UNIVERSIDADE DE LISBOA

Faculdade de Medicina

MUNIX

A new method of motor unit number estimation

José Filipe Oliveira Castro

Mestrado em Neurociências

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ABSTRACT:

Since 1971, when McComas described the first neurophysiological technique for the estimation of the number of motor units in a muscle (MUNE), several other methods have been developed over the following decades. Each technique has its own advantages and disadvantages, but at the moment none of them has gathered enough consensus to arise as a predominant method.

In neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS), the possibility of monitor the loss of motor units throughout the course of the disease would be of the outmost importance, in particular in the context of clinical trials. In present time, the neurophysiological methods that we have at our disposal are not the most adequate ones to follow the progression in this kind of diseases.

Motor unit number estimation techniques would be an excellent measure of the loss of motor units in these patients. However, all of the techniques described have limitations that prevented them to become a primary endpoint in clinical trials.

In this work, we describe a new technique for estimating the number of motor units in a muscle that it's called Motor Unit Number Index (MUNIX). We assess test-retest variability and evaluate the suitability of this technique as a potential marker of disease progression in ALS.

A group of 15 normal subjects was studied two times by two different raters to assess intra and inter rater variability. Overall reliability results were reasonably good (MUNIX megascore ICC=0.740).

A group of 11 ALS patients was studied over 9 to 12 months at regular intervals. We compared MUNIX to other known disease progression markers such as compound muscle action potential (CMAP), ALSFRS-R and muscle strength, and to another MUNE technique. MUNIX declined significantly with time ($p < 0.001$) and had higher progression rates than ALSFRS-R and muscle strength ($p = 0.005$).

We also compared MUNIX with multiple point stimulation MUNE in the abductor digiti minimi of ALS patients. MUNIX showed a significantly higher progression rate, with a steeper decline than that other MUNE method, showing that it

can a suitable technique for estimating the number of motor units and to monitor its loss in the course of neurodegenerative diseases.

Key-words: MUNIX, MUNE, Motor unit, Amyotrophic Lateral Sclerosis, ALS, Disease progression

RESUMO:

Desde 1971, quando McComas descreveu o primeiro método neurofisiológico para estimar o número de unidades motoras num músculo (MUNE), vários outros métodos foram desenvolvidos. Cada técnica possui as suas vantagens e desvantagens mas, até agora, nenhuma reuniu aceitação generalizada de forma a se assumir como o método predominante.

Em doenças neurodegenerativas, como a Esclerose Lateral Amiotrófica (ELA), a possibilidade de avaliar a perda de unidades motoras durante o curso da doença é de extrema importância, particularmente em ensaios clínicos. Actualmente, os métodos neurofisiológicos de que dispomos não são os mais adequados para quantificar a progressão deste tipo de doenças.

As técnicas de estimativa de unidades motoras assumem-se como uma excelente medida de avaliação de perda de unidades motoras nestes doentes. No entanto, todas estas técnicas apresentam limitações que as impedem de serem consideradas como um *endpoint* primário em ensaios clínicos.

Neste trabalho, descrevemos um novo método para estimar o número de unidades motoras num músculo, denominado Motor unit number index (MUNIX). Fomos avaliar a variabilidade intra- e inter-utilizador, bem como a sua adequação como um potencial marcador de progressão de doença na ELA.

Um grupo de 15 indivíduos saudáveis foi avaliado duas vezes por dois avaliadores independentes de forma a avaliar a reprodutibilidade do método. Globalmente, a reprodutibilidade do método foi bastante satisfatória (ICC Megascore MUNIX=0.740).

Um grupo de 11 doentes com ELA, foi avaliado durante 9 a 12 meses em intervalos regulares. Comparámos o MUNIX com outras medidas de progressão da doença já descritas, tais como o potencial de acção muscular composto (CMAP), a escala ALFRS-R e o grau de força muscular, assim como com outro método de estimativa de unidades motoras. O MUNIX progrediu significativamente ao longo do tempo ($p < 0.001$) e teve uma maior taxa de progressão comparativamente à ALSFRS-R e ao grau de força muscular ($p = 0.005$).

Posteriormente, comparámos o MUNIX com o *multiple point stimulation* MUNE no músculo abdutor do 5º dedo nos doentes de ELA. O MUNIX revelou uma taxa de progressão significativamente maior, com um declínio mais linear do que este outro método de MUNE, demonstrando que é uma técnica adequada para estimar o número de unidades motoras e avaliar a sua perda ao longo do curso de doenças neurodegenerativas.

Palavras chave: MUNIX, MUNE, unidade motora, Esclerose lateral amiotrófica, ELA, progressão de doença

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1. INTRODUCTION

1.1 Motor unit

History and anatomical studies

The first time the concept of "motor unit" was mentioned in the literature was in 1925, by Sir Charles Sherrington (Liddell and Sherrington, 1925). He defined motor unit as the "axon-motoneuron and its adjunct muscle fibers" and drew attention for its all-or-none responsiveness. Four years later, Sherrington (1929) redefined motor unit as "the axon and the group of muscle fibers it activates. Each such "motor unit" has centrally, of course, a nerve-cell of which a group or "pool" represents the muscle in the spinal cord". In fact, over 80 years have passed since the dawn of this groundbreaking concept, and we still define motor unit as the aggregate of an axon and the muscle fibers it innervates.

Eccles and Sherrington (1930), with their optical recording system, were the first to investigate the number of motor units in muscles of the cat hindlimb. The dorsal nerve root ganglia were excised and, after the time needed for the sensory fibers to degenerate has passed, the surviving myelinated fibers were added up and assumed to be motor. They obtained values around 640 for the semitendinosus, 430 for the medial gastrocnemius and 250 for the soleus. Albeit this pioneer study, and even taken in consideration the recognition of the distribution of myelinated nerve fibers in two groups, the authors failed to distinguish between the thinner (γ) axons that supply the small muscle fibers in the muscle spindles, and the thicker (α) axons that innervate skeletal muscle fibers. Only a few years later (Leksell, 1945) this problem was acknowledged.

The first anatomical studies of human motor units were by Feinstein (Feinstein et al., 1955). Although it was a tedious task to count stained cross-sections of large myelinated fibers from cadavers' nerves, the greatest difficulty was to decide the proportion of α-motor and sensory axons. After comparing the counts made with a cross-section of a patient who died after having a severe case of poliomyelitis, the authors proposed the ratio of 60:40 (α-motor/sensory axons)

This ratio was used in the vast majority of anatomical studies that were reported in the following decades. In table 1 (Sica and McComas, 2003) we can see an overall of anatomic studies in various human muscles

Table 1: Anatomical estimation of numbers and sizes of motor units in various human muscles *(Sica and McComas, 2003)*

Muscle	Reference	Nº of motor units	Muscle fibers/unit
External rectus	Feinstein et al. (1955)	2970	9
Superior rectus (2)	Christensen (1959)	1779	23
Temporalis	Carlsöö (1958)	1331	936
Masseter (2)	Carlsöö (1958)	1425	2373
Platysma	Feinstein et al. (1955)	1096	25
Biceps Brachii	Christensen (1959)	3552	163
Brachioradialis	Feinstein et al. (1955)	333	>410
Opponens Pollicis	Christensen (1959)	6047	13
Thenar (median n.)	Lee et al. (1975)	203	--
First dorsal interosseous	Feinstein et al. (1955)	119	340
First lumbrical	Feinstein et al. (1955)	96	108
Abductor digiti minimi (10)	Santo Neto et al. (1985)	380	190
Opponens digiti minimi (4)	Carvalho et al. (1988)	158	100
Flexor digiti minimi (10)	Santo Neto et al. (1998)	130	108
Sartorious (2)	Christensen (1959)	740	300
Rectus femoris	Christensen (1959)	609	305
Gracilis	Christensen (1959)	275	527
Semitendinosus	Christensen (1959)	712	713
Med. + lat. Gastrocnemii	Christensen (1959)	778	2037
Med. Gastrocnemius	Feinstein et al. (1955)	579	1934
Tibialis anterior	Feinstein et al. (1955)	445	562
Plantaris (5)	Carvalho (1976)	204	372

Note: Unless otherwise indicated, values derived from single muscle

Although the results of these studies are more or less similar, they are different enough to prevent the designation of a true standard value for the number of motor units in human muscles. On top of that, is now clear from other anatomical studies (Boyd and Davey, 1968), that the ratio of motor to sensory axons varies greatly between muscles. Therefore, the 60:40 ratio proposed by Feinstein is, at the best, uncertain.

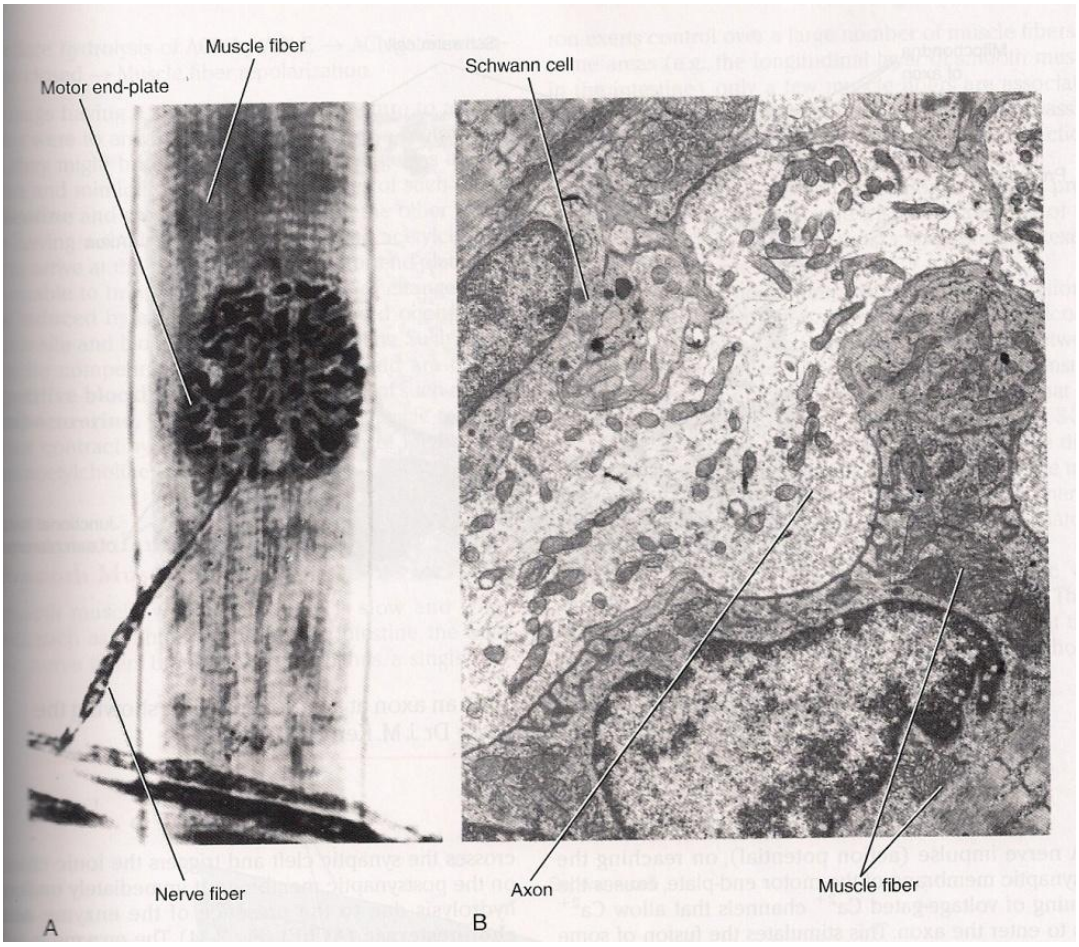
Nevertheless, these histological studies can serve as baseline comparison for the physiological motor unit number estimation (MUNE) techniques that have been developed.

