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Extra Virgin Olive Oil Intake Delays the Development of Amyotrophic Lateral Sclerosis Associated with Reduced Reticulum Stress and Autophagy in Muscle of SOD1G93A Mice

Olive oil in SOD1G93A Mice

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease associated with mutations in antioxidant enzyme Cu/Zn-superoxide dismutase 1 (SOD1). Albeit there is no treatment for this disease, new insights related to an exacerbated lipid metabolism have been reported. In connection with the hypermetabolic lipid status, the hypothesis whether nature of dietary fat might delay the progression of the disease was tested by using a transgenic mouse that overexpresses the human SOD1G93A variant. For this purpose, SOD1G93A mice were assigned randomly to one of the following three experimental groups: 1) a standard chow diet (control, n=21), 2) a chow diet enriched with 20% (w/w) extra virgin olive oil (EVOO, n=22), and 3) a chow diet containing 20% palm oil (palm, n=20). They received the diets for 8 weeks and the progression of the disease was assessed. On the standard chow diet, average plasma cholesterol levels were lower than those mice receiving the high fat diets. **Mice fed an EVOO diet showed a significant higher survival and better motor performance than control mice.** EVOO group mice survived longer, showed better motor performance and larger muscle fiber area than animals receiving palm. Moreover, the EVOO-enriched diet improved the muscle status as shown by expression of myogenic factors (*Myod1* and *Myog*) and autophagy markers (*LC3* and *Beclin1*), as well as diminished ER stress through decreasing *Atf6 and Grp78*. Our results demonstrate that EVOO may be effective in increasing survival rate, improving motor coordination together with a potential amelioration of ER stress, autophagy and muscle damage.

Key words · Extra virgin olive oil · Palm oil · Amyotrophic Lateral Sclerosis · Reticulum stress · autophagy · Cu/Zn-superoxide dismutase 1 · SOD1G93A mice

Abbreviations used: apo, apolipoprotein; FPLC, fast performance liquid chromatography; HDL, high density lipoproteins; LDL, low density lipoproteins; P, post natal day; TG, triglycerides; VLDL, very low density lipoproteins.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset disease characterized by the selective loss of motor neurons in the brain and spinal cord, resulting in progressive paralysis and death. In one fifth of familial ALS, the disease has been found associated with mutations in the major antioxidant enzyme Cu/Zn-superoxide dismutase 1 (SOD1) [1-3], and transgenic mice overexpressing mutant forms of SOD1 [4] are considered good animal models for ALS since they show most of features of the human disease. Studies using these mice have proved that mutant SOD1 (mSOD1) triggers ALS by attacking motor neurons, astrocytes and microglia [5]. These mice also show a hypermetabolic state mainly of muscular origin [6] and involving a rapid removal of lipids [7]. Consequently, the increase in the energy content of the diets prolonged lifespan and maintained motor neuron [6, 8], while restricting calorie intake worsened the disease [9]. Furthermore, by generation of a transgenic mouse model expressing a mutant SOD1G93A selectively in skeletal muscle, it has been demonstrated that skeletal muscle is a primary target of SOD1G93A-mediated toxicity and that oxidative stress triggers muscle atrophy [10].

A higher adherence to a Mediterranean-type diet has been associated with lower Alzheimer disease risk and may be protective against stroke and other neurological disorders [11-13]. The Mediterranean diet is characterized by high fat, mainly from olive oil, complex carbohydrates in the form of cereals and legumes, high fiber in the form of fruit and vegetables, and the limited consumption of animal proteins, representing a design tested by populations in the Mediterranean Basin for more than 2000 years. Olive oil, as the main source of fat in the traditional Mediterranean diet, has been object of considerable attention regarding to its potential benefits. In this regard, its intake has been shown to

improve cardiovascular risk factors, such as lipid profiles, blood pressure, postprandial hyperlipidemia, endothelial dysfunction, oxidative stress, and antithrombotic profiles [14]. Using genetically modified mice that spontaneously develop atherosclerosis, we have proved that extra virgin olive oil was highly beneficial when consumed judiciously and in diets of low cholesterol content [15].

As above mentioned, the studies suggesting that high-fat diets may have an effect in delaying the development of the ALS did not address the influence of different sources of fat. In addition due to the described beneficial effects of extra virgin olive oil, it would be plausible that feeding this oil may also own a positive effect on the ALS outcome. To test the hypothesis, we fed SOD mice high fat diets containing extra virgin olive oil or palm oil and evaluated their clinical performance. To determine the mechanisms involved, we performed an analysis of muscle gene expression.

2. Material and Methods

2.1 Animals

Animals were purchased from The Jackson Laboratory (B6SJL-Tg(SOD1-G93A)1Gur/J) and hemizygotic transgenic mice were obtained by mating transgenic males with F1 hybrid females (B6SJL). Transgenic mice were identified by PCR assay of DNA extracted from the tail with specific primers for human SOD1 (according to The Jackson Laboratory protocol). Animals were provided with food and water *ad libitum* and housed under a standard light:dark (12:12 hour) cycle in the *Servicio General de Apoyo a la Investigación*-SAI of the Universidad de Zaragoza. All of the procedures were approved by the in-house Ethics Committee for Animal Experiments of the Universidad de Zaragoza. Animal care and experimentation were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE on the protection of animals used for experimental and other scientific purposes.

