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Changes in Motor Unit Loss and Axonal Regeneration Rate in Sporadic and Familiar Amyotrophic Lateral Sclerosis (ALS) – Possible Different Pathogenetic Mechanisms?

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1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal, neurodegenerative disorder affecting upper and lower motor neurons; it's the commonest of the motor unit (MU) diseases in Europe and North America, characterized by a broad spectrum of clinical presentations [1, 2]. Striking asymmetry and selective involvement of individual groups of muscles, especially of hand and forearm, are typical early features of the disease. On average, delay from onset of symptoms to diagnosis is about 14 months and expected survival commonly ranges from months to a few years [3].

Five to ten percent of cases are familial and about 20% of these families have point mutations in the Cu/Zn superoxide dismutase-1 (SOD-1) gene. In mammals, there are three SOD isoenzymes [4]: the cytosolic SOD1 (Cu/Zn-SOD), whose mutations are associated with familiar ALS, the mitochondrial Mn-SOD (SOD-2) and the secreted extracellular SOD (SOD-3). Most mutations in SOD-1 gene are autosomal dominant in inheritance, but there is one confirmed autosomal recessive mutation, predominant in Scandinavian ancestry, the D90A mutation in exon 4 [5].

More than 130 mutations in SOD-1 have been identified so far [6, 7]. Superoxide dismutase (SOD-1) is a well characterized enzyme, which exists as a homodimer whose sequence of 153 amino acids is remarkably well conserved across species. Sporadic and familial forms of the

disease are clinically indistinguishable, suggesting they may share common mechanisms, but the pathogenic mechanisms underlying disease's induction in familial cases are still largely controversial. The prevailing hypothesis is that familial ALS, SOD-1 positive, could be caused by a neuronal damage due to a gradual accumulation of a toxic product SOD-1 derived; this cumulative damage leads to a disruption of the cytoskeleton and organelle trafficking within motor neuron dendrites. As the amount increases, a critical threshold may be reached, which overwhelms cellular homeostasis resulting in fast cell death [8, 9]. Aggregates do not exclusively occur in neurons, but also in glial cells, raising the question whether mutant SOD-1 expression in neurons is sufficient *per se* to induce pyramidal degeneration and sustain disease evolution over time [10-12]. Little is known about the differences both in motor unit loss and axonal regeneration rate between sporadic and familial ALS and whether these changes underlie different pathogenetic mechanisms could represent a fascinating topic of debate.

2. Motor unit changes in familial ALS: What did we learn from animal studies?

To date, the more exhaustive study on the morphological differences between wild-type (WT) and transgenic SOD-1 motorneurons was made by Amendola and Durant [13]. By analyzing the arborizations of motorneurons in SOD-1^{G85R} mutant mice, they showed:

- i. a dramatic increase in the total dendritic length;
- ii. a significant proliferation of dendritic branches;
- iii. a greater dendritic membrane area, as confirmed by intracellular recordings revealing a lower input resistance when compared with WT cells.

However, it's unclear whether these changes represent early compensatory modifications or a disease mechanism. Previous evidence emphasized an increased ratio of inhibitory to excitatory synapses in organotypic slice cultures derived from embryonic spinal cords of SOD1^{G93A} mice [14] and a dampening in cholinergic transmission was also described in the lumbar spinal cord from adult SOD1^{G93A} mice [15]. Conversely, an intrinsic hyperexcitability of mutant SOD1^{G93A} spinal motorneurons was found in culture and in organotypic slice cultures [16, 17]. However, as the cells were not recorded from until they had been cultured for several weeks, the exact time course and progression rate of these changes are still largely obscure. Recently, Bories and colleagues showed motorneuron dysfunction appears centrally long before axonal degeneration [18], suggesting a pivotal role of these morphological changes in the core of disease mechanism.

Schwindt and Crill [19-21] proved that motorneurons have persistent inward currents (PICs) able both to potentiate and prolong synaptic firing rate after supraspinal input stopped: these currents are mainly generated in the dendritic regions, suggesting that motorneurons dendrites are not passive but active integrators of motor control. These conclusions fit with data in animals showing an increase in dendritic arborization in SOD-1 mutant mice compared with WT cells. High energy demands, due either to altered motorneuron

excitability or dendritic overbranching, destabilize calcium homeostasis [22-24]. These all changes make motor cells more susceptible to the axon transport, mitochondria and metabolic dysfunctions prominent in ALS, as motorneurons become heavily dependent on mitochondria for Ca⁺⁺ buffering [25, 26].