Physiology of the motor units

Skeletal muscle fibers are innervated by large myelinated nerve fibers derived from alpha motor neurons (α-MNs) of the spinal cord and brainstem. The cell bodies of the α-MNs are localized in the anterior gray horns in the spinal cord or in the motor nuclei in the brainstem. The axon of each motor neuron exits the spinal cord through a ventral root (or through a cranial nerve from the brainstem) and traverses progressively smaller branches of peripheral nerves until it enters the muscle it controls. As each myelinated fiber enters a skeletal muscle, it branches many times, each branch terminating on a muscle fiber at a site named neuromuscular junction.

Each motor unit innervates exclusively muscle fibers of the same type. The number of branches depends on the size of the motor unit and can go from 100 up to 1000 muscle fibers scattered over the muscle, depending mostly on the function of this muscle. In muscles with more refined motions, less muscle fibers each unit innervates, and vice-versa.

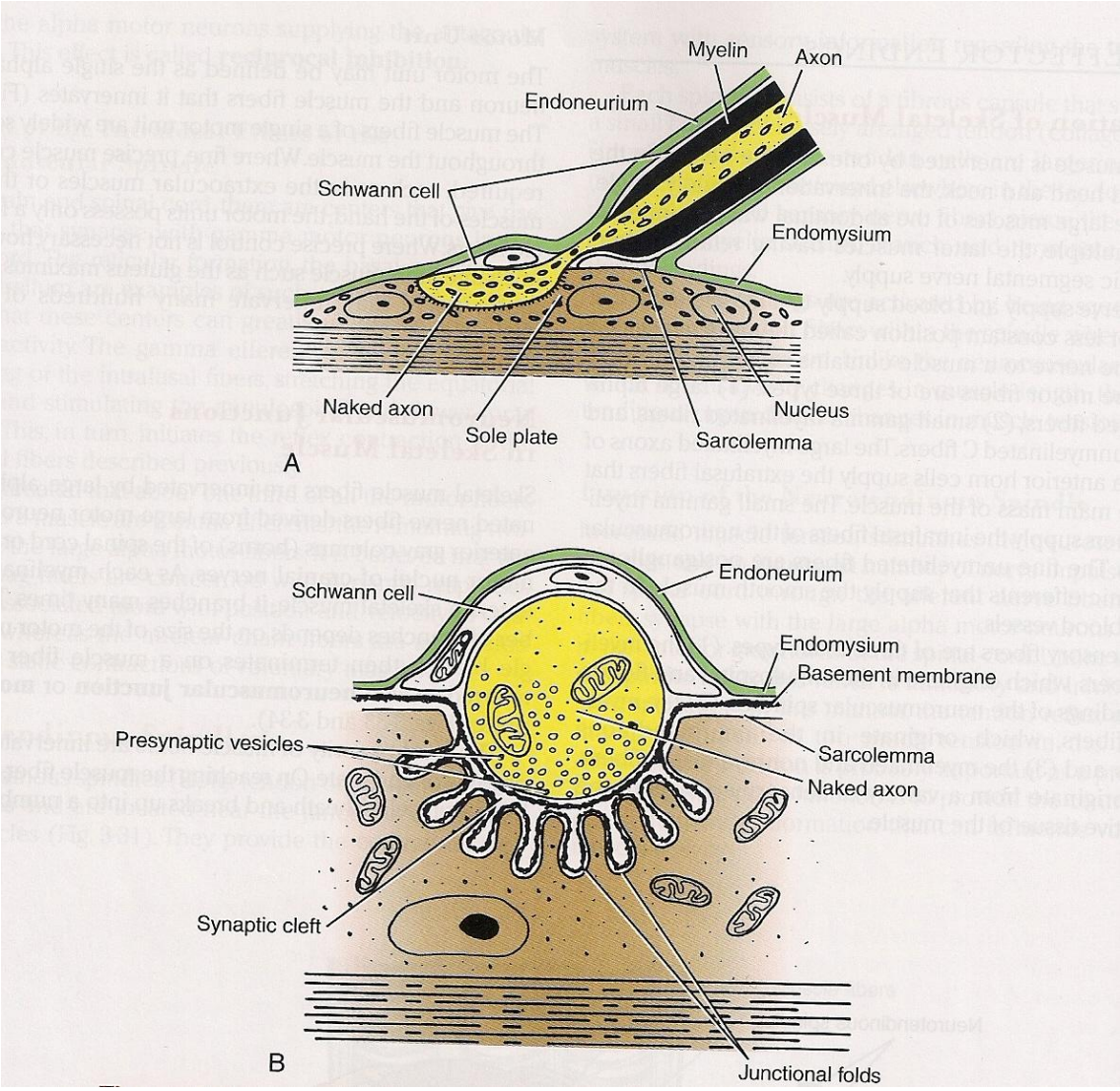
Figure 1
A - Photomicrograph of a motor end plate showing terminal branching of a nerve fiber.
B - Electron micrograph of a terminal axon at a motor end-plate
Clinical Neuroanatomy; Richard Snell, 2010



Near the motor end-plate, the nerve branch ends as a naked axon (presynaptic membrane), with no myelin surrounding it, since the Schwann cells serve only as a cap, never projecting into the synaptic cleft. At this point, the axon is slightly expanded, and has many mitochondria and acetylcholine (ACh) vesicles.

At this site, the surface of the muscle fiber is slightly elevated due to the accumulation of granular sarcoplasm and the presence of numerous mitochondria and nuclei.

Figure 2:
A - A skeletal neuromuscular junction
B - Enlarged view of a muscle fiber showing the terminal naked axon
Clinical Neuroanatomy; Richard Snell, 2010



When an action potential travels through the nerve and arrives at the presynaptic membrane, Ca^{2+} enter the axon, via Ca^{2+} voltage dependent channels, and begins a cascade that leads to the release of ACh to the synaptic cleft.

The ACh then binds to postsynaptic-nicotinic-type ACh-gated channels and Na^{+} ions flow into the muscle cell giving origin to an action potential that spreads along the sarcolemma into the contractile myofibrils. This leads to the release of Ca^{2+} ions from the sarcoplasmic reticulum, which, in turn, causes the muscle to contract.

Since a single action potential in an α-motor neuron can activate dozens, or even hundreds, of muscle fibers synchronously, the resulting currents sum to generate an electrical signal that is easily recorded outside the muscle itself. The superficial recording of this large electrical field, generated by the activation of the muscle fibers, is the basis of Electromyography (EMG), Nerve conductions studies (NCS) and MUNE techniques.

1.2 Amyotrophic Lateral Sclerosis

Despite the fact that MUNE techniques could be useful in many neurodegenerative diseases like Amyotrophic Lateral Sclerosis (ALS), Spinal muscular atrophy (SMA), Poliomyelitis or even different types of Peripheral neuropathies, the focus of this work will be on ALS.

Definition

ALS, also known as Motor neuron disease (MND) or Lou Gehrig's disease, is a fatal neurodegenerative disorder of the motor system, characterized by progressive muscular paralysis reflecting degeneration of motor neurons is the primary motor cortex, brainstem and spinal cord (Wijesekera and Leigh, 2009). The term "Amyotrophy" represents the loss of muscle fibers due to denervation caused by degeneration of the anterior horn cells. "Lateral sclerosis" represents the replacement of the corticospinal tract by gliosis as the result of cortical motoneurons degeneration (Rowland and Shneider, 2001).

Diagnostic and classification criteria

The diagnosis of ALS can be challenging. Not only there are a number of potentially mimicking diseases (*e.g.* Cervical radiculomyelopathy), but also there is no specific biomarker. Therefore, the diagnosis is based on a collection of some very characteristic clinical findings in combination with examinations to document signs of lower and/or upper motor neuron signs and to rule out other conditions.

The first set of clinical criteria for the diagnosis of ALS was developed in 1994 by the World Federation of Neurology (Brooks, 1994), the "El Escorial" diagnostic criteria. These criteria were revised a few years later (Brooks et al., 2000), the "Airlie House" criteria. According to this last set of criteria, patients can be classified as summarized in table 2 (Brooks et al., 2000).

Table 2: Summary of Revised El Escorial Research Diagnostic Criteria for ALS *(Wijesekera and Leigh, 2009)*

The diagnosis of ALS requires: 1 Evidence of LMN degeneration by clinical, electrophysiological or neuropathological examination; 2 Evidence of UMN degeneration by clinical examination, and 3 Progressive spread of symptoms or signs within a region or to other regions, as determined by history or examination, Together with the absence of: [1] Electrophysiological and pathological evidence of other disease that might explain the signs of LMN and/or UMN degeneration, and [2] Neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs
Categories of clinical diagnostic certainty on clinical criteria alone Definite ALS • UMN signs and LMN signs in 3 regions Probable ALS • UMN signs and LMN signs in 2 regions with at least some UMN signs rostral to LMN signs Probable ALS – Laboratory supported • UMN signs in 1 or more regions and LMN signs defined by EMG in at least 2 regions Possible ALS • UMN signs and LMN signs in 1 region (together), or • UMN signs in 2 or more regions • UMN and LMN signs in 2 regions with no UMN signs rostral to LMN signs

UMN (Upper Motor Neuron) signs: clonus, Babinski sign, absent abdominal skin reflexes, hypertonia, loss of dexterity.
LMN (Lower Motor Neuron) signs: atrophy, weakness. If only fasciculation: search with EMG for active denervation.
Regions reflect neuronal pools: bulbar, cervical, thoracic and lumbosacral.