2.2 Dietary Trial

Two-month old female SOD1G93A mice on the same genetic background (B6SJL) and hemyzygous for the mutation were assigned randomly to one of the following three experimental groups: 1) a control group (n=21) fed a standard chow diet, 2) an olive-oil group (n=22) fed a diet enriched in (20%, w/w) extra virgin olive oil from Empeltre cultivar (Muniesa, Belchite, Spain), and 3) palm oil group (n=20) fed a diet containing 20% palm oil. The standard mouse chow diet was Teklad Mouse/Rat Diet no. 2014 (Harlan Teklad; Harlan Ibérica, Barcelona, Spain). To avoid the potential confounding effects of variation between batches of chow, 25 kg from a single batch were reserved and used to

prepare diets and feed experimental groups throughout the experiment. Weekly diets were stored in N_2 at –20ºC and their composition analyzed, as previously described [16]. The chemical composition of the diets is shown in Table 1. Olive oil and palm oil diets were isocaloric. These high-fat diets were lower in carbohydrates, had slightly lower protein content and a higher percentage of fat and vitamin E than the chow diet. Chow diet had a monounsaturated fatty acid content of 20.5 % of total fat and a higher amount of polyunsaturated than saturated fatty acids (P/S ratio 3.4). The high-fat diets contained a similar amount of polyunsaturated fatty acid and differed in the amount of monounsaturated and saturated fatty acids. The olive oil-enriched diet the P/S ratio was 2.9, which indicates an important decrease in saturated fatty acids compared to the palm oil-containing diet. Likewise, the olive oil diet was enriched in phenolic compounds. The nutritional intervention was well tolerated and lasted until the animal death.

2.3 Behavioral Tests

In a subset of 10 females per experimental group, motor coordination, strength and balance were assessed using a rotarod (Panlab, Barcelona, Spain). Animals were placed onto the cylinder at a constant speed of 14 rpm. Also, neuromuscular strength was tested by the hanging-wire test. Each mouse was placed on a wire lid of a conventional housing cage and the lid was turned upside down. The latency from the beginning of the test until the mouse stood with at least two limbs on the lid was timed. In both tests, the animals had three attempts to stand for a maximum of 180 seconds per trial, and the longest latency was recorded. The onset of symptoms was scored as the first day that a mouse could not remain on the hanging wire for 3 min. Behavioral tests were performed weekly during the morning,

beginning at postnatal day 77 (P77) until the death of the mice. During late-stage disease, mice were assisted to access to food and water. Euthanasia was performed when mice failed to right themselves within 20 seconds of being placed on their side [17].

2.4 Plasma Determinations

Those surviving mice from the three experimental groups not used for the behavioral tests were sacrificed by suffocation in CO_2 at 120 days of age (terminal stage) and after a four-hour-fasted regimen, and blood was drawn from the heart. Gastronemius and tibial anterior muscles were obtained, and a piece of them was immediately frozen in liquid N_2 for RNA isolation while the other was preserved in buffered formaldehyde for histological analysis.

Plasma total cholesterol (TC), and triglyceride (TG) concentrations were measured in a microtitre assay, using commercial kits from Thermo (Madrid, Spain). Apolipoproteins A1, A4 and B were quantified by enzyme-linked immunosorbent assays with specific polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, and Biodesign, Saco, ME, USA) as previously described [18]. Plasma lipoprotein profiles were also determined in 100 μL of pooled plasma samples by fast protein liquid chromatography (FPLC) gel filtration [16] using a Superose 6B column (Amersham Pharmacia, Barcelona, Spain). The contents of cholesterol, phosphatidylcholine and sphingomyelin in each fraction were assayed as described [19].

2.5 HDL reactive oxygen species

The presence of reactive oxygen species (ROS) in this lipoprotein fraction was assessed by measuring the conversion of 2',7'-dichlorofluorescein diacetate (DCFH-DA) into

fluorescent dichlorofluorescein (DCF) [20]. Briefly, the FPLC-separated HDL fractions (5 μg of cholesterol) were incubated, at 37º C with 2 μg of DCF, in 25 μl of 0.1% sodium azide and 100 μl of PBS, up to a total volume of 150 μl [21]. The fluorescence was measured after 3 h of incubation at 485 nm excitation and 535 nm emission wavelengths in a microplate reader (SPECTRAfluor Plus, TECAN).

2.6 RNA Preparation and Analysis

Muscle RNA was isolated using Tri Reagent (Sigma). DNA contaminants were removed by TURBO DNase treatment using the DNA removal kit from AMBION (Austin, TX). RNA was quantified by absorbance at A260/280 (the A260/280 ratio was >1.75). The integrity of the 28S and 18S ribosomal RNAs was verified by agarose formaldehyde gel electrophoresis followed by ethidium bromide staining and the 28S/18S ratio was greater than 2. The mRNA expression was assayed by quantitative real time RT-PCR (RT-qPCR) analysis. Equal amounts of DNA-free RNA from each sample were used for first-strand cDNA synthesis and the PCR reactions were performed using the SuperScript II Platinum Two-Step RT-qPCR Kit with SYBR Green (Invitrogen, Madrid, Spain) or TaqMan (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions and following the methodology previously described [22-25]. The relative amount of mRNAs normalized expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method.

2.7 Histological Analysis

Samples of muscle stored in buffered formaldehyde were embedded in paraffin. Sections (4 μm) were stained with hematoxylin-eosin, observed with a Nikon microscope and images

were captured. Morphometric analyses of fiber area were evaluated blindly using Scion Image software (Scion Corporation, Frederick, Maryland, USA).