In humans, these data were only in part reproduced by the pioneering study of Aggarwal [7, 27]. By evaluating MU changes in 87 subjects carrying mutations in SOD-1 gene, he showed that asymptomatic carriers of the SOD1 mutations, different from patients with sALS, have no significant difference in the number of motor neurons when compared with age and sex matched controls; as symptoms develop, a sudden and catastrophic loss of MU occurs. However, the significance of these differences is still largely misunderstood.

3. Use of Motor Unit Number Estimation (MUNE) and Macro-electromyography (Macro-EMG) in the diagnosis and management of ALS: A brief historical overview

Clinical neurophysiology in ALS plays a fundamental role both in the diagnosis of suspected disease and in the assessment of its severity and progression, offering a promising tool to quantify muscle involvement and evaluate response to therapy [28-30]. Electromyography (EMG) investigation, usually performed with concentric needle electrodes [31], plays an essential role in the diagnosis and monitoring of ALS [32-34]. Amplitude, duration, area, shape, stability on repeated discharges of MU and activity at full effort are parameters conventionally used to evaluate disease's stage. EMG may also assess the presence of activity of the denervation-reinnervation process and number of functioning motor units by evaluating recruitment-activation pattern [28, 35]. In Motor Neuron Diseases (MND), standard needle electromyography often reveals evidence of chronic reinnervation (increased motor unit action potential amplitudes and duration, with reduced recruitment), eventually associated with fasciculations and signs of denervation activity in progress, but provides little information about the extent of both motor neuron loss and axonal regeneration. The supramaximal CMAP amplitude also provides little evidence of the extent of motor neuron loss and normal CMAP amplitudes might mistakenly suggest that motor neuron loss has not occurred yet [36, 37].

A particular method to evaluate the full MU is the so-called macro-EMG [38-41]. This technique provides information from a larger area of the muscle than traditional needle EMG methods. The signal is recorded by most of the fibers inside the entire MU and is often employed to follow the degree of reinnervation. That represents a quantitative technique and can be applied to follow progression and study of putative therapies [33, 42] by evaluating size of individual MU [39, 43].

Among quantitative electrodiagnostic (EDX) techniques, the methodology of Motor Unit Number Estimation (MUNE) has been previously and widely employed in measuring loss of functioning MU in ALS patients [36, 44-49].

MUNE is very sensitive in documenting disease progression in ALS. Some studies combining MUNE and standard electromyography showed a highly significant correlation between motor unit loss, clinical quantitative features and changes in compound motor action potential (CMAP) amplitude over time [50]. That is not surprising considering their different targets; while MUNE assesses motor unit loss, changes in CMAP amplitude and duration also account for collateral reinnervation. A few longitudinal studies using MUNE in some ALS patients have been reported that MUNE decreases as the disease progresses and that MUNE is a very reliable and reproducible method in patients with ALS [36, 51-55]. Its inter-individual and intra-individual reproducibility linearly increases as disease progresses, making this technique particularly useful in the symptomatic stage of the disease [36, 55-57].

We routinely use the standard incremental technique, known as the McComas technique. Despite some limitations in comparison with statistical MUNE (alternation of motor unit, inability to recognize small motor units, small sample size), it is more reliable and less complex; in addition, statistical MUNE cannot identify instable MUPs since it is based on the assumption that variability is due solely to the number of motor units responding in an intermittent manner [58]. More recently, Shefner and colleagues proposed a new method to follow over time motor unit loss in patients with ALS [59]: nerves were stimulated at 3 specified locations and 3 increments were obtained at each location. Average single motor unit action potential (SMUP) amplitude was calculated by adding the amplitude of the third increment at each location and dividing by 9; SMUP was divided into maximum CMAP amplitude to determine the MUNE. This approach needs further validation, but has some unquestionable advantages: it's easy to perform, well tolerated by patients and specialized equipment is not necessary. Most important, by applying the multipoint method MUNE values decline rapidly in patients with ALS, although the rate of decline is similar to that obtained with the standard incremental technique.