In 2008, a consensus meeting was held by a group of experts and a new set of rules to define the electrophysiological diagnosis of ALS was recommended, the Awaji criteria (de Carvalho, 2008). These criteria simplified the previous ones and highlighted the importance of the fasciculation potentials in the diagnosis of ALS. Table 3 summarizes the modifications introduced by the recent Awaji recommendations.

Table. 3 – Comparison between the revised El Escorial criteria and the Awaji set of recommendations

1. Principles of the Revised El Escorial Criteria
The diagnosis of ALS requires
A: evidence of lower motor neuron (LMN) loss (reduced interferential pattern on full contraction and increased firing rate) B: evidence of reinnervation (motor units of large amplitude and longer duration) C: fibrillation and sharp-waves
2. Principles of the Awaji-shima Consensus Recommendations
The diagnosis of ALS requires
A: evidence of lower motor neuron (LMN) loss (reduced interferential pattern on full contraction and increased firing rate) B: evidence of reinnervation (motor units of large amplitude and longer duration) C: fibrillation and sharp-waves OR fasciculation potentials (fibrillation and sharp-waves are required in weak limb muscles).
3. Number of muscles affected by region
Cervical and Lumbar-sacral region A minimum of two muscles innervated by different roots and nerves
Bulbar and Thoracic region A minimum of one muscle

Clinical features

The first author to clearly recognize ALS as a clinico-pathological entity was the renowned French neurologist Jean Martin Charcot in 1869 (Charcot, 1869).

Roughly, two thirds of typical ALS patients present with a spinal form of the disease. The initials symptoms are typically focal muscular weakness of insidious onset, either proximally or distally in upper and/or lower limbs. Muscle wasting may precede focal weakness, and sometimes fasciculations or cramps may appear months before any weakness. These symptoms are usually asymmetrical (monomelic) at start, but eventually progress to the other limbs, and most patients go on developing bulbar and respiratory symptoms.

Bulbar onset patients usually notice difficulty speaking clearly or swallowing, which eventually evolves to severe dysarthria or dysphagia. Limb symptoms eventually occur within some months of the first complaints.

A smaller proportion of patients (≈ 3%) present with respiratory weakness without significant limb or bulbar symptoms.

Clinically, all of these patients present some of the following symptoms or signs:

- Focal muscle atrophy
- Fasciculations
- Spasticity
- Pathologically brisk tendon reflexes
- Hoffmann's sign
- Babinski's sign
- Dysarthria
- Dysphagia
- Fasciculations and wasting of the tongue

Respiratory failure and other pulmonary complications are usually the cause of death in ALS patients.

Epidemiology

The majority of ALS cases are sporadic. Only about 5-10% of cases have any kind of familiar history of ALS (Anderson, 2003).

The incidence of sporadic ALS is, in average, 1.9-2.1 per 100000/year in Europe and North America without significant differences between the different countries (Worms, 2001; Logroscino et al, 2010). A constant finding in studies is the higher number of men affected, with a M/F ratio of around 1.5:1 (Abhinav et al., 2007; Logroscino et al., 2008; Worms, 2001).

The mean age of onset for sporadic ALS varies between 55-65 years with a median age of onset of 64 years (Haverkamp et al., 1995; Leigh, 2007). Only around 5% of cases have an onset before 30 years of age (Haverkamp et al., 1995), although

juvenile sporadic ALS cases are being progressively more recognized (Gouveia e de Carvalho, 2007). Bulbar onset is commoner in women and in older age groups of patients (Haverkamp et al. 1995).

Although the incidence of ALS is thought to be regionally uniform, there are some clusters in some regions in the Western Pacific where the prevalence may be 50-100 times higher than elsewhere. The Guam island, the West Papua and the Kii peninsula in Japan are the three largest areas of increased incidence. These patients have ALS associated with Parkinsonism and Dementia (Armon 2003). Despite the decrease in incidence of ALS in these areas over the past 40 years, the cause of these clusters are still unveiled (Steele and McGeer, 2008; McGeer and Steele, 2011).

Etiology

The actual cause of ALS is still unidentified, despite some genetic risk factors have been acknowledged. At present time, most authors believe that a complex interaction between genetic and environmental aspects is the causal factor for motor neuron degeneration (Shaw, 2005)

Pathogenesis

The precise molecular pathway leading to degeneration of motor neurons in ALS is still unknown. But, taking as example other neurodegenerative disorders, most likely this will be an intricate interaction among multiple pathogenic cellular mechanisms.

Since the purpose of this work is not to review exhaustively ALS, we will only list some cellular mechanisms that have been shown to be deregulated in tissues of ALS patients:

Protein aggregation and endoplasmic reticulum stress
Excitotoxicity
Oxidative stress
Proteasome Inhibition and Autophagy
Mitochondrial dysfunction and Apoptosis

Neuroinflammation

Impaired axonal transport

Deficits in neurotrophic factors and dysfunction of signalling pathways

Transcriptional dysfunction

Genetic factors

Histopathological features

Regarding pathologic features, the hallmarks in ALS include the degeneration and loss of motor neurons with astrocytic gliosis and the presence of intraneuronal inclusions in degenerating neurons and glia.

In the upper motor neurons, there is a depopulation of the Betz cells in the motor cortex, variable astrocytic gliosis, and axonal loss within the descending pyramidal motor pathway connected with myelin pallor and corticospinal tract gliosis.

In lower motor neuron pathology, there is degeneration of the ventral horns and brainstem motor neurons leading to the death of these cells. The remaining neurons are atrophic and may contain intraneuronal inclusions such as Ubiquitinated (TDP-43) inclusions (Neumann et al., 2006), Bunina bodies (Okamoto et al., 2008), and Hyaline conglomerates (Wood et al., 2003).

Diagnosis and monitoring of disease progression

There are a variety of investigations that can help in the diagnosis of ALS either by documenting neurogenic or active denervation signs, or by excluding other conditions that can mimic ALS.

Electrophysiological studies

Electrophysiological studies are the most important investigation to rule out other mimicking conditions and to confirm the diagnosis of ALS. Neurogenic changes and/or signs of active denervation such as fibrillation potentials, positive sharp waves and fasciculations potentials have a fundamental role in the current criteria for ALS diagnosis (de Carvalho et al. 2008).

Nerve conduction studies

NCS allow the exclusion of peripheral nerve pathologies such as Multifocal motor neuropathy, that can mimic ALS presentation. These studies are generally normal or near normal, except for the amplitude of the compound muscle action potential that can be diminished (Brooks et al., 2000).

Conventional electromyography

Needle EMG is the most selective tool for demonstrate signs of lower motor neuron dysfunction. It can identify widespread loss of motor units even before it is clinically detectable.

Active denervation signs such as fibrillation potentials and positive sharp waves, and chronic denervation signs such as large motor unit potentials with increased duration, reduced interference pattern with higher firing rates and unstable motor unit potentials, are only demonstrable through needle EMG.

Fasciculation potentials are a significant feature of ALS. Not only they have an upgraded value in the diagnosis (de Carvalho et al., 2008), but they can offer some understanding of the pathophysiology of ALS.

Transcranial magnetic stimulation (TMS)

TMS offers a non-invasive and reliable method of assessing upper motor neuron function. Changes in cortical motor threshold and cortical silent period can be documented with this technique and have shown to be correlated with disease progression (de Carvalho and Swash, 2010).

Quantitative electromyography

MUNE techniques are special neurophysiological methods that estimate the number of functional motor units on a giving muscle (Bromberg, 1993; Daube, 2006).

The neurophysiological index is a mathematical derivation of three standardised neurophysiological measurements, representing aspects of the effects of denervation and reinnervation and of the excitability of anterior horn cells (Swash and de Carvalho, 2004, Cheah et al., 2011).

These methods, while not perfect, are sensitive to quantify disease progression and have both been applied in clinical trials (de Carvalho and Swash, 2005; de Carvalho et al. 2005).

Neuroimaging studies

The main role of neuroimaging in ALS is to exclude structural lesions that may mimic ALS symptoms.

Magnetic resonance imaging (MRI) may identify changes in the corticospinal tracts of ALS patients (Luis et al., 1990), but the role of this or other more advanced neuroimaging modalities in very limited in clinical practice. Nevertheless, there are recent studies regarding the use of neuroimaging in identifying potential biomarkers of disease progression (Turner et al., 2009) and in detection of changes before disease onset (Ng et al., 2008).

Clinical scales

There are many clinical rating instruments to evaluate ALS patients that can assess disease status, follow progression and serve as endpoints in clinical trials.

At the time, the commonly used and available instruments for the assessment of disease status and progression in ALS include the Norris scale (Norris et al., 1984), the Appel scale (Apple et al., 1987) and ALSFRS (ALS CNTF Treatment Study Phase I–II Group, 1996).

The ALSFRS is a functional scale, proposed in 1996, designed to assess patients' abilities to carry out activities of daily living (ADL) grouped in four categories: bulbar, upper extremity, lower extremity function and gross body function. Although this scale was demonstrated to be robust and reliable (Cedarbaum and Stambler, 1997) it granted disproportionate weighting to limb and bulbar, as compared to respiratory dysfunction (Cedarbaum et al. 1999). Therefore, the ALSFRS-R, which incorporates additional assessments of dyspnea, orthopnea, and the need for ventilatory support, was proposed three years later (Cedarbaum et al. 1999).

This scale is a sensitive and reliable score that has been largely used in clinical trials (Cedarbaum and Stambler, 1997) and has been proved to be predictive of survival (Kaufmann et al., 2005).

Management

ALS is considered an incurable disease, so treatment is mostly symptomatic. The majority of symptoms that arise during its natural course are treatable, and patients should be managed by a multidisciplinary team focusing on improving quality of life and patient's autonomy. Table 4 resumes the most common symptomatic treatments in ALS (Radunovic et al., 2007).