2.8 Statistical Analysis

The results are expressed as means ± SD. Comparisons were made using one-way ANOVA and Mann-Whitney *U*-test. Correlations between variables were sought using the Spearman's correlation tests. Kaplan-Meier analysis was performed to determine disease onset and survival time. All calculations were performed using SPSS version 15.0 software (SPSS, Chicago, IL). Significance was set at $P < 0.05$.

3. Results

3.1 Increased cholesterol and diminished ROS levels in EVOO diet

The effects of the experimental diets on plasma lipid parameters in SOD1G93A mice are shown in Table 2. Mice consuming the high lipid diets showed increased plasma total cholesterol and APOA1 levels and no significant change in their plasma triglycerides. Neither did plasma APOA4 and APOB experience any significant change. The distribution of APOA1 and APOA4, cholesterol, phosphatidylcholine and sphingomyelin among the plasma lipoproteins analyzed by FPLC is shown in supplementary figure 1. No striking changes were observed in any of these parameters. Interestingly, the content of ROS in HDL was lower in animals consuming high fat diets and particularly in animals consuming EVOO (panel F).

3.2 EVOO diet prolongs the lifespan and improves the motor behavior of SOD1G93A mice

Statistical significant differences in survival rate were observed between chow and EVOO diet ($p<0.05$) (figure 1A). Mice fed with a chow diet showed a mean lifespan of 127.5±4.47 days, whereas mice fed with an EVOO diet survived 142.87±2.92 days. Moreover, disease onset of palm oil group (89.83±2.15 days) was significant earlier than chow diet group (106.11±3.94) or EVOO group (107.33±6.68) (figure 1C).

According to the rotarod test (figure 2A), significant differences between chow diet and EVOO group were observed at P112 ($p<0.05$). From P112 to P126, EVOO group mice showed better motor performance than control group. In contrast palm oil group showed early symptoms at P84 and significant differences from chow diet group were detected from P91 to P119 ($p<0.05$, $p<0.001$). Furthermore, palm oil group did not show any

improvement in motor behavior from the beginning of the assay.

In relation to hanging-wire test (figure 2B), the motor symptoms appeared earlier in palm oil group (P84), although the decline began to be significant at P105 and continued until P133 ($p<0.001$, $p<0.01$, $p<0.05$). No statistical significant differences were observed between chow and EVOO diet.

Taking together these results, the higher survival rate together with the significant improvement in motor behavior observed in EVOO-enriched diet mice, suggested that EVOO could diminish the muscle damage.

3.3 EVOO-enriched diet increase the muscle fiber area in SOD1G93A mice

In figure 3, panels A, B and C, representative images of muscle fibers coming from mice receiving the different diets are displayed. Animals receiving the palm diet showed a lesser size of muscle fibers. Quantitative analyses, shown in panel D, confirmed that this difference was statistically significant when compared to the mice receiving the EVOO-enriched diet.

3.4 EVOO diet improve levels of myogenic factors and autophagy markers

Myogenic regulatory factors (MRF), biomarkers of disease progression and autophagy expression genes were measured in skeletal muscle tissue from 120 day old mice to determine whether muscular mRNA changes were involved in clinical and histological changes (figure 4). Several myogenic regulatory factors, *Pax7*, *Myod1*, *Myf5* and *Myog* genes were analyzed (figure 4A). Interestingly, *Myod1* and *Myog* transcripts were significantly elevated in palm oil group ($p<0.05$, $p<0.01$). Moreover, *Myog* was

significantly overexpressed compared to EVOO group ($p<0.05$). According to biomarker of disease progression's genes, *Chrna1* and *Col19a1* levels were significantly upregulated in palm oil group compared to EVOO group ($p<0.05$) (figure 4B). *Chrna1* levels were also found significantly overexpressed in palm oil group as compared to control ($p<0.05$). The high levels of *Myod1*, *Myog* and *Chrna1* observed in the palm oil group suggested that palm oil diet did not compensate for muscle damage as efficiently as the EVOO diet.

In relation to autophagy markers, *LC3* and *Beclin1* transcripts were significantly decreased in EVOO group ($p<0.05$) (figure 4C). Moreover, *LC3* was significantly overexpressed in palm group compared to EVOO group ($p<0.05$).

3.5 EVOO diminishes ER stress through Atf6 and Grp78

In order to look for a potential ER stress involvement, the expression of the three described pathways was evaluated: *Atf6*, *Ire1* and *Perk*. As shown in figure 5A, mice consuming EVOO showed significantly decreased levels of *Atf6* expression compared to control and palm groups. The latter group showed significantly increased levels of *Grp78* when compared to mice receiving the EVOO-containing diet. Analysis of correlation of both expressions, *Atf6* and *Grp78*, showed a positive and significant value (figure 5C) suggesting a coordinate regulation of this cascade of ER stress despite the different diets supplied. Conversely, *Grp78* expression was negatively associated with area of fiber (figure 5D). On the other hand, *Ire1* and *Perk* mRNA expressions (data not shown) did not show any significant change. Collectively, these data suggest that olive oil administration is improving ER stress through the *Atf6* and *Grp78* branch.

Analysis of proliferating mitochondria marker, *Pgc1a*, revealed a significant decrease in

palm-fed mice (0.7 ± 0.1) when compared to the control group (1.0 ± 0.2). No change was observed in mice receiving the EVOO diet (1.0 ± 0.5).

4. Discussion

The present work describes a nutritional intervention with two high fat diets on an ALS mouse model where the nature of fat has a profound influence on the survival and motor behavior of the mice. To explain these findings, a study of muscle pathology has being carried out and provided a difference in muscle fiber area between both high fat diets. Furthermore, mRNA expression has evidenced that changes in expression of myogenic and reticulum stress genes are involved in the differential response between both high fat diets.

