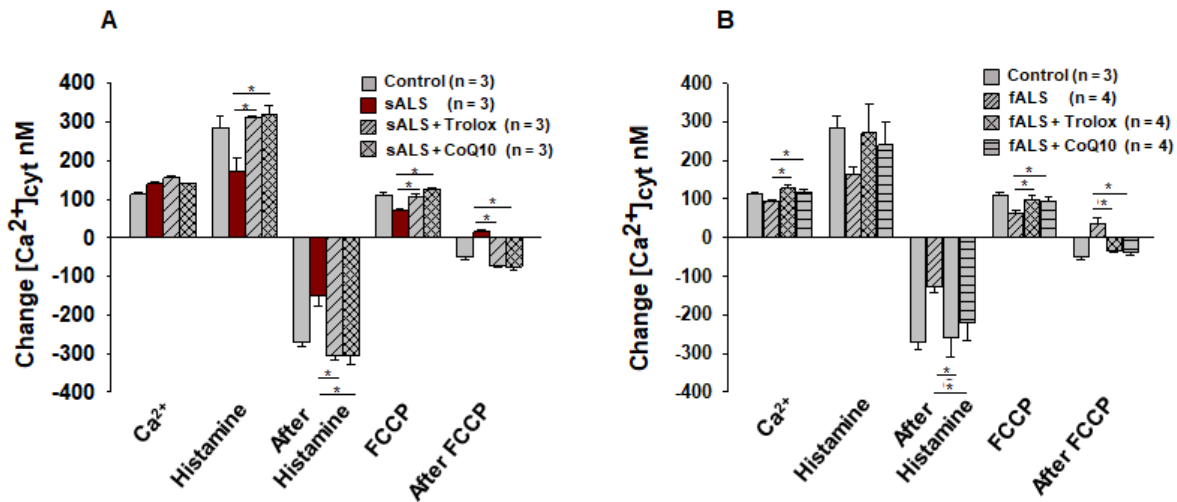
### 3.2. Modulation of cytosolic $\text{Ca}^{2+}$ by antioxidants.

The altered mitochondrial functions are prerequisites for oxidative stress (Cunha-Oliveira et al 2020). To reverse the effects of oxidative damage, ALS-derived fibroblasts were incubated with the antioxidants Trolox (Babahajian et al 2019) and CoQ<sub>10</sub> (Chaturvedi, Beal 2008), which have neuroprotective potential. The same experimental run was carried out as described in the previous section but with additional pretreatment of cells with the antioxidants.

As seen in Figure 2A, in the presence of 2 mM  $\text{Ca}^{2+}$ , the sALS+Trolox and sALS+CoQ<sub>10</sub> samples had slightly elevated  $[\text{Ca}^{2+}]_{\text{cyt}}$  with respect to the healthy control. However, the signals were quite similar to the untreated sALS. The responses of sALS+Trolox and sALS+CoQ<sub>10</sub> fibroblasts to Histamine and FCCP differed significantly from the untreated ALS cells. Moreover, both ALS-antioxidants signals were approximately equivalent to those of healthy fibroblasts (Figure 2A).

The Figure 2B shows the results of  $[\text{Ca}^{2+}]_{\text{cyt}}$  alterations in fALS fibroblasts pretreated with antioxidants. In the presence of 2 mM  $\text{Ca}^{2+}$ , both fALS+Trolox and fALS+CoQ<sub>10</sub> samples had by approximately 20 % higher  $[\text{Ca}^{2+}]_{\text{cyt}}$  than the untreated fALS, and they were almost identical to the healthy fibroblasts. The stimulation of  $\text{Ca}^{2+}$  transport by Histamine increased the values of  $[\text{Ca}^{2+}]_{\text{cyt}}$  in both ALS groups bringing them to proximity of healthy fibroblasts. The dynamics of  $[\text{Ca}^{2+}]_{\text{cyt}}$  changes after the addition of FCCP

demonstrated an improvement of mitochondrial metabolism by anti-oxidative agents, particularly an increase of matrix calcium capacity (Figure 2B). Overall, our data indicate that Trolox and CoQ<sub>10</sub> are capable of stabilizing the Ca<sup>2+</sup> homeostasis of ALS affected fibroblasts almost to the level of healthy cells.



**Figure 2. Modulation of cytosolic [Ca<sup>2+</sup>]<sub>cyt</sub> in ALS fibroblasts by pretreatment with antioxidants. (A).** Healthy control; untreated and treated with Trolox and CoQ<sub>10</sub> sALS fibroblasts. **(B).** Healthy control; untreated and treated with Trolox and CoQ<sub>10</sub> fALS fibroblasts. Data are means ± S.D. (n = individuals). \* p < 0.02.