Table 4: Treatment of symptoms in ALS *(Radunović et al., 2007)*

	Drugs	Other treatments
Cramps	Carbamazepine[12,19] Phenytoin[12,19] Quinine (removed from US market)	Physiotherapy[17,19] Physical exercise[12,19] Massage[12,19] Hydrotherapy[17,19]
Spasticity	Baclofen Tizanidine Dantrolene Botulinum toxin type A[77,78]	Physiotherapy[76] Hydrotherapy Cryotherapy
Excessive watery saliva	Atropine[17,19] Hyoscine hydrobromide[17,19] Hyoscine butylbromide[17,19] Hyoscine scopoderm[79] Glycopyrronium[17,19] Amitriptyline[73]	Home suction device[19] Dark grape juice Sugar-free citrus lozenges Nebulisation[12,19] Steam inhalation[17,19] Injections of botulinum toxin into parotid glands[80–84] Irradiation of the salivary glands[85,86]
Persistent saliva and bronchial secretions	Carbocisteine Propranolol[87] Metoprolol[87]	Home suction device[12,19] Assisted cough insufflator–exsufflator[88] Rehydration (jelly or ice) Pineapple or papaya juice Reduced intake of diary products, alcohol, and caffeine Butter
Excessive or violent yawning	Baclofen	
Laryngospasm	Lorazepam	Reassurance
Pain	Simple analgesics Non-steroidal anti-inflammatory drugs Opioids	Comfort (seating, sleeping, day and night care)
Emotional lability	Tricyclic antidepressant[89] Selective serotonin-reuptake inhibitors[89] Levodopa Dextrometorphan and quinidine[90]	
Communication difficulties		Speaking techniques[91] Low-tech augmentative and alternative communication tools[91] Voice amplifiers Light writers Scanning systems operated by switches Brain–computer interfaces[92]
Constipation	Lactulose Senna	Hydration Increased fibre intake
Depression	Amitriptyline Citalopram	Psychological support, counselling
Insomnia	Amitriptyline Zolpidem	Comfort, analgesia
Anxiety	Lorazepam Midazolam	Psychological support, counselling
Fatigue		Modafinil[93]

Nutritional management is of major significance in ALS. Since dysphagia is a common symptom, leading to increased risk of malnutrition, dehydration and weight loss, special care should be given to nutritional status. Eventually, enteral feeding most be considered, with percutaneous endoscopic gastrotosmy (PEG) being the standard of care (Leigh et al., 2003). PEG has been suggested to maintain a good nutritional status and prolong survival in ALS patients (Mazzini et al., 1995).

Weakness of respiratory muscles develops as the disease progresses and is a significant indicator of survival. It ultimately leads to respiratory complications, being the main cause of death in ALS (Gil et al., 2008). Erect forced vital capacity and vital capacity along with percutaneous nocturnal oximetry are the most commonly used tests to assess respiratory function. The latter can be useful to determine the need for non-invasive positive-pressure ventilation (NIPPV) (Pinto et al., 2003). NIPPV has been shown to improve survival and quality of life (Pinto et al., 1995; Bourke et al., 2006) and is the preferred therapy to alleviate symptoms of respiratory insufficiency.

A vast number of clinical trials with various therapeutic targets have been reported in the literature. There are, currently, more than 150 trials registered with ALS as a target condition (http://clinicaltrials.gov). Despite this large number of studies, only riluzole has proven a modest effect on survivability (Bensimon et al., 1994; Lacomblez et al., 1996; Miller et al., 2007). One of the possible reasons for the lower success rate in ALS clinical trials may be the may be with the outcome measures chosen for these studies. Up until now, survival time and functional outcome have been chosen as the primary endpoints. Although these measures are of indisputable importance, they may be insensitive for screening new drugs (Costa et al., 2010). Consequently the need for sensitive biomarkers, like neurophysiological measurements or molecular biomarkers, is growing day by day.

1.3 Motor unit number estimation

Ascertaining the number of axons innervating a specific muscle is of primordial importance in clinical neurophysiology. MUNE allows for a quantitative measure of the function of motor axons and it may be sensitive to mild degrees of axonal loss. Further than that, MUNE techniques are not influenced by the compensatory reinnervation process following denervation due to lower motor neuron degeneration, as opposed to motor amplitude.

MUNE can provide significant information about the structure, organization and function of the brainstem and spinal cord motor system. Moreover, MUNE offers the chance to study the effects of age and muscle denervating diseases on motoneuron populations. It also can be used to establish the natural history of these disorders and to assess therapeutic efficacy of clinical interventions.

Although theoretically MUNE allows various exciting possibilities, the lack of a standard anatomical determination of the number of motor units in a muscle has hampered the use and development of MUNE techniques. As previously mentioned in this work, anatomical studies of motor units are also, at best, estimates of the true number of motor units.

The advent of physiological MUNE techniques happened in 1971 when McComas described his incremental stimulation method (McComas et al., 1971a). Despite being proposed over 40 years ago, the MUNE field was hindered during nearly 20 years because of the initial application of the incremental stimulation technique in muscle diseases such as myotonic, Duchene or limbgirdle dystrophies (McComas et al., 1971b; McComas et al., 1971c; Sica and McComas, 1971). Because much was yet to be learned regarding motor unit properties, the low values of MUNE recorded in these patients muscles were attributed to some type of dysfunction in their motoneurons. This hypothesis for the pathogenesis of muscle diseases was proven wrong, and the MUNE field lost the attention it deserved.

In the next two decades, other techniques were described (Brown and Milner-Brown, 1976; Lee et al., 1975; Brown et al., 1988; Stashuk et al., 1994; Daube, 1988, 1995) but the crucial step was given in 1993 when Bromberg (Bromberg, 1993) studied the reliability of MUNE and considered its application for evaluating ALS patients.

These techniques will be explained in the next pages along with their advantages and limitations.

Before we look at each individual method, we have to understand several basic assumptions about electrical characteristics that are made by MUNE techniques.

All of these methods measure the average size (amplitude and/or area) of single motor unit potentials (SMUP), as well as the size (amplitude and/or area) of compound muscle action potential (CMAP) obtained by supra-maximal stimulation of a motor nerve. The MUNE is calculated by dividing the size of the maximal CMAP by the average size of the SMUP. These methods assume that each motor unit has a similar size and that it is the same size each time it is activated. It is preferable to use negative peak area or amplitude instead of peak-to-peak amplitude due to the effects of temporal dispersion in phase cancellation. If peak-to-peak amplitude is used, it may lead to an inflation of the average value and, consequently, to an underestimation of MUNE.

What distinguishes these 5 techniques is the method used to obtain the SMUPs used to calculate the average SMUP.

The first assumption is that the electrical activity recorded is derived from a single muscle. If a single motor unit potential is actually generated by a muscle at a distance from the recording electrodes its amplitude will be misleadingly small, leading to an overestimate of the motor unit number. For that reason, SMUPs with waveforms mostly positive in polarity, or with area<25 μV/ms or amplitude<10 μV are considered to arise from distant muscles and discarded (Bromberg, 2007).

The second assumption is that the SMUP responses are, in fact, derived from a single motor unit. It is reasonable that two or more axons have similar thresholds causing a single response. This of course, can lead to erroneous MUNE values. As the number of stimulus increases though, this joint response will tend to decrease, giving different SMUP morphologies with each stimulus. This alternation phenomenon increases as the current stimulus increases due to higher probability of stimulating more and more axons. This is one of the reasons why is difficult to directly identify more than 10 motor units in a muscle when stimulating a single point along the nerve.

The third assumption is that a sample of 10 or 20 SMUP that are used to determine the average size of the SMUP are representative of the entire population of

SMUPs. Taking into account the classical studies regarding electrical stimulation of peripheral nerves (Erlanger and Gasser, 1937), we know that the largest fibers with higher conduction velocities have the lowest threshold. This can present a bias on the selection of the motor units used to calculate the average SMUP size. However, the clinical data reported in various studies using various techniques (McComas et al., 1971; Doherty and Brown, 1993; Doherty et al., 1994) suggested that percutaneous electrical stimulation of motor axons provided an unbiased sample of SMUP.

Incremental stimulation

Incremental stimulation MUNE was the first physiological technique to be described (McComas et al., 1971). Despite its limitations, it was a major breakthrough in neurophysiology.

It was applied to the extensor digitorum brevis (EDB), with a surface strip electrode placed so as to completely cover the end-plate zone of the muscle, and a reference electrode placed over the sole, considered to be an "inactive" spot.

Consecutive, manually adjusted electrical stimuli (duration - 50 μsec; repetition rate - 0.25 Hz) were applied to the deep peroneal nerve at a site just above the ankle through a bipolar surface electrode (cathode distal to the anode).

With progressive increases in stimulus intensity, incremental increases of the CMAP size were perceived. Each consecutive increment of the CMAP was considered to represent the addition of a SMUP as its threshold was reached. About 10 quantal increments were obtained in order to get a somewhat representative sample of SMUPs.

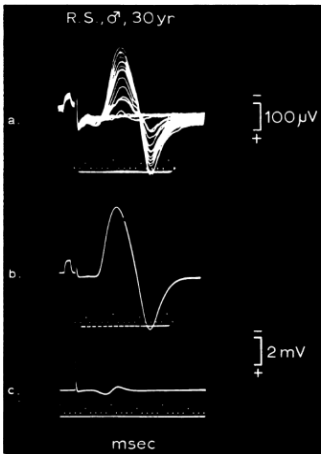


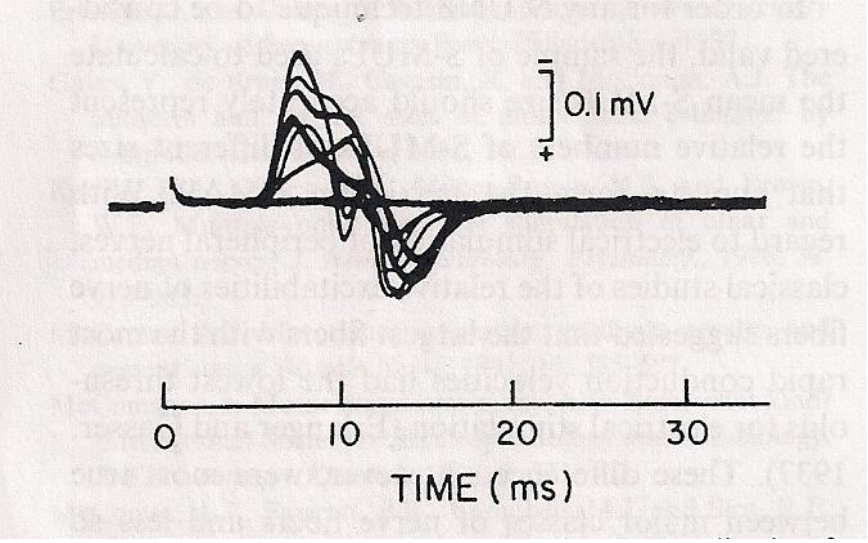
Figure 3: Incremental stimulation MUNE *(McComas et al., 1971a)*

The average amplitude of SMUP was then calculated and maximal amplitude of the CMAP divided by it in order to estimate the number of motor units.

This is a rather simple and elegant method that can be most effective when there is a reduction in the number of motor units in the studied muscle. In this case, the individual steps with stimulus current changes can be quite easily identified. On the contrary, in a young and healthy subject with a large number of motor units, the thresholds of motor axons quite often overlap, making quantal increments very difficult to identify.

When the thresholds of two or more axons overlap at a given stimulus intensity a set of stimuli can evoke 2^n-1 increments to the CMAP, where *n* is the number of axons with that threshold overlap. These steps (alternations) represent the possible arrangements of SMUPs, which in turn represent the variation, from stimulus to stimulus, of the motor units responding. This can lead to an underestimation of the SMUP size and, consequently, to an overestimation of the number of motor units.