In an attempt to reproduce in mice the average intake of fat in humans of Western society, we provided animals with extra virgin olive oil or palm oil as 20% (w/w) of the diet, but deprived of dietary cholesterol, to reproduce the classical Mediterranean diet. A dietary pattern characterized by a high fat content, mainly derived from olive oil, and low cholesterol [14]. We have found that a dietary fat supplement of extra virgin olive oil, administered at a level comparable to that of human energy intake, resulted in an amelioration of ALS pathological findings and delay in their onset. These results are in agreement with the benefits observed using high fat diets [6, 8], but it adds a new aspect regarding the nature of fat used. The use of EVOO was clearly more beneficial in SOD1G93A mice than that of palm oil. In this regard, two aspects are different: the higher supply of palmitic acid and the decrease of oleic acid in the latter oil. These two components have been observed to behave differently. In particular, oleate has been found to prevent palmitic-induced insulin resistance, inflammation [26] and endoplasmic reticulum stress [27] in cultured muscle cells. Our data of animals receiving EVOO clearly indicate a decrease of endoplasmic reticulum stress markers, *Atf6* and *Grp78* but not *Ire1* and *Perk,* when compared with animals receiving palm oil (Figure 5). The unfolded protein

response (UPR) through signal transduction pathways transfers information about protein folding status in the ER. It is mainly executed by three distinct signal cascades involving inositol requiring (IRE1), PKR-like ER kinase (PERK), and activating transcription factor (*Atf6*) [28] and may participate in the pathogenesis of ALS [29]. The present results suggest that the *Atf6* network is differentially expressed in this dietary trial in agreement with the changes in *Grp78* that detects the accumulation of misfolded proteins in the ER and collaborates with *Atf6* [28]. PGC-1alpha has been considered as a novel and clinically relevant disease modifier of human and experimental ALS [30]. In our results, the supplement of palm oil also decreased the expression of this mitochondrial proliferation marker. Overall, our results indicate that both ER and mitochondria are important contributors to the muscle cell fitness in ALS. This is in agreement with the proposed network connecting the sarcomere integrity to mitochondrial oxidative metabolism [31], and the important role of skeletal muscle as a primary target of muscle atrophy [10] and of ALS toxicity [10, 32, 33].

Following with the neurodegenerative conditions observed in ALS, the loss of motor neuron connections in the neuromuscular junction is likely due to an energetic deficit in the mutant muscle that leads to pathological conditions [22]. In this regard, the present work proposes a dietary intervention to correct this deficit. Interestingly, mice receiving EVOO-enriched diet significantly survived 15 days longer than control animals, although no significant differences were found in the onset of symptoms. In addition to this longer lifespan observed in animals receiving the EVOO-enriched diet, the motor coordination and balance of these treated mice improved along disease progression over mice fed a chow diet. Nevertheless, mice receiving palm oil-enriched diet showed shorter mean survival of 4

days, significant earlier disease onset (Figure 1) and a significant lower motor coordination compared to control mice along the disease progression (Figure 2). Moreover, the neuromuscular strength in EVOO-enriched diet mice resembled the one observed in control group while this strength was significantly and progressively declined in palm-oil enriched diet mice until the terminal stage (Figure 2). Overall, these data are suggesting that EVOO diets displayed general benefits when compared to control group and specific ones when compared to palm-enriched diets, and that not all high-fat diets may have the same outcome. Clinical trials carried out in patients have addressed the influence of hypercaloric diets by comparing carbohydrates versus fat [34]. In this trial, source of fat was a commercial formula containing marine and borage oils and was found safe. A second trial, NCT01016522 *(*www.clinicaltrials.gov/), has been conducted to evaluate the safety of ketogenic diets in ALS patients using a commercial formula as well. In these settings, our results may provide guidance for improving these human interventions.

Interestingly, a recent study focused on the role of SOD1 in the ER stress response suggested that SOD1 could act as a switch to prompt the ER stress response through its binding to the cytosolic carboxyl-terminal of Derlin-1, a component of the ER-associated degradation machinery [29]. In particular, the binding of mutant SOD1 to Derlin-1 could trigger ER stress through the disruption of the ER-associated degradation machinery and therefore could contribute to motor neuron death [29]. Taking into consideration the higher and significant survival rate in EVOO-enriched diet mice together with the significant improvement of their behavior function over palm oil-enriched diet mice, our results suggested that EVOO-enriched diet could ameliorate the ER stress response induced by mutant SOD1 expressed in mice. This would agree with the *in vitro* protection of

endoplasmic reticulum stress observed for the main phenolic compound of EVOO, hydroxytyrosol [35].