#### 4. Discussion

To explore the nuances of the mechanisms of Ca<sup>2+</sup> regulation associated with the sporadic and familial ALS we performed the stress analysis of skin fibroblasts obtained from the ALS patients. The rationale behind the evaluation of cellular mechanisms under the induced Ca<sup>2+</sup> stress lies in our earlier studies of skeletal muscle mitochondria isolated from the wild type and transgenic mice with Huntington's disease (Gizatullina et al 2006). In that study, only the condition of an elevated cytosolic Ca<sup>2+</sup> enabled us to distinguish otherwise hidden metabolic differences between the pathologic and healthy samples. Using the same approach, in the current study, the dynamics of [Ca<sup>2+</sup>]<sub>cyt</sub> changes were evaluated fluorimetrically using the stimulation of cells with Histamine to provoke high cytosolic Ca<sup>2+</sup> and then uncoupling of mitochondria.

To mimic the physiological extracellular concentration of Ca<sup>2+</sup>, the suspensions of fibroblasts were preincubated with 2 mM Ca<sup>2+</sup> (Peacock 2010; Carafoly, Krebs 2016) prior to challenging with the stressors. The mechanisms involved in elevation of cytosolic Ca<sup>2+</sup> consist of influx through the plasma membrane transporters and voltage-dependent ion channels, and of Ca<sup>2+</sup> release from intracellular stores, with the endoplasmic reticulum being the major Ca<sup>2+</sup> harbor. In its turn, the elevated [Ca<sup>2+</sup>]<sub>cyt</sub> activates the mitochondrial mechanism of gas pedal (Szibor et al 2020) producing ATP via the pyruvate mediated oxidative phosphorylation (OXPHOS). This supports the ATP-driven Ca<sup>2+</sup> pumps of endoplasmic reticulum and plasma membrane for carrying out the removal of the accumulated excess Ca<sup>2+</sup> from cytosol. The mitochondrial contribution to the normalization of cytosolic Ca<sup>2+</sup> also extends to the uptake of cytosolic Ca<sup>2+</sup> and stimulation of the rates of OXPHOS.

We found that all  $\text{Ca}^{2+}$  related parameters in ALS fibroblasts are very different from those in healthy cells. The Histamine-induced transport of  $\text{Ca}^{2+}$  into the ALS cells was 30-45 % weaker than that in healthy cells. The post-stimulation decrease of  $[\text{Ca}^{2+}]_{\text{cyt}}$  in both ALS subtypes was not efficient either, but nevertheless, sALS cells eliminated  $[\text{Ca}^{2+}]_{\text{cyt}}$  somehow better than fALS cells (Figure 1D, After Histamine). These data are indicative of mis-regulated mechanisms of  $\text{Ca}^{2+}$  homeostasis in ALS pathogenesis, one of which is the perturbed metabolism of mitochondria.

The experimental setup used in this work, an uncoupling of mitochondria under the elevated cytosolic  $\text{Ca}^{2+}$ , is an approach for indirect qualitative assessment of mitochondria ability to accumulate and retain calcium in the matrix. The reduced calcium retention capacity was observed in ALS fibroblasts which upon addition of FCCP release lower amounts of  $\text{Ca}^{2+}$  than the healthy cells (Figure 1D, FCCP). Moreover, the mitochondria of both ALS subtypes were not able to sequester back the elevated free  $\text{Ca}^{2+}$  which kept spontaneously releasing during the course of measurement. Our observation correlates with the data of another study that revealed the reduced mitochondrial polarization in fALS and sALS fibroblasts (Walczak et al 2019).

To eliminate the oxidative stress mediated by malfunctioning mitochondria, the ALS fibroblasts were treated with Trolox and CoQ<sub>10</sub> before evaluating the  $\text{Ca}^{2+}$  signaling. Both compounds were equally effective against the sporadic and familial ALS cells, almost completely reverting the dynamics of  $[\text{Ca}^{2+}]_{\text{cyt}}$  modulations to the healthy control background. These data are in agreement with our previous finding of the recovery of mitochondrial functions in sALS and fALS skin fibroblasts by antioxidants (Debska-Vielhaber et al 2021).

**Conclusion:** In this study we demonstrated the dysregulated mechanisms of cytosolic calcium control and mitochondrial  $\text{Ca}^{2+}$  retention in ALS skin fibroblasts. The observed impairments are shown to be rescued by means of antioxidants that could be considered a promising therapeutic approach to treat the ALS disorder (Chaturvedi, Beal 2008; Babahajian et al 2019; Debska-Vielhaber et al 2021).

### Acknowledgments:

This work was supported by the grant (No. 26009, 2015-2016) from Stiftung für Medizinische Wissenschaft, Germany, to TG. The authors thank Kerstin Kaiser and Jeannette Witzke for their excellent technical assistance.

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