Figure 4: Alternation phenomena in a series of 100 constant intensity stimuli
(Doherty et al., 2003)



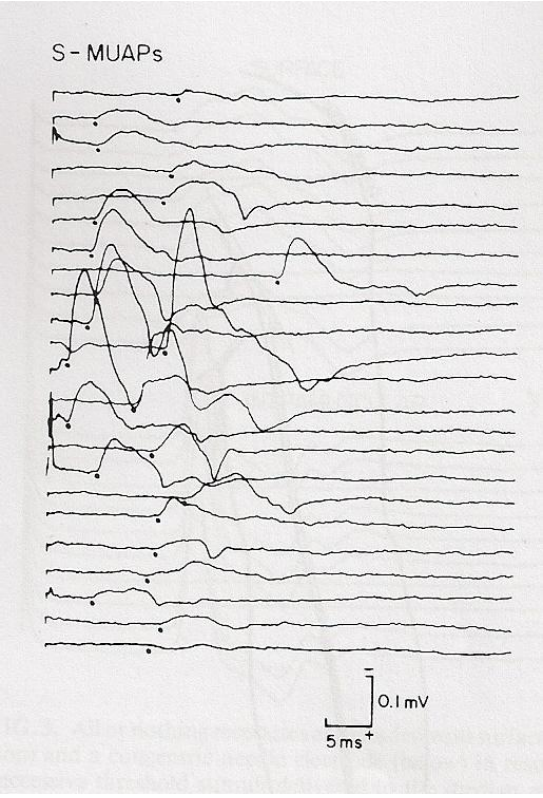
Another pitfall is the operator bias in the choice of the increments that actually represent an addition of a SMUP to the CMAP.

Multiple point stimulation

In order to surpass the problem of alternation, stimulation of multiple points along the peripheral nerve path was suggested, originating the multiple point stimulation technique (Brown and Milner-Brown, 1976; Kadrie et al., 1976). These two first papers gave somewhat different results in obtaining a more representative SMUP sample and this method was not considered suitably for MUNE. However, these studies shed some light into the recruitment pattern of motor units.

The modification introduced by Brown and Milner-Brown, was to stimulate the motor nerve in various (10 to 20) sites along the length of the nerve and record only the first all-or-nothing motor unit potential. The stimulus is given at 1 Hz with 50 to 100 μs duration with the cathode distal. Starting from a site just proximal to the motor point, the first single reproducible, all-or-nothing, free of alternation SMUP is found. After the first SMUP is found, the cathode is moved a short distance distally and the process is repeated until at least 10 SMUPs are recorded.

Figure 5: SMUPs obtained by multiple point stimulation MUNE
(Doherty et al., 1995)



This simple change in methodology avoided completely the problem of alternation and seemed to be a great advance in the MUNE field. Despite thse promising features, multiple point stimulation was left aside until 1993 when Doherty and Brown used this method to study the number and size of motor units in adults (Doherty and Brown, 1993). This technique was used by Felice in ALS patients and controls, in an elegant study that proved the utility of MUNE in the assessment of ALS (Felice, 1995).

An adaptation of this technique was described by Wang (Wang and Delwaide, 1995), which consists in recording only two or three clearly identifiable SMUPs in each point of stimulation in order to avoid alternation, and at the same time allowing for the increase of the SMUP sample. However, with this alternative method, there may be an increased probability of recruiting the same motor unit in different stimulation sites.

Advantages

The advantages of multiple point stimulation technique are: the average SMUP size is based on real motor units and not a statistical estimate or an estimate derived by algorithms intended to correct for alternation; there is no alternation; near motor thresholds stimuli are well tolerated by the subjects.

Disadvantages

The possibility of recording the same SMUP when stimulating at different sites along the nerve, is one of the most striking issues in multiple point stimulation MUNE. A formal method to detect duplicate SMUP is the collision technique, as described by Aoyagi (Aoyagi et al., 2000). However, collision studies are not viable in a clinical setting, so the primary means of identifying duplicate SMUPs is the comparison of waveforms signatures. Another drawback of this method is that in only applicable to distal muscles as it is required at least 50-100 mm of the motor nerve to allow collection of at least 10 SMUPs.

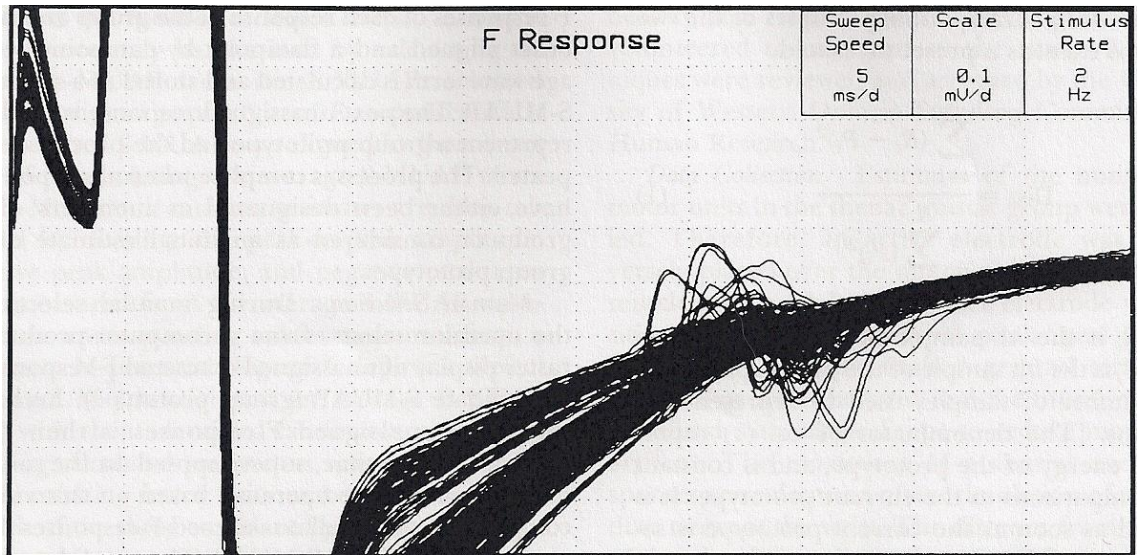
F-Response method

Following some studies on F-responses elicited by submaximal stimuli (Komori et al., 1991; Doherty et al., 1994) and the interpretation of these responses as a single motor unit potential, Stashuk and colleagues (Stashuk et al., 1994) proposed a method

of estimating the number of motor units based on the automated analysis of the F-responses.

This technique applies series of 200-300 successive submaximal stimuli (10-50% of maximal CMAP) to a motor nerve at a rate of 2 Hz, recording 100 ms of surface EMG signal on a muscle innervated by that nerve. An F-response is considered to be representative of a discharge of a single motor unit, when 2 or more responses with identical shape, size and latency are recorded within the set of 200-300 stimuli.

Figure 6: Superimposition of 300 CMAPs and related F-Waves, used for the calculation of F-Wave MUNE
(Stashuk et al., 1994)



The recorded traces are then analyzed either automatically or manually in order to identify the SMUPs. The manual method can take up to 3h for scanning the entire set of responses, thus making it impractical in the clinical setting. An algorithm was then developed (Stashuk et al., 1994) in order to automatically select the F-responses considered being SMUPs.

When a representative sample of SMUPs is collected (at least 10), the average size is calculated, and used to estimate the number of motor units by dividing the size of the CMAP by the size of the average SMUP.

Advantages

This technique is carried out with a minimum of operator intervention, reducing a possible operator bias in choosing SMUPs. The low intensity of the stimulus applied is well tolerated by the subject.

Disadvantages

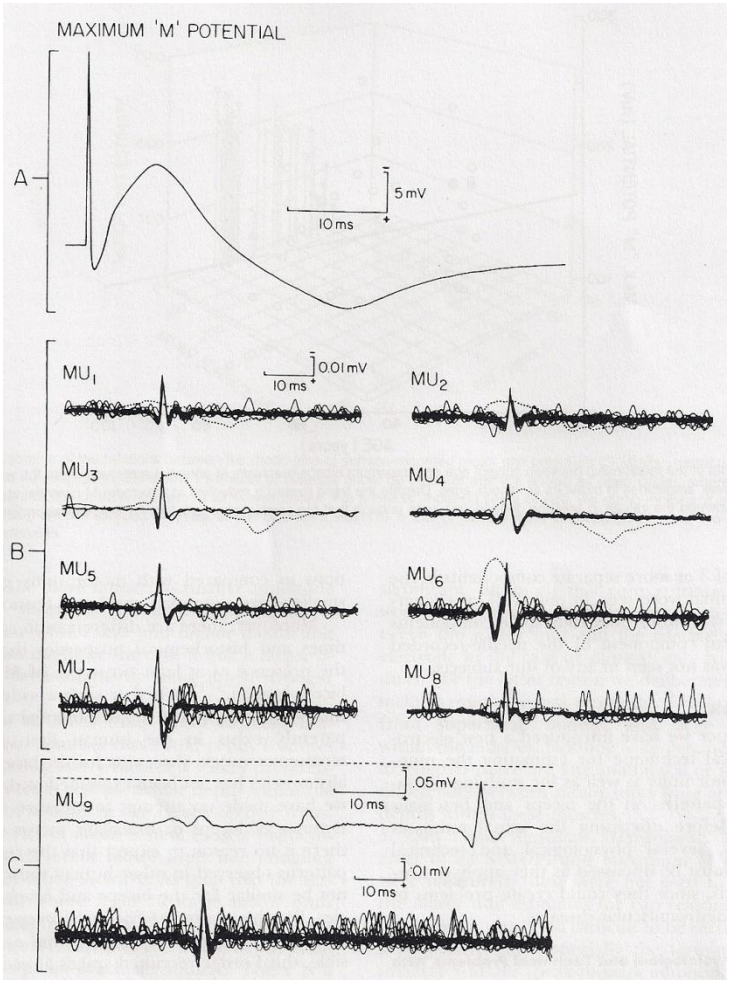
The F-responses may be derived by the activation of more than one motor unit, leading to an overestimation of the average size of the SMUP. Alternation phenomena cannot be fully excluded despite the robustness of the algorithm. This method requires special software that may not be available in every EMG machine. Finally, in some pathological condition, such as ALS, the physiology of the F-responses may be altered (e.g. hiperexcitability of the anterior horn cells, reduced number of responses) leading to an increased difficulty in recording the SMUP sample.

Spike-Triggered Averaging

One of the first major studies with spike-triggered averaging technique was by Brown and colleagues who applied this method to the biceps brachii muscle in healthy subjects (Brown et al., 1988).