According to these results and taking into consideration that skeletal muscle is one of the main targets of the disease, our previous studies in this tissue suggested a deregulation of different genetic biomarkers along disease progression [22, 23]. Consequently the next step forward in our study was focused on the analysis of genetic biomarkers' expression in the skeletal muscle of SOD1G93A mice fed a standard chow diet, EVOO or palm-enriched diet. For this purpose we tested the expression levels of seven genes related to the skeletal myogenesis and integrity, *Pax7, Myod1*, *Myf5*, *Myog*, *Rrad*, *Chrna1* and *Col19a1* (Figure 4). A significant increase of *Myod1*, *Myog* and *Chrna1* was observed in palm oil-enriched diet mice, whereas in EVOO-enriched diet mice, the expression levels of the tested genes tended to reach control levels. Furthermore, an upregulation of the myogenic regulatory factors, such as *Myod1* and *Myog*, was previously found coincident with increase muscle damage and denervation markers *Rrad* and *Chrna1* respectively [23], in the same animal model. This result suggested that palm oil-enriched diet would favor skeletal myogenesis in a greater extent than in EVOO-enriched diet mice. In addition, palm oil-enriched diet mice showed a significant upregulation of *Col19a1* compared to EVOO group and in a setting of ER stress. It is known that a downregulation of the *Col19a1* expression in skeletal biopsy samples of SOD1G93A mice correlated inversely with longevity [22], suggesting that the survival rate of animals that overexpressed this gene was lower than the observed in animals in which this expression was found downregulated. This result was coincident with the higher survival rate observed in mice receiving EVOO-enriched diet. Therefore in our study, the significant upregulation of *Myod1*, *Myog Chrna1* and *Col19a1* in mice fed palm

oil-enriched diet suggests that the latter diet could not compensate muscle damage as efficiently as the EVOO-enriched diet. In fact, EVOO-enriched diet diminishing ER stress was accompanied by an amelioration of muscle damage (Figure 3).

In clear connection with ER homeostasis, autophagy and ER are in a reciprocal interaction since autophagy activation can be regulated by ER Ca^{2+} stores [36]. It has been also reported that the SOD1G93A expression is sufficient to induce muscle atrophy associated with oxidative stress through activation of the autophagy [10]. Furthermore, the autophagy, process that leads to the degradation of cytoplasmatic components inside lysosomes, also participate in mutant SOD1 clearance in muscle cells combined with the proteasome activity [37]. It has been described that certain olive oil´s components can modify proteolysis [38], so the EVOO-enriched diet may influence the autophagy process in our experimental approach. To this purpose, we have tested two markers of autophagy, *LC3* and *Beclin1*, which are essential for autophagosome formation; in particular, *LC3* is used to monitor autophagy processes [39]. Our previous experiments suggested that the expression of *LC3* and *Beclin1* was increased at terminal stages in muscle of SOD1G93 mice (data not shown). Here we found that EVOO-enriched diet not only induced a significantly downregulation of the expression of *LC3* and *Beclin1* genes (Figure 4C) but also increased the fiber area (Figure 3). Therefore, these results indicate lower muscle atrophy in these mice and suggest that the EVOO treatment showed protective effects in the muscle.

In conclusion, our results establish an important role for quality of fat on the progression of ALS in SOD1G93A mice. In addition, they provide new insights on the molecular pathways influencing neurodegenerative progression and may contribute to

emphasize the relevance of Mediterranean diet in this pathology. Although our findings from mice may not be directly applicable to humans, their relevance is particularly important considering the devastating progression of the disease and the high security of the nutritional intervention proposed.

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Table 1. Composition of the experimental diets provided to mice.

	Chow	Extra virgin olive oil	Palm oil
Energetic Content (kJ/g)	13	18	18
Carbohydrate	57	51	51
Protein	16	12	12
Fat	3	22	22
Vitamin E (IU %)	10	22	21
Cholesterol (mg %)	30	25	25
Fatty acids,			
Lauric (12:0)	0.1	0.1	0.1
Myristic (14:0)	0.02	0.0	0.0
Palmitic (16:0)	15.8	15.0	20.7
Margaric (17:0)	0.2	0.2	0.2
Stearic (18:0)	1.7	1.7	2.4
Arachidic (20:0)	0.1	0.2	0.2
Behenic (22:0)	0.02	0.0	0.0
Lignoceric (24:0)	0.1	0.1	0.1
Palmitoleic (16:1)	0.02	0.2	0.0
Oleic (18:1)	20.1	31.5	24.3
Gadoleic (20:1)	0.4	0.4	0.3
Linoleic (18:2n-6)	57.9	47.7	48.7
Linolenic (18:3n-3)	3.5	2.9	2.9
Saturated	18.0	17.3	23.7
Monounsaturated	20.5	32.1	24.7
Polyunsaturated	61.4	50.6	51.6
P/S ratio	3.4	2.9	2.2
Total phenolic compounds (mg/kg)	0.1	45	2

Dietary components are expressed as % (w/w). Other components of the chow diet are crude fiber 4.5 % and minerals 6.8 %. A total dry matter of 87.5%. P/S ratio = polyunsaturated / saturated fatty acid ratio.

Table 2. Plasma lipid parameters following the different experimental diets

	Control (n=11)	EVOO (n=12)	Palm (n=6)
APOA1 (AU/L)	76 ± 9	87 ± 8[a]	92 ± 8[a]
APOA4 (AU/L)	3.8 ± 1.1	5.5 ± 1.6	5.8 ± 1.0
APOB (AU/L)	0.5 ± 1.0	0.6 ± 1.0	0.9 ± 1.0
Total cholesterol (mg/dL)	100 ± 7	112 ± 11[a]	124 ± 13[b]
Triglycerides (mmol/L)	0.4 ± 0.1	0.5 ± 0.3	0.5 ± 0.2

Values are means ± standard deviations. Mice were fed chow or experimental diets for 8 weeks and fasted 4 hours before blood sampling. Statistical analysis was done using non-parametric one-way ANOVA according to Kruskal-Wallis test and unpaired Mann-Whitney U-test as post-hoc test. Different superscripts ([a] vs Control, [b] vs EVOO) are significantly different from each other at $P < 0.05$. AU, arbitrary units.