Use of Macro-EMG is limited to muscles from which electrical activity can be elicited without any interference from other muscles [60]; moreover, it's difficult to perform it in the hands during the course of the disease due to the strong wasting of the intrinsic hand muscles. Because of these limitations, our twenty-years experience led us to combine the two techniques in order to improve diagnostic sensitivity each other.

4. Different motor neuron impairment and axonal regeneration rate in patients with sporadic or familial Amyotrophic Lateral Sclerosis with SOD-1 mutations

4.1. Background and methodological considerations

In a previous study [61] we found that ALS patients with SOD-1 mutations have a higher number of MU at moment of diagnosis when compared with sporadic cases, as previously emerged from the work of Aggarwal in pre-symptomatic SOD-1 mutations carriers [27, 62]. Compared with previous studies, our innovatory ideas were:

- i. taking into consideration simultaneously Macro-EMG and MUNE changes in proximal and distal muscles in the same sample of patients;
- ii. following all our patients with a one-year follow-up;
- iii. evaluating Macro-EMG and MUNE changes both in sporadic and familiar cases (sALS and fALS).

In the group of 15 symptomatic SOD-1 mutation carriers, two were found to have a point mutation in exon 4, codon 100, GAA to GGA-Glu100Gly; two were found to have a point mutation in exon 4, codon 113, ATT to ACT-Ile113Thr; five were found to have a point mutation in exon 5, codon 148, GTA to GGA-Val148Gly, and six with homozygous for aspartate to alanine mutations in codon 90 (homD90A), representing the most common SOD-1 mutation with a typical recessive fashion inheritance. Sixty ALS patients (34 males: mean age \pm SD 60.0 \pm 15.5 years; 26 females: mean age \pm SD 62.0 \pm 9.2 years) were enrolled in the study and examined basally (T0) and every 4 months (T1, T2, and T3). Fifteen of these patients are familial (SOD-1 mutation carriers, 9 males: mean age \pm 1SD 46.3 \pm 14.8 years; 6 females: mean age \pm 1SD 49.0 \pm 8.5 years). Macro Motor Unit Potentials (macro-MUPs) were derived from Biceps Brachialis (BB) muscle; MUNE was performed both in BB and Abductor Digiti Minimi (ADM) muscles of the same side. Thirty-three healthy volunteers (13 females and 20 males, mean age: 57.7 \pm 13.8 years) served as controls. All patients had probable or definite ALS, according to the well known criteria of the World Federation of Neurology [18]. The sample group of patients included cases with a disease duration from clinical onset of symptoms to the time of the first examination less than 48 months (mean \pm SD: 12.2 \pm 11.0 months). Twenty-two patients presented a bulbar onset and the remaining a spinal one. As concerns symptoms and signs, among SOD-1 mutation carriers 10 have the spinal type, while only 5 patients have the bulbar type. Forty patients were in treatment with riluzole (Rilutek[®], 50 mg) at a mean daily dosage of 100 mg throughout the period of EDX follow-up.

Standard macro-EMG method was applied [39]. The SFEMG recording surface was exposed 7.5 mm from the tip and the recording was made using two channels: the first one in whom the SFEMG activity was displayed (using the cannula as reference) and used to identify the MU and trigger the averaging procedure (band-pass filter for this channel: 500-10KHz); fiber density (FD) of the triggering single fibre electrode was recorded. The second channel averaged the activity from the cannula until a smooth baseline and a constant macro MUP was obtained (Filter pass-band: 5-10KHz). We measured from the averaged signal the total area between the curve and the baseline, the maximal peak-to-peak amplitude (macro-MUP) during the total sweep time of 70ms [63]. Results were expressed as individual area values from at least 20 trials. The relative macro amplitude was expressed as the obtained mean value [39]. Fibre density was expressed as number of time locked spikes obtained on the SFEMG channel [64]. In twenty-nine patients (subgroup 1: 19 males and 10 females; mean age \pm 1SD: 60,0 \pm 11,8 years; spinal/bulbar onset: 22/7; mean disease duration 29,7 months) macro-EMG was repeated after 4 months (T1). Among the second subgroup, eleven patients (subgroup 2: 8 males and 3 females; mean age \pm SD: 57,0 \pm 12,8 years; range 30–72 years; spinal/bulbar onset: 10/1; mean disease duration 31 months) were re-tested after 8 months (T2) and in 8 (subgroup 3; 7 males

and 1 female; mean age \pm SD: $58,0 \pm 13,6$ years; spinal/bulbar onset: 7/1; mean disease duration 37 months) after 12 months from the first examination.