This method involves recording electrical activity from two channels. Using voluntary isometric contraction, motor units are recorded in one of the channels with a combination of an amplitude window discriminator and a needle electrode, isolating one motor unit from the rest of the electrical activity. The motor unit spike is then used as a trigger to record the surface SMUP time-locked with the chosen motor unit, extracting this signal from the asynchronous surface detected EMG activity. The surface recordings where then averaged (200 to 500 traces) until no further changes in the shape of the SMUP occurred. Filter settings were 500Hz-5KHz for the intramuscular recordings and 0.1Hz-2KHz for the surface recordings.

Figure 7: Spike-Triggered Averaging MUNE in Biceps brachii muscle
(Brown et al., 1988)



10 SMUPs where recorded and used to calculate the average SMUP size. The maximal CMAP, obtained by supramaximal stimulation of the musculocutaneous nerve at or just distally to the posterior axillary fold, was divided by the average SMUP, giving the estimate of the number of motor units.

Advantages

This method does not suffer from alternation problems and it can be applied to most muscles, including proximal ones that are not accessible by other techniques. The use of intramuscular needle recordings may provide information regarding motor unit firing patterns, fiber density, jitter, blocking or other pathopysiological phenomena of reinnervation or instability of neuromuscular transmission.

Disadvantages

This method can be very lengthy for collecting a representative sample of SMUPs. It is somewhat uncomfortable for the subject as intramuscular needles are not painless. It requires the collaboration of the subject, by maintaining a steady contraction. The measurements of the negative area of the SMUPs can be tough because of the difficulties in establishing the onset and baseline of the SMUPs. It involves special software. Finally, there may be a bias in the selection of the SMUPs towards low threshold motor units because of the physiological order of motor unit activation, giving lower values of SMUP size.

Decomposition-Enhanced Spike-Triggered Averaging

In order to overcome some of the problems with the Spike-triggered averaging method, an algorithm was developed for combining EMG signal decomposition with Spike-triggered averaging (Stashuk and Brown, 1994).

The goal of this improvement is to analyze segments of 20 to 60 s of EMG signal during moderate isometric contraction and extract the motor units signal recorded from intramuscular needles. In this manner, the operator is not required to manually select each intramuscular motor unit potential as a trigger for the surface SMUP, decreasing the time needed for each assessment, and providing a larger sample of SMUPs, and at higher levels of contraction.

The original algorithm used for this method was designed for use with concentric needle electrodes and during isometric constant or slowly changing force contractions. The EMG signal was first filtered by a first-order differential filter (McGill et al., 1985) in order to attenuate most of the distant volume conducted EMG signal. This enhances the detection of motor unit action potentials (MUAP). The following step is the application of a multipass clustering algorithm to the set of MUAP recorded in a 5 s interval corresponding with the maximal level of motor unit recruitment. This allows for the estimation of the number of MUAP trains in the composite signal and the computation of the prototypical MUAP shape for each train. The Spike-triggering averaging technique is then applied, in order to collect SMUP from the surface electrodes.

Advantages

This method can greatly enhance the Spike-triggering averaging technique by increasing the number of SMUPs obtained from each intramuscular detection, reducing the time required for the collection of the entire set needed for MUNE calculation. It reduces the level of subject cooperation that is needed for the test. It allows for the study of higher threshold motor units, reducing the bias of the Spike-triggering averaging technique towards the selection of low threshold motor units.

Disadvantages

Regardless of how capable the decomposition algorithm may be, the complex interference pattern recorded by the surface electrode often makes it quite difficult to clearly identify SMUPs. This prevents the use of negative peak area, obliging the use of peak-to-peak or negative amplitude that may lead to an erroneous estimation of the number of motor units. Intramuscular needles are still used, causing some discomfort to the subjects. Finally, special software is required for these analyses, which may not be available in every EMG machine.

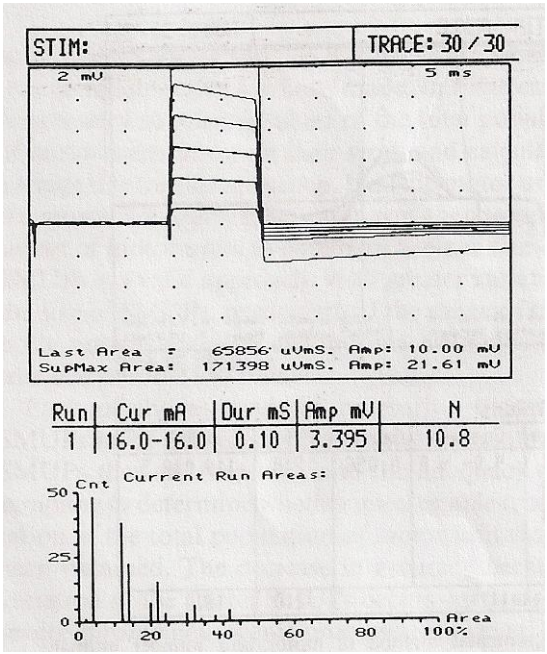
Statistical method

The first time the statistical MUNE technique was reported in the literature was in 1988 (Daube, 1988), but it was only in 1995 that Jasper Daube described formally is method (Daube, 1995).

The statistical method relies on the know relation between the variance of multiple measures of step functions and the size of the individual steps when these steps have a Poisson distribution. In a pure Poisson distribution the measures decrease at higher values and the variance of these measurements is equal to the size of the individual components making up each measurement.

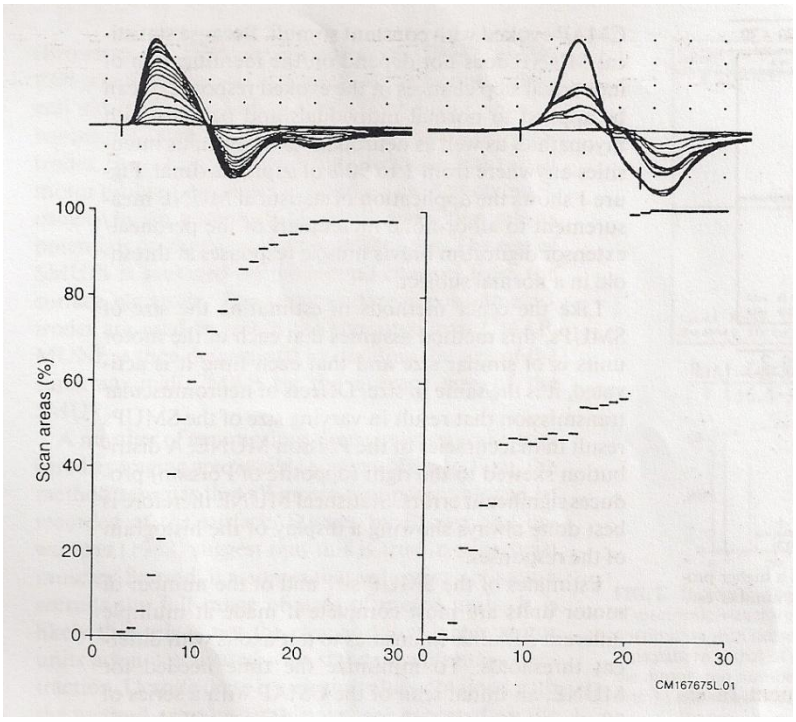
In a set of 30 constant submaximal stimuli there will be variability in the CMAP response related to the inherent differences of thresholds of individual axons. Given that the differences on the CMAP follow a Poisson distribution, the variance of this distribution will correspond to the average size of the SMUP.

Figure 8: Series of motor responses to increasing stimuli, following a Poisson distribution
(Daube et al., 1995)



Firstly, a scan of 30 stimuli with increasing equal increments is done in order to identify unusually large steps in the CMAP.

Figure 9: Normal (left) and abnormal (right) CMAP scanning curves
(Daube et al., 1995)



If all steps on the scan are small, as it is in normal subjects, stimulus intensity eliciting response windows 10-20%, 25-35%, 40-50% and 55-65 % of maximal response are chosen, and 4 to 10 trials of 30 stimuli with that intensity are applied. The results of the different trials are then averaged, thus obtaining the estimation of number of motor units.

On the other hand, in subjects with denervation and reinnervation such as ALS, if the CMAP scan detects true gaps (>10 % of total range), these gaps are attributed to a single SMUP, and a series of operational guidelines is needed in order to account for these large motor units (see Bromberg, 2007).

There is still a large debate regarding technical details around this method. Not only the window size (5 or 10 %) and the placement of the windows along the scan curve are a matter of discussion, but there is no agreement in using Poisson or binomial distribution on the statistical method (Blok et al., 2005; Bromberg, 2007).

Advantages

It is a relative fast technique (around 15 minutes per muscle), without significant discomfort for the patient as it only uses submaximal electrical stimulation. It is applicable to almost every muscle where a CMAP is obtainable. Alternation is not a problem in this method. Also, as it uses a wide range of stimulus intensities, motor axons with different thresholds are recruited, thus providing with a representative sample of the SMUPs.

Disadvantages

There are a wide number of operator variables on this method. Up until today, there is a lack of a broad consensus on these variables and its implications on MUNE calculations. Special software is required for this technique, which is not available in every EMG machine.

<u>High density MUNE</u>

Van Dijk and collaborators proposed a new method of MUNE mixing elements from the Incremental stimulation and Multiple point stimulation techniques, with 120 channel high-density surface electromyography (HD-EMG). This combination may

resolve the problem of alternation to a large extent, allowing a collection of a larger SMUP sample, hence increasing the MUNE accuracy.

The HD-EMG allows the decomposition of recorded submaximal CMAPs into the contributions of single motor units by adding spatial information to the obtained waveforms, thus distinguish individual SMUPs.

Despite the advantage of increasing the SMUP sample, this method has several drawbacks. When the number of SMUPs contributing to a given submaximal CMAP is high (more then 4), the accuracy and reliability of the SMUPs detection decreases significantly. It requires special electrodes and software that may not be easily available. Finally, each assessment can take more than 60 minutes, making it not viable in a clinical setting.