Figure 1. Survival and disease symptom onset of SOD1G93A mice.

Kaplan-Meier analysis of survival (A) and clinical symptom onset (B) revealed significant differences between the diets. Mice fed an EVOO diet showed a higher and significant survival than control mice ($p<0.05$) whereas disease symptom onset occurred significantly earlier in palm oil group ($p<0.05$) (C). n=10 mice per group.

Figure 2. Effect of different diets on the progression of the disease of SOD1G93A mice.

Motor performance was evaluated by rotarod (A) and hanging-wire (B) tests. Data are shown as mean ± SEM. Student's *t*-test was performed to compare control and treatment groups: a**,** $p<0.05$ (EVOO vs control group) and b, $p<0.05$ (palm oil vs control group).

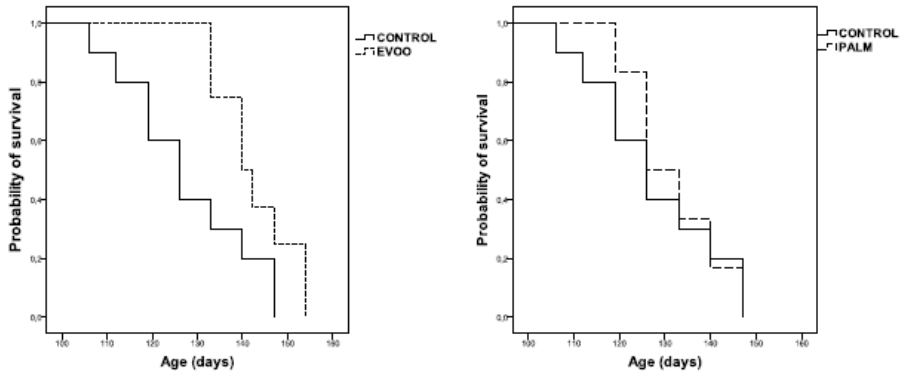
Figure 3. Analyses of cross-sections taken from muscular fibers. Panels A, B and C correspond to representative images coming from mice receiving control, EVOO and palm diets, respectively. Panel D summarizes fiber areas in SOD1G93A mice following different the experimental diets. Data are mean ± SD for each group. Statistical analysis was done using non-parametric one-way ANOVA according to Kruskal-Wallis test and unpaired Mann-Whitney U-test as post-hoc test. ^{b}, $p< 0.05$ vs EVOO.

Figure 4. Gene expression of myogenic regulatory factors (MRFs), biomarkers of disease progression and autophagy in skeletal muscle of SOD1G93A mice.

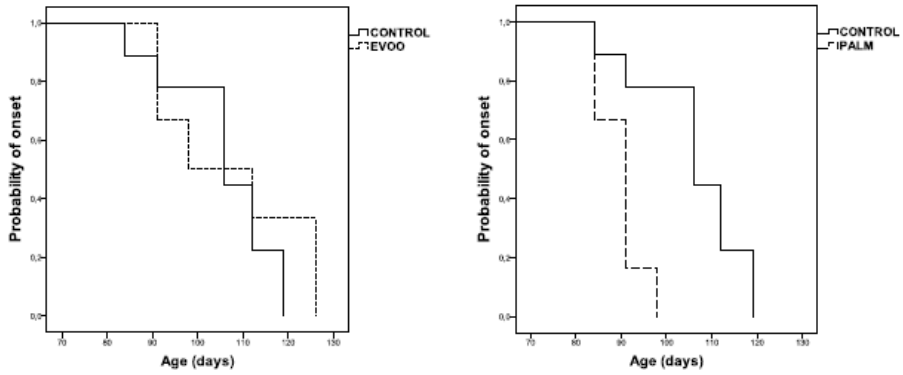
Relative expression levels of MRF (A), biomarkers of disease progression (B) and autophagy (C) compared between mice fed a chow, EVOO and palm oil diet. Values are means ± standard deviations. Student's *t*-test: *$p<0.05$ and **$p<0.01$.

Figure 5. Reticulum stress marker expressions in the different experimental conditions. A, *Atf6* and B, *Grp78* mRNA expressions, respectively. Values are means ± standard deviations. Unpaired Mann-Whitney U-test as post-hoc test. *p<0.05. Panel C, relationship between *Atf6* and *Grp78* expressions. Panel D, relationship between muscle fiber area and *Grp78* expressions. Correlation was calculated according to Spearman's test. Values corresponding to all experimental groups have been included, where empty, black squares and grey diamonds correspond to chow, EVOO- and palm oil-containing diets, respectively.

A



B



C

	CONTROL (n=10)	EVOO (n=10)	PALM (n=10)
Survival (days)	127.51 ± 4.47	142.87 ± 2.92[a]	131.83 ± 4.21
Onset (days)	106.11 ± 3.94	107.33 ± 6.68	89.83 ± 2.15[b]