MUNE technique was performed on the same Keypoint[®] EMG equipment (Medtronic Dantec, Copenhagen) provided with specific software for data acquisition and processing at same time and immediately after macro EMG on the same test session. The used technique relayed on manual incremental stimulation of the motor nerve, known as the McComas technique [46]. The use of specific software for MUNE detects “alternation”, eliminates subjectivity and the sampling of artifactually small motor units in ALS patients [36, 46, 54]. Percutaneous stimuli were delivered over musculocutaneous nerve immediately below axilla, recording from BB muscles, and ulnar nerve at the wrist by recording from the ADM muscle of the same upper limb [36]. Signals were detected with common surface electrodes, Ag/AgCl type, tapered on the cutis over target muscles with a common muscle-belly tendon montage.

4.2. Main findings and possible explanations

MUNE values in ALS patients were behind normal limits in 55 (91.7%) and within normal limits in 5 (8.3%) in biceps brachialis (BB) muscle; in 58 (96.7%) and in 2 (3.3%) in ADM muscle, respectively [36]. In brief, we can summarize our findings in two main points:

- i. MUNE revealed a normal amount of motor units in fALS at the moment of diagnosis, followed by a dramatic loss of motor units, more pronounced than in patients with sALS (see Figure 1, top panels);
- ii. Macro-EMG in SOD-1 fALS showed increased fiber density and area values when compared with patients with sALS, likely suggesting a paradoxical more effective axonal sprouting in fALS (Figure 1, bottom panels).

Functioning MUs number progressively decreased in both muscles throughout the entire follow-up period. In ALS MUNE exhibited a parallel trends in proximal and distal muscles (BB and ADM), independently of disease duration; mean step area, instead, increased more in BB, especially in patients with longer disease duration. The MUNE's results as concerns patients with fALS, SOD-1 positive, were 80.2 ± 7.8 (T0), 21.8 ± 2.2 (T1), 16.8 ± 1.0 (T2) and 16.5 ± 2.2 (T3) for BB and 42.8 ± 6.6 (T0), 18.4 ± 3.1 (T1), 15.3 ± 2.1 (T2) and 9.0 ± 2.1 (T3) for ADM. Curiously, SOD-1 fALS patients showed a higher number of functioning motor units in the early stage of disease ($p < 0.001$) and a more dramatic drop in later phases (Figure 1). These results suggest a normal pool of motor units in asymptomatic familiar ALS carriers [27]. No electrodiagnostic difference was found between patients with different SOD-1 point mutations. Moreover, we did not find any significant difference between spinal and bulbar-onset fALS in terms of surviving MU, for both BB and ADM muscles ($p > 0.05$, Figure 2), as well as between males and females ($p > 0.05$, Figure 3).

In sALS patients at T0, both Macro-motor Unit Potentials (Macro-MUPs) area and fiber density (FD) were above upper normal limits (for a global overview of Macro-EMG in healthy subjects, see Sartucci et al., 2007 and 2011): macro-MUP area was 4397.6 ± 255.9 μ Vms, mean FD 2.0 ± 0.2 (a summary of results is given in Figure 2). The macro-MUP area was abnormal in 57 (95.0%) and normal in 3 (5.0%) patients; in SOD-1 carriers baseline values of MUP area and FD matched