Bayesian statistical method

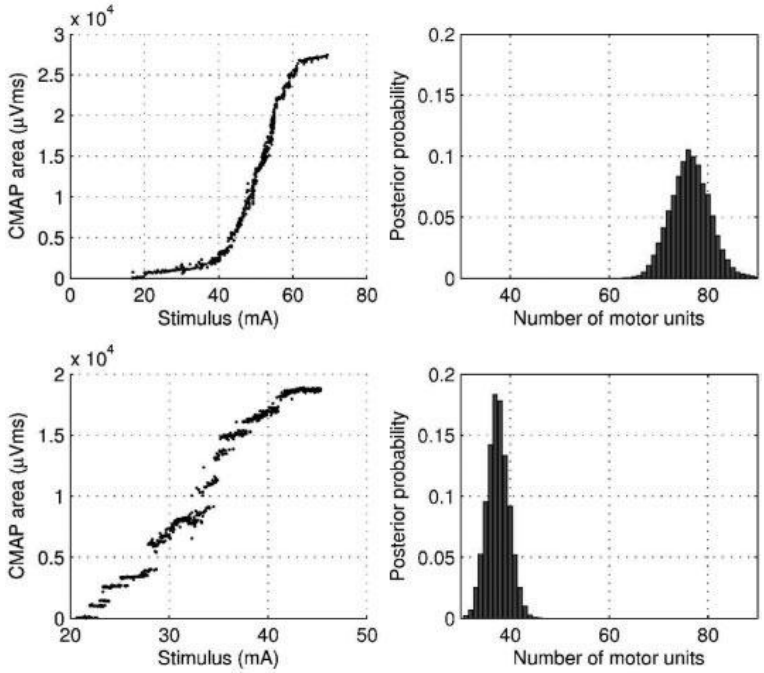
One of the most intricate, yet promising, techniques recently proposed is the Bayesian approach to the statistical MUNE (Ridall et al., 2006; Henderson et al., 2007).

This method uses data from the entire stimulus–response curve of a particular nerve collected by gradually increasing the stimulus intensity over at least 500 stimuli.

A Bayesian model is then applied to this data, as described by Ridall (Ridall et al., 2006) in order to obtain a probability that a certain number of motor units in that muscle is true.

The use of Bayesian statistics allow for the incorporation of a number of variables into the equation, avoiding the assumptions made by the Statistical method - the single MUAPs have the same size and that the units firing probabilistically, for a given stimulus, have the Poisson distribution. Instead, the Bayesian method incorporates probabilistic motor unit firing and motor unit size variability into the model.

Figure 10. Stimulus–response curves (left) and the calculated motor unit number and distribution of motor units (right) in a normal subject (top) and an ALS subject (bottom). In the posterior distribution the most likely number in the normal subject was 79 (71–89) and in the ALS subject 29 (25–34).
Henderson et al., 2007



This method also has some assumptions:

- Motor units fire independently of each other in an all or nothing response and the response to each stimulus is independent of the response to previous stimuli. Motor unit firing only occurs if the stimulus intensity exceeds a variable threshold. The threshold for each unit is distributed as a Gaussian variable with its own mean threshold and precision parameter. The mean threshold is defined as the stimulus at which a unit has a 50% probability of firing. The precision parameter defines the range over which the unit exhibits probabilistic firing. The probability of a unit firing as a function of the stimulus can therefore be represented by a sigmoidal curve known as an "excitability curve" (Brown and Milner-Brown, 1976).
- Each motor unit upon firing emits an action potential in the muscle characterized by an area or amplitude which is independent of the stimulus and normally distributed about a mean particular to that unit with a variance common to all units. (These means can then be allocated a suitable distribution to describe their between-unit variability.)
- The measured CMAP area is the superposition of the muscle action potentials (described by Assumption2) of those units that respond to a stimulus (as described in

Assumption 1) together with a component from the baseline noise, which itself is normally distributed with its own mean and variance.

Although this assumptions are well debated in the original paper (Ridall et al., 2006), more studies are needed in order to verify if these are undisputable. Correlation of data obtained by this method with MUNE values calculated with other methods is also desired.

1.4 MUNIX

MUNIX stands for "Motor unit number index" and it is a new method for estimating the number of motor units in a muscle. It was described in 2004 (Nandedkar et al., 2004), and it is being tested in a multicenter study, in which our laboratory is included, not only in control subjects (Neuwirth et al., 2011a; Neuwirth et al., 2011b), but also in ALS patients (Unpublished data). This method uses a mathematical model based on the CMAP and the surface EMG interference pattern (SIP) to derive an index related to the number of motor units, and not the actual number. However, in a previous study (Neuwirth et al., 2010) MUNIX values from the abductor pollicis brevis (APB) were correlated with previously published data using other MUNE techniques.

This method is a three-step process. Firstly, the CMAP of the desired muscle is recorded by stimulating supramaximally the appropriate motor nerve with standard nerve conduction techniques. As MUNIX calculations relies on the CMAP amplitude and area, it is important to ensure that the maximum CMAP amplitude is recorded. Non optimal electrode placement can give low CMAP values and underestimate MUNIX. The negative phase of the CMAP is used to compute amplitude, area and power (the area and power are calculated by summating the absolute and square of the sample values, respectively, and multiplying it by the sampling interval for the measurement).

The second step consists on the recording of the SIP, with each epoch containing 300 ms of surface EMG signal.

The patient is instructed to maintain an isometric contraction at 9 rising levels of force, starting on minimum and ending on full contraction. The force per se is not measured, as it would be impracticable, but the operator offers manual resistance to the patients movement, thus helping the recruitment of different levels of force. Each level of force will roughly correspond to 10% increments, from 10 to 100%, giving the patient a short rest before the maximal contraction. Either the CMAP and the SIP are recorded using a filter setting of 3-3000 Hz.

The SIP epochs are analyzed in order to identify artifacts such as high frequency noise, power line frequency interference, baseline shift. Also tremor may occur, causing a nearly synchronous firing of motor units with high amplitude bursts. These situations

can lead to erroneous MUNIX calculations and recordings containing these artifacts should be rejected.

There are some criteria for a SIP epoch to be accepted:

- SIP area > 20 mV/ms
- Ideal Case of Motor Unit Count < 100
- SIP area / CMAP area > 1

For the final step, all of the signals are imported to an independent analysis software for the MUNIX calculation. The mathematical model used for MUNIX computation is described next.

MUNIX Mathematical model

Let us assume an ideal case in a given muscle where all motor units are identical, with the same SMUP waveform, amplitude, area and power, where *N*= number of motor units; *Mp*= power of a single SMUP; and *Mr*= area of a single SMUP. Since the CMAP is the sum of all SMUPs, assuming there is no temporal dispersion, the CMAP waveform will be a magnified image of the SMUP.

Giving these postulations, the CMAP area will be given by:

$$CMAP\ area\ (Cr) = N\ x\ Mr$$

and the CMAP power will be (note that the power is proportional to the square):

$$CMAP\ power\ (Cp) = N\ x\ N\ x\ Mp$$

Considering a slight voluntary contraction, when the subject activates few motor units, and assuming that the SMUPs do not superimpose, the SIP measurements will be given by:

$$SIP\ area\ (Sa) = D\ x\ Mr$$

$$SIP\ power\ (Sp) = D\ x\ Mp$$

where D represents the number of SMUP discharges.

With some algebraic manipulation of the aforementioned relationships, one can easily verify that:

$$N = \frac{CMAP\ Power\ x\ SIP\ Area}{CMAP\ Area\ x\ SIP\ Power}$$

This formula is called an *Ideal case motor unit count (ICMUC)* to reflect the ideal conditions used for its calculation. However, these assumptions can be reasonable met when the SIP is recorded at a low force contraction, with few motor units discharging at a low rate.

When the force of contraction increases, larger motor units will be recruited and superimposition of SMUPs will also occur, giving higher amplitude signals. This will lead to a decrease in the ICMUC. So, to compare ICMUC values between subjects, standardization of force would be necessary. However, this can be a very laborious and tedious task, impracticable in the clinical setting. Instead, one can use the SIP area as reflection of force.

A plot of the ICMUC vs. SIP area would reflect the number and size of the motor units recruited at the each force level. The following equation models the relationship between these variables in order to facilitate comparison and quantitation:

$$ICMUC = A\ x\ (SIP\ Area)^{\alpha}$$

The values of A and α are obtained through a linear regression between the recorded ICMUC and SIP area values.

For the purpose of comparisons between laboratories, one as to define at what SIP area MUNIX calculation is made. The value of 20 mV/ms was then proposed by the authors (Nandedkar et al., 2004). Despite this value may seem a bit arbitrary, there are some practical reasons for it to be chosen. This SIP area is achieved with slight contraction, where the motor units recruited are small, with somewhat similar size and without significant superimposition, thus approaching as much as possible the ideal conditions of the model. If different SIP areas are used for the calculation, MUNIX values will differ, making it obvious that this computation is in fact an index and not a direct estimate of the number of motor units.

MUSIX

A measure that can be easily obtained after the MUNIX calculations is the average size of a motor unit on the studied muscle. This value is called motor unit size index (MUSIX), is measured μV in and it is obtained according to the formula:

$$MUSIX = CMAP\ Amplitude/MUNIX$$

In contrast with most MUNE techniques that estimate the average size of the SMUP first and then the MUNE, this method calculates MUNE first and then SMUP average size.

Advantages

MUNIX is a non-invasive method that allows for a quick estimation of the number of motor units in a given muscle. In average only 5 minutes are needed for a muscle to be assessed. Also, it is not a very challenging method regarding technical difficulties. It is not discomfort for the subject as it only requires one CMAP to be obtained by electrical stimulation. It can be applied to any muscle, distal or proximal, where a CMAP can be obtained. Finally, it can be easily done in most EMG machines, since the software used to analyze the data is independent from the EMG software and can be executed in any computer.

Disadvantages / Limitations

For MUNIX measurements some degree of patient cooperation is required, which is not always achieved due to tremor , spasticity or cognitive dysfunction. The index given by this method is not an estimate of the true number of motor units, hindering the comparison with other MUNE techniques.

When the motor units have a bimodal distribution it is not possible to achieve a full range of force levels. In this situation, the SIP will have low amplitudes at slight efforts and very large-amplitude at moderate and high efforts. This combination yields a higher MUNIX that would be expected for that muscle. When this bimodal distribution appears, changing the "*SIP area > 20 mV/ms criteria*" to 50 mV/ms will reduce significantly its influence on MUNIX calculations.

When the CMAP amplitude is very small the recording of SIPs can contain volume-conducted activity from nearby muscles (Nandedkar and Barkhaus, 2007). For this reason, when the CMAP in a muscle is < 0.5 mV, that muscle is considered not suitably for MUNIX measurements.

2. OBJECTIVES

Primary:

To assess the test-retest variability of a novel neurophysiological technique (MUNIX) for the estimation of the number of motor units in healthy subjects.

Secondary:

To evaluate the suitability of this technique as a potential marker of disease progression in ALS.

3. STUDY POPULATION

The study population was be divided in two groups: a group of healthy subjects and a patient group (patients with ALS).

Healthy subjects group

This group comprised 15 healthy individuals older than 20 years without any medical or neurological disorders that might influence MUNIX measurements (e.g. peripheral nerve dysfunction, neuromuscular disorders, diabetes, oncological diseases or drug treatment with neurotoxic drugs).