Fig. 1

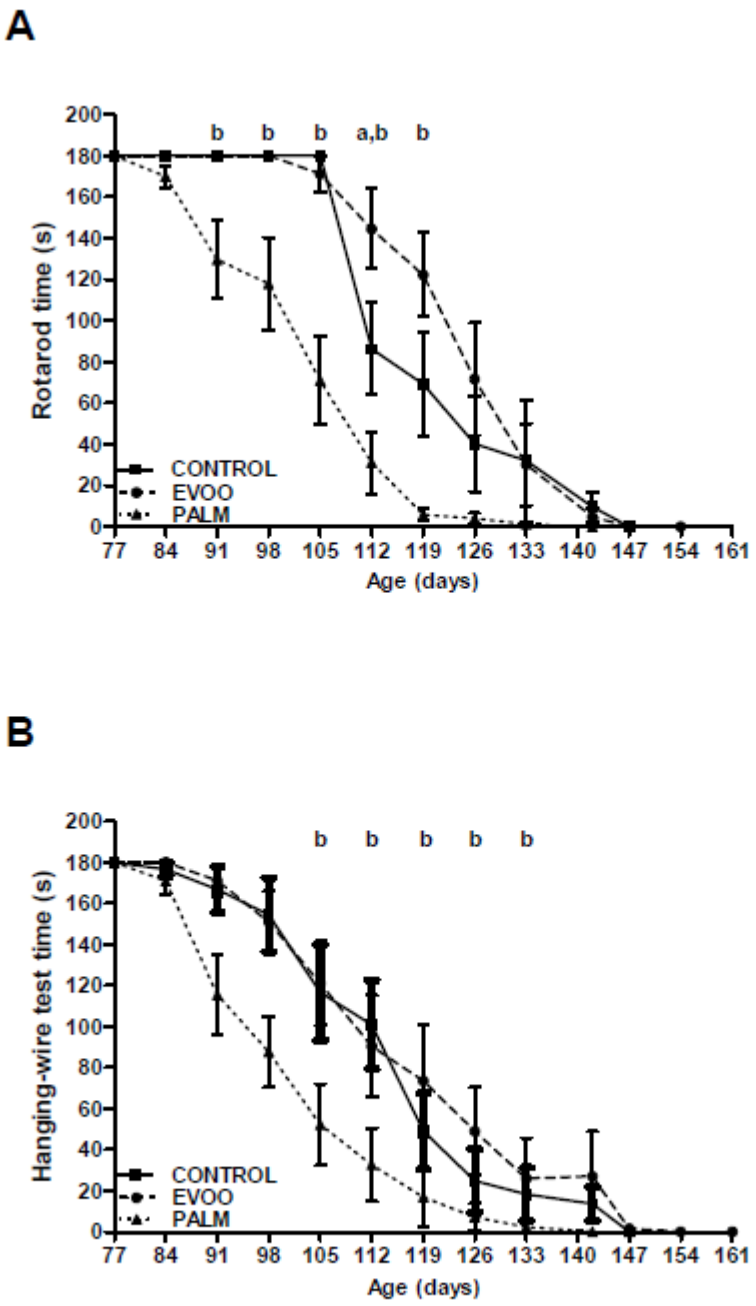
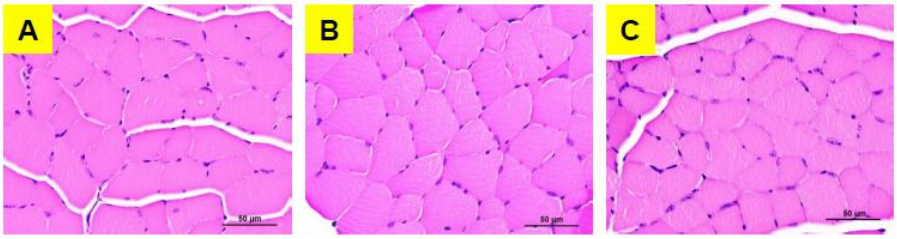