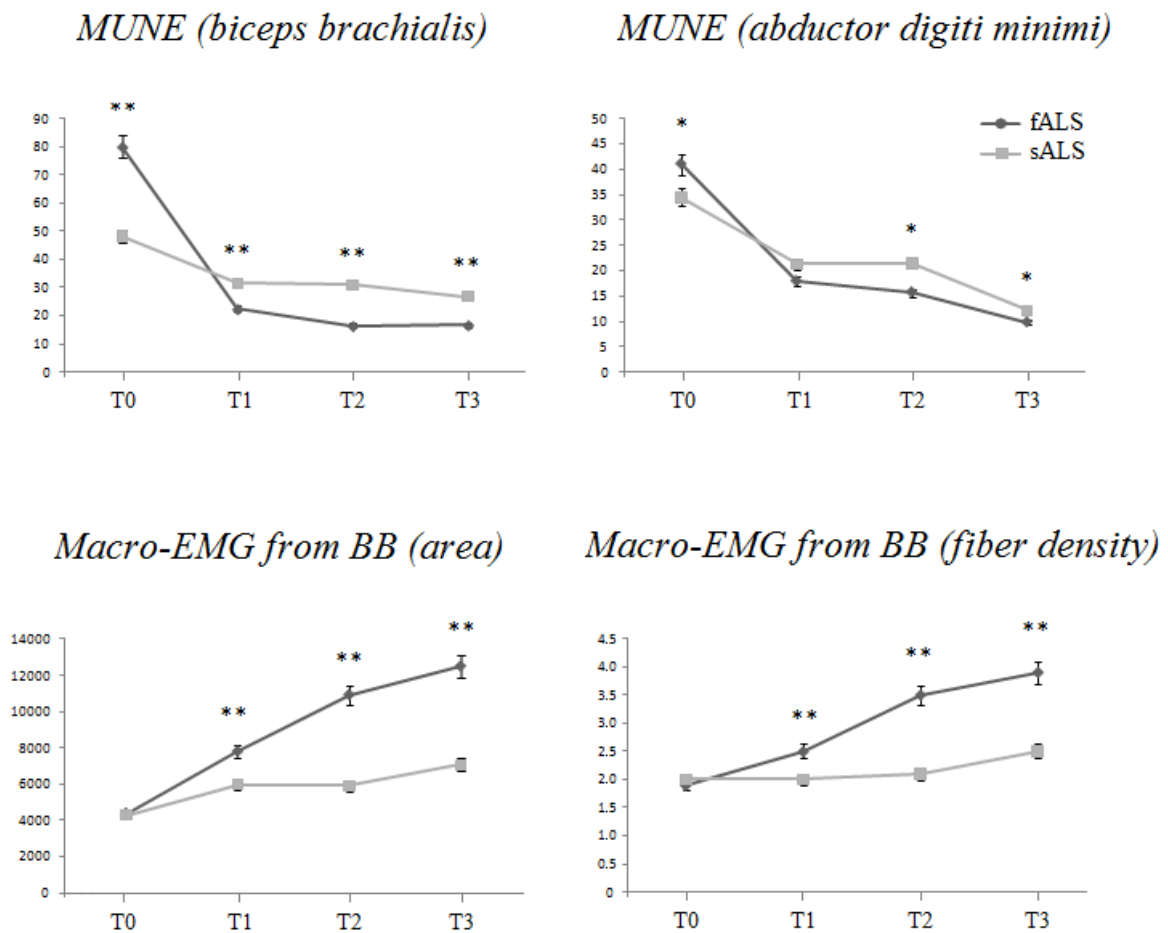


Figure 1. The top row shows MUNE values both for biceps brachialis, on the left, and abductor digiti minimi muscles, on the right, at different time points (at the moment of diagnosis and after 4, 8 and 12 months: T0, T1, T2 and T3). At the moment of diagnosis, motor units number is higher for familiar cases (black lines, fALS) compared with sporadic ones (gray lines, sALS). Bottom row shows time-trend of Macro-EMG parameters (fiber density, area) over time. All the values increase more steeply in familiar than in sporadic forms (black and gray lines, respectively), strengthening the idea that in the first group there is a paradoxical more effective axonal sprouting (modified from Bocci et al., *Int J Mol Sci* 2011; * $p < 0.05$; ** $p < 0.01$).

with those of sALS patients ($4378.9 \pm 319.6 \mu\text{Vms}$ and 1.9 ± 0.3 , for Macro-MUPs area and FD respectively; $p = 0.815$ and $p = 0.147$). In sALS, Macro-MUPs area resulted progressively increased at every time, especially at T3, compared with T0 (Figure 1, bottom panels): Area: +45.3% (T1); +49.0% (T2); +83.6% (T3); FD showed a trend to increase up to T3: +3.5% (T1); +15.4% (T2); +22.4% (T3). Interestingly, in SOD-1 carriers there was a much steeper increase at T1, T2 and T3 in respect to sporadic forms, as concerns both Macro-MUPs area and FD values. Macro-MUPs area was 7791.0 ± 953.4 , 10922.8 ± 1123.7 and 12499.3 ± 1874.4 ($p < 0.01$) μVms and mean FD 2.5 ± 0.3 , 3.5 ± 0.6 and 3.9 ± 0.5 ($p > 0.01$). As a whole, these results account both for a more severe involvement of alfa-motorneurons pool and a paradoxical more effective axonal sprouting in fALS compared with sALS.