Fig. 2



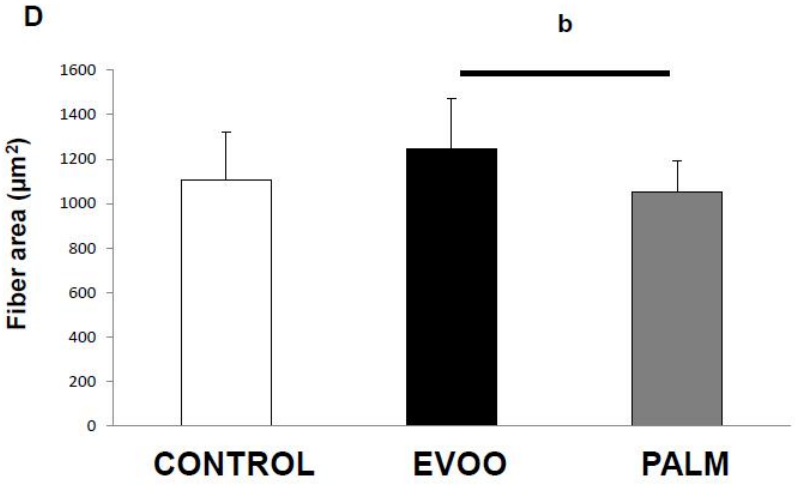


Fig. 3

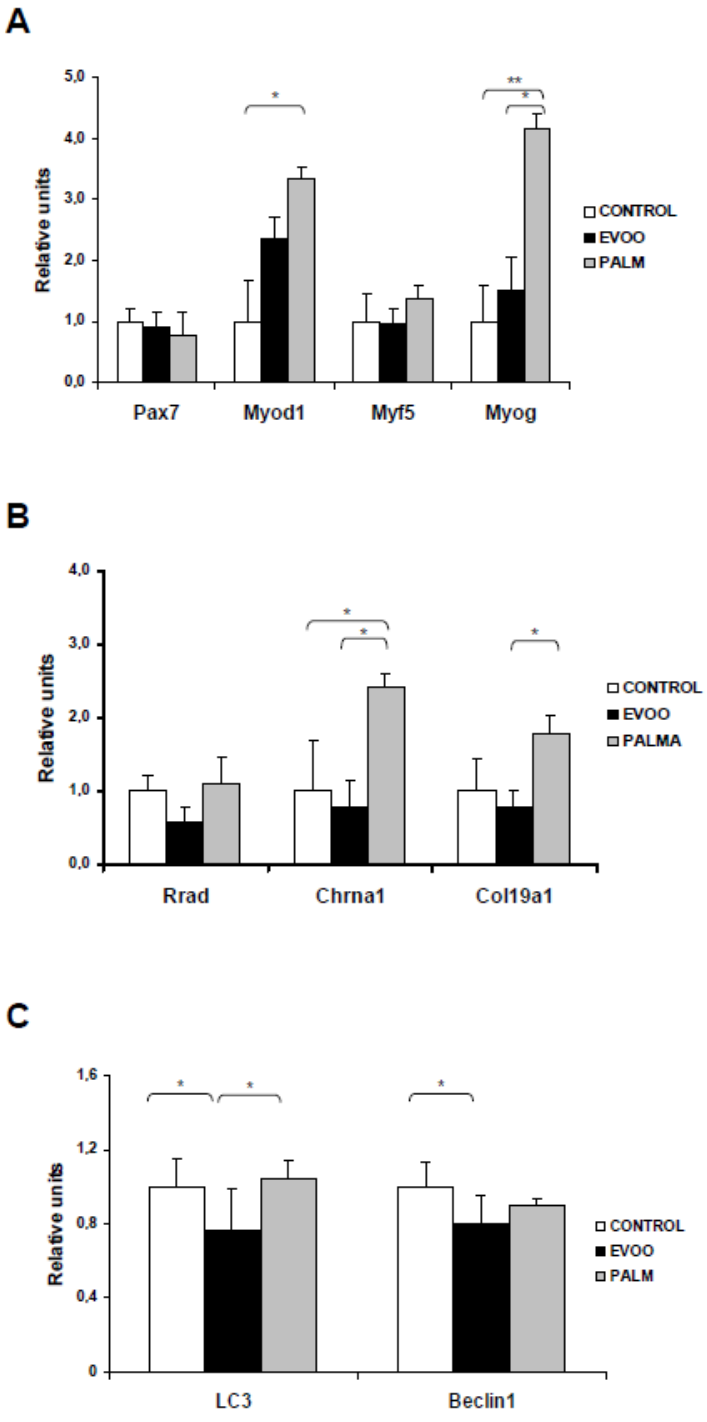


Fig. 4

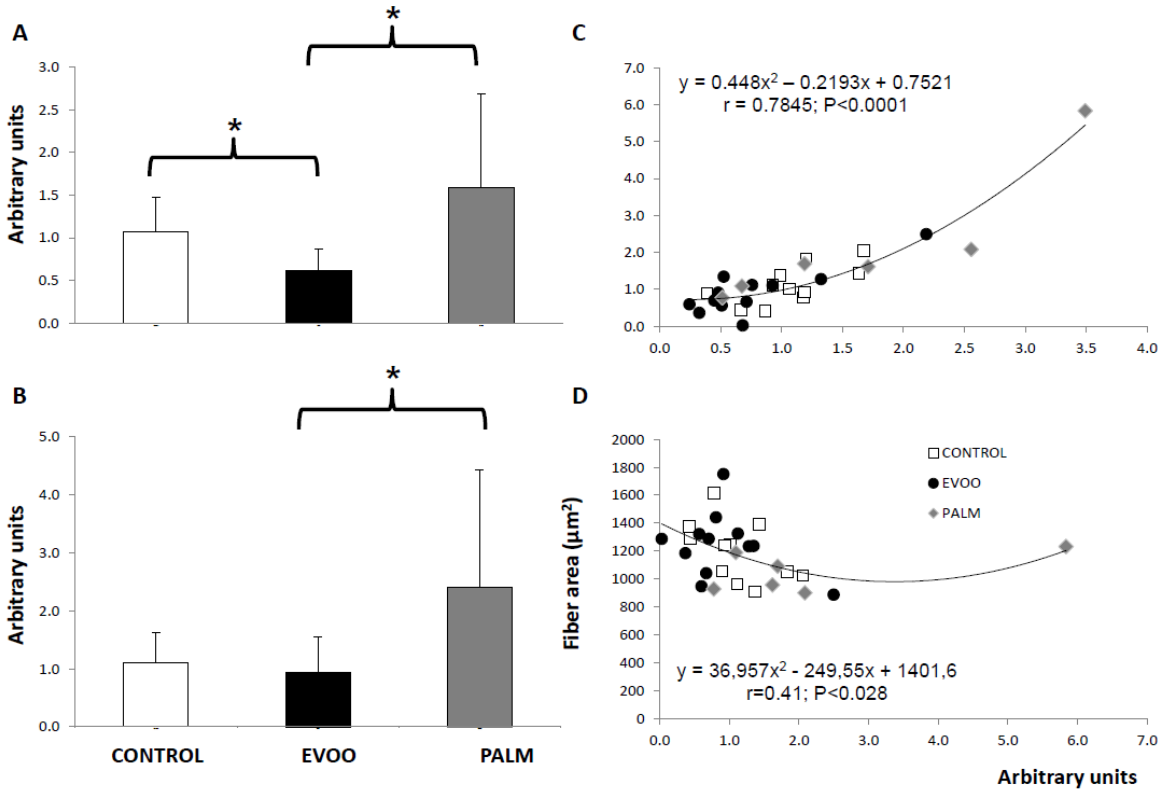


Fig. 5