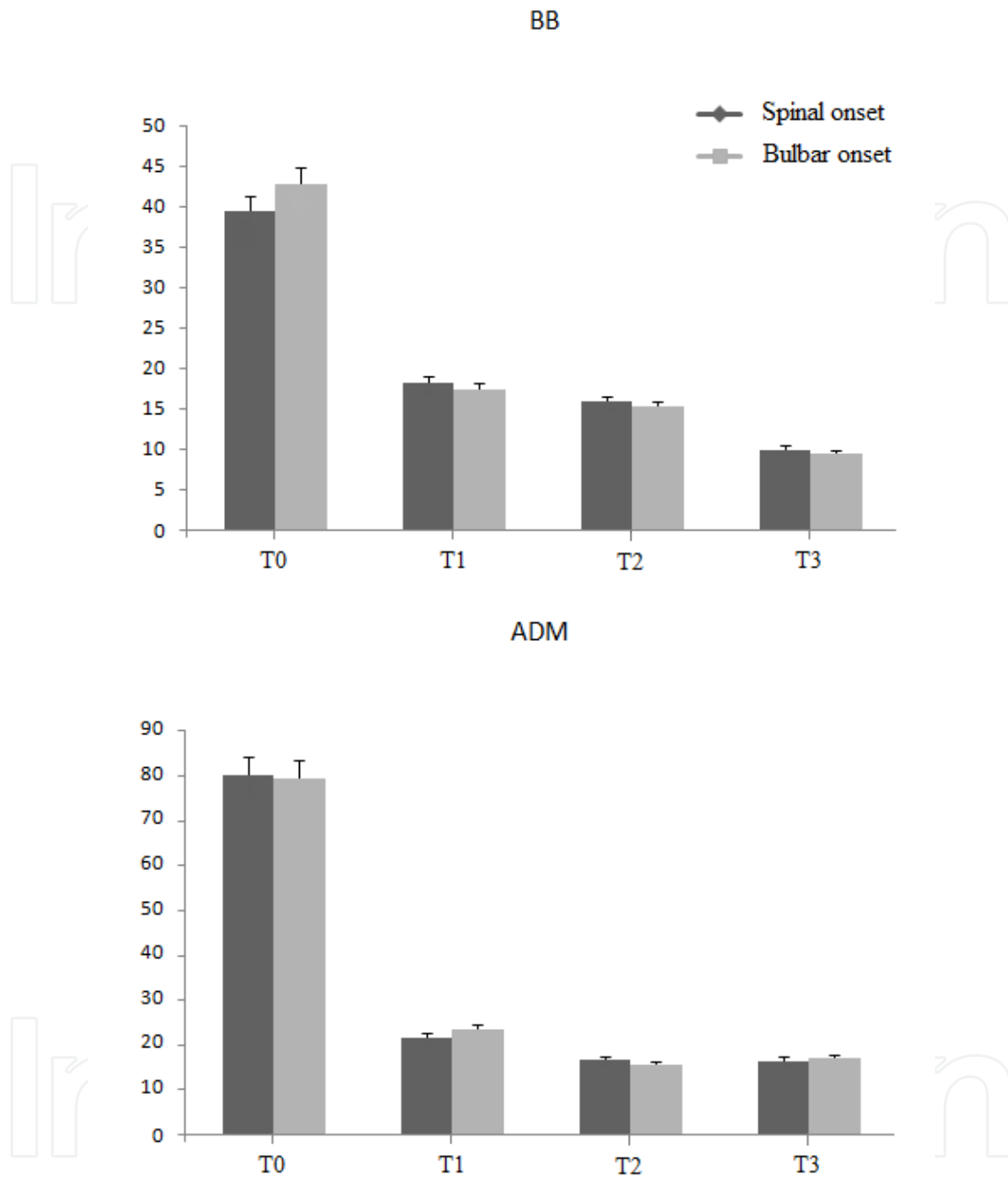


Figure 2. Histogram highlighting MUNE values in both BB (at the top) and ADM (bottom histogram) muscles at every time of follow-up, in males (gray columns) and females (black columns); the top row shows the evolution of motor unit loss in the familiar form, whereas the bottom one the trend in sporadic cases (modified from Bocci et al., *Int J Mol Sci* 2011).

Macro-MUP area and FD were beyond upper normal limits, as expected, in ALS [63, 65]. Our results indicate that carriers of SOD-1 mutations have a higher number of motor units at moment of diagnosis when compared with sporadic cases. On the other hand, in sALS the

macro-EMG parameters progressively increased, displaying a gradual increment of correlation up to 8 months, suggesting that the process of MU rearrangement begins to fall after 8 months of disease course. In familiar SOD-1 form there isn't a specific time interval in which the axonal regeneration and the collateral sprouting can balance the neuronal damage. Paradoxically, despite faster loss of motor units, in fALS we have undisclosed a more effective axonal sprouting in the few surviving motor fibers. Compared with sporadic forms, in SOD-1 fALS the substantial lack of a fleeting stabilization of motor unit number within eight months from clinical onset, as emerged from MUNE, could indicate that damage of cell types different from motor neurons is a critical factor for the progression of corticospinal degeneration [66, 67]. Our results strengthen the idea that accelerated disease progression does not alter the timing of disease onset. These data are consistent with those reported by Yamanaka and colleagues [67]: using chimeras derived from embryonic cells of SOD-1^{G37R} mice, they postulated that multiple cell types drive non-cell-autonomous onset of motor degeneration. That could also explain the wide variability in terms of age of onset, clinical presentation and rate of progression in familiar forms of ALS. This is in line with previous papers showing a differential pyramidal tract degeneration in homozygous SOD-1^{D90A} ALS and sALS [68-70]; e.g., Blain and colleagues have recently reported a marked reduction in fractional anisotropy in the corticospinal tract in patients with sALS and fALS, despite similar levels of upper motor neurons dysfunction and overall clinical disability [68]. ALS is featured by repetitive cycles of denervation/reinnervation and the mechanism lead to a variation in FD within a given motor unit [33, 42]. SOD-1 carriers had a full complement of motor neurons during the asymptomatic phase, indicating that SOD-1 mutation carriers have normal survival of motor neurons until sudden catastrophic cell death occurs. This significant gradual preclinical loss does not occur in SOD-1 mutation carriers. Despite the small sample of fALS patients, we also tried to detect significant differences in motor unit pool between spinal and bulbar forms, for both BB and ADM muscles (Figure 2). Interestingly, we did not found any difference suggesting the rate and amount of motor units decrease are approximately similar in proximal and distal muscles. That confirms the non length-dependent and all-or-none nature of pathological processes underlying progression of fALS. A possible explanation could be based on an epigenetics approach: it has been proposed that epigenetic silencing of genes vital for motor neuron function could underlie ALS [44,45]. The promoters of genes thought to be implicated in sALS, SOD-1 and VEGF, or that of MT-Ia and MT-II (the most common human isoforms of the metallothionein (MT) family of proteins), have been found with inappropriate methylation levels [46]. There's an increasing interest in this field, despite no conclusive remark has been collected in human models so far. That's likely due to the discrepancy between humans patients and animal models, in terms of disease and pre-symptomatic phase duration, absence of sensitive biological markers and different pathogenesis. Our findings agree with those described by Aggarwal both in symptomatic and asymptomatic SOD-1 mutation carriers [27]: symptomatic fALS could represent an all-or none process and it is not the final result of a slow attrition of motor neurons.

Another interesting finding is about the lack of significant differences in motor unit depletion over time between females and males in SOD-1 type, both in fALS and sALS form (see Figure 3): the antioxidant effects of estrogens and their proved role in preventing glutamate related

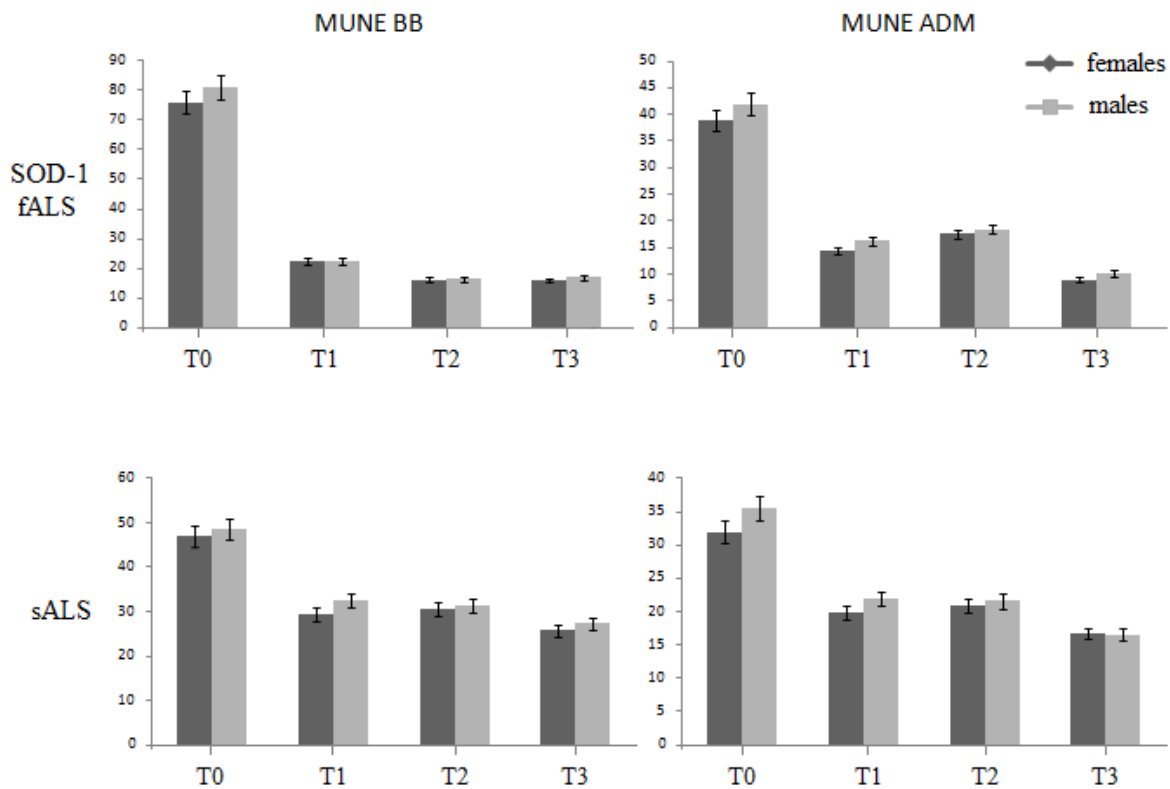


Figure 3. Histogram highlighting MUNE values in both BB (left) and ADM (right) muscles at every time of follow-up, in males (gray columns) and females (black columns); the top row shows the evolution of motor unit loss in the familiar form, whereas the bottom one the trend in sporadic cases. The lack of significant differences between males and females, in sporadic as well as in familiar forms, is consistent with results recently reported in recent literature [71, 72]. (modified from Bocci et al., *Int J Mol Sci* 2011: * $p < 0.05$; ** $p < 0.01$).

toxicity in vitro [71, 72] could not delay both the early retraction of nerve terminals from neuromuscular end-plates and the dying-back of axons during asymptomatic phase in vivo.

5. Conclusions and future directions

Although our preliminary results cannot be directly compared with those found in animals, these data could expand current knowledges about morphological and functional differences between mutant and wild type motoneurons in ALS.

We speculate that overbranching occurs not only in dendrites but also in the few surviving axons. This increased complexity of axonal arborization, compared both with healthy and sALS subjects, is still largely undervalued and whether that represents a pointless neuroprotective response of nervous system or a disease mechanism is an intriguing matter of debate. However, as suggested in animal models [73], our Macro-EMG data seem to suggest that overbranching might be one way to mitigate loss of function along corticospinal pathways. These evidences highlight a novel hypothesis for the adult onset of fALS symptoms, namely

that they result from age-related factors (e.g., neuron loss or other traumatic insults) that cause a breakdown of homeostatic compensatory processes for neuronal hyperactivity.

Further studies are needed to solve these dilemmas, especially in familiar forms different from those related to mutations pertaining to Cu/Zn superoxide dismutase gene. Particularly, it could be very interesting if a combined MUNE/Macro-EMG protocol was applied to subjects carrying mutations in C9ORF72 gene; these patients, although very rare in the Mediterranean area, typically have upper motor neuron-predominant variants, show memory and executive dysfunctions and account for about 30% of the cases of fALS [74-77]. Most important, the increasing interest in C9ORF72 mutations are due to the frequent association with extra-pyramidal features and Frontotemporal Dementia spectrum.

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