Informed consent was obtained from all subjects.

The subjects were subdivided into two age groups, between 20 and 59 years and 60 years or older in order to take into account the physiological loss of motoneurons associated with aging (Doherty et al., 1993).

Patients group

This group included patients with ALS/MND. These patients fulfilled the category for possible, probable lab-supported, probable or definite ALS regarding to the revised El Escorial criteria. All were diagnosed as ALS according to Awaji guidelines. The patients had a minimum follow-up of 9 months, with visits approximately every 3 months.

Symptom onset, defined as onset of weakness, muscle wasting, fasciculations, cramps (not present before), dysarthria, dysphagia, dyspnea, falls or disturbance of fine movements must be less than 18 months of baseline visit.

Patients with any history of medical or neurological disorders that might influence MUNIX measurements (e.g. peripheral nerve dysfunction, neuromuscular disorders, diabetes, oncological diseases or drug treatment with neurotoxic drugs), were excluded.

Informed consent was obtained from all subjects.

4. METHODS

For the purpose of studying inter and intra rater variability, the healthy subjects group were evaluated twice by two separate investigators in an alternating fashion with a break of 30 minutes minimum between each assessment. Electrodes and marks were completely removed so that any traces of electrode placement were erased. This group was composed by 9 subjects with less than 60 years old, and 6 subjects with more than 60 years old. The division into two age groups takes into account physiological loss of motoneurons at a higher age and that the onset of ALS peaks in the 6th decade (Doherty et al., 1993).

In both ALS patients and healthy subjects, the following muscles were assessed: abductor pollicis brevis (APB), abductor digiti minimi (ADM), biceps brachii (BB), tibialis anterior (TA), abductor hallucis (AH) and extensor digitorum brevis (EDB) after supramaximal distal stimulation of the median, ulnar, musculocutaneus, tibial and peroneal nerves, respectively. In ALS patients the clinically less affected side was examined. If both sides were affected symmetrically, the right side was chosen. Since the loss of motoneurons is often focal in ALS, measurements in multiple muscles (proximal and distal; upper and lower limbs) will probably reflect the amount of functioning motor units more accurately. For that purpose, the MUNIX and CMAP megascores were calculated by aggregating the results of individual muscles in a subject.

In ALS patients the following clinical data was collected: gender, age, region of onset and disease duration The ALSFRS-R scale (Cedarbaum et al. 1999) was applied at the time of MUNIX calculation. Before performing MUNIX measurement, manual muscle testing according to the Expanded Medical Research Council Scale for Manual Muscle Testing (MRC) was performed in each investigated muscle.

ALS patients were evaluated approximately every 3 months (± 4 weeks) for a period of 9 to 12 months. Multiple point stimulation MUNE (Brown and Milner-Brown, 1976; Kadrie et al., 1976) in ADM was also performed in every visit for the purpose of comparison between the two techniques.

The same surface electrodes (Cardinal Health, Madison, WI, USA, disposal ground and 2 disc electrodes, 15mm diameter, Ref 019-415200) were used throughout the study. Measurements were performed using a Keypoint® EMG machine.

For the measurements, subjects were positioned in a comfortable, supine position. MUNIX measurements were performed according to the manner previously described in this work (see 1.4 MUNIX).

Particular attention was paid to electrode placement and limb position, in order to ensure consistency between repeated measures. The tested muscle was fully relaxed and in neutral position. Skin surface was always cleaned properly before applying electrodes. Positioning of the stimulation and recording electrodes and distances between the active and reference electrode were standardized.

Abductor pollicis brevis

The active electrode was positioned in the thenar eminence; the reference electrode was positioned on the distal phalanx of the thumb; ground electrode was placed over the back of the hand. Electrical stimulation was applied on the median nerve just above the wrist, at 7 cm from the active electrode.

Abductor digiti minimi

The active electrode was positioned on the hypothenar muscle; the reference electrode was positioned on the distal phalanx of the 5^{th} finger; ground electrode was placed over the back of the hand. Electrical stimulation was applied on the ulnar nerve just above the wrist, at 7 cm from the active electrode.

Biceps Brachii

The active electrode was positioned on the middle of the long head of the BB; the reference electrode was positioned on the medial epicondyle; ground electrode was positioned on the interior surface of the arm. Electrical stimulation was applied on the musculocutaneous nerve in the axillary fold.

Tibialis anterior

The active electrode was positioned on the proximal middle third of TA; the reference electrode was positioned on the patella; ground electrode was positioned on

the interior surface of the leg. Electrical stimulation was applied on the peroneal nerve, posterior to the head of the fibula.

Abductor hallucis

The active electrode was positioned over the middle portion of the abductor hallucis; the reference electrode was positioned on the first toe; ground electrode was positioned on the internal malleolus. Electrical stimulation was applied to the tibial nerve posterior to the internal malleolus.

Extensor digitorum brevis

The active electrode was positioned over the extensor digitorum brevis; the reference electrode was positioned on the 5^{th} toe; ground electrode was positioned on the dorsum of the foot. Electrical stimulation was applied to the peroneal nerve just above the ankle.

The recording electrode position was always adjusted in order to achieve maximal amplitude with minimum rise time and a sharp negative takeoff of the CMAP. In reproducibility investigations (healthy subjects group) the amplitude of the CMAP was maximized in each occasion, without referring to previous values. In serial investigations (ALS group) the amplitude of the previous assessment was used as the target amplitude. If CMAP amplitude was less than 0.5 mV, this muscle was excluded. For the SIP recordings, the activation of each muscle was carefully assessed, in order to avoid the recruitment of neighboring muscles, in particular in weak ALS patients. Special attention was paid to temperature (always higher than 29 degrees on the dorsum of hands and 27 degrees on the dorsum of feet).

All the electrophysiological tests (MUNIX, MUNE and CMAP) were performed by the author.

Statistical analysis

For statistical analysis of variability (intra and inter-rater test–retest reliability), a two-way random, single measure intraclass correlation coefficient (ICC) was calculated. The ICC represents the variability over measurements of every subject divided by the

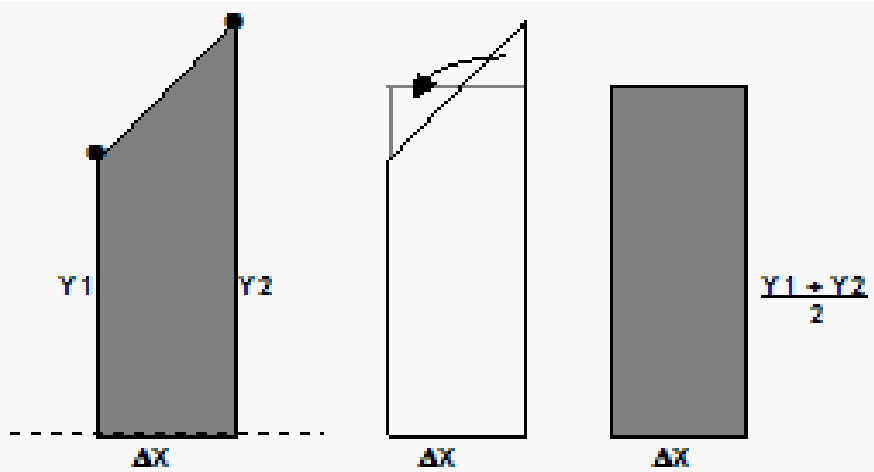
total variability of all subjects. The reliability of the mixed effects model was verified routinely by inspecting the distribution of the residuals using quantile plots. No systematic deviation from normality was detected. Intra- and inter-rater variability were estimated from mixed effect models with subject, examiner or visit as crossed random effects without fixed effects. ICC is the percentage of the inter subject variability compared to the total variability. High ICC values near 1.0 indicate that the raters have measured similarly.

For the analysis of progression of the studied variables over time in the ALS population, a Repeated measures ANOVA was applied. Repeated measures ANOVA compares the average score at multiple time periods for a single group of subjects, determining whether or not changed has occurred over time.

Since these variables have different scales, there was a need to normalize the results in order to compare them. For that purpose, the value that was obtained at the initial assessment was considered 100% for that patient. Subsequent results, obtained in return visits, are expressed as a percentage of the baseline value.

In order to quantify this progression, the Area under the disease progression curve (AUC) was calculated for each variable, according to the Trapezoid rule, using the program GraphPad Prism 5®.

Figure 12: Trapezoid rule for AUC calculation



The AUC was then normalized, by comparison with an AUC of a normal, non-progressive subject (always the same results on return visits), thus reflecting a progression rate of the disease.

5. RESULTS

5.1 Healthy subjects group

Our healthy subjects group was comprised by 15 individuals, 9 with less than 60 years and 6 with more than 60 years. In the total cohort there were 7 male and 8 females. The mean age was 45.2 ± 20.7 years (range 22-76; median age 31). In the group <60 there were 5 males and 4 females with a mean age of 30.4 ± 10. years (range 22-57; median age 28). In the group >60 there were 2 males and 4 females with a mean age of 67.3 ± 6 years (range 61-76; median age 66.5).

5.2 Descriptive analysis of the results in the Healthy subjects group

In table 5 are listed the mean values and standard deviations of CMAP and MUNIX of all measurements (four measurements per muscle).

Table 5: Mean values of MUNIX and CMAP in healthy subjects

n = 15	**Mean**	**SD**	**Range**
MUNIX APB	143.1	47.0	78-260
MUNIX ADM	168.7	45.2	86-270
MUNIX BB	150.1	65.3	58-299
MUNIX TA	149.0	33.1	86-222
MUNIX AH	215.1	127.0	37-521
MUNIX EDB[a]	110.6	59.4	39-247
MUNIX MEGASCORE[a]	811.7	209.5	560-1348
CMAP APB	8.2	2.0	5-14.4
CMAP ADM	10.9	1.8	7.7-15.4
CMAP BB	6.4	2.8	2.3-13.6
CMAP TA	7.2	1.4	3.9-10.2
CMAP AH	14.2	7.1	4.5-31
CMAP EDB[a]	7.1	3.4	3.1-15.5
CMAP[a]	47.0	9.0	34.9-66.1

a - EDB, CMAP MEGASCORE and MUNIX MEGASCORE: n = 8 subjects

In table 6 are listed the mean values and standard deviations of CMAP and MUNIX of all measurements (four measurements per muscle), in the youngest group.

Table 6: Mean values of MUNIX and CMAP in youngest controls

n = 9	**Mean**	**SD**	**Range**
MUNIX APB	165.7	46.4	78-260
MUNIX ADM	183.6	45.7	116-270
MUNIX BB	177.9	67.5	79-299
MUNIX TA	158.1	30.6	101-222
MUNIX AH	280.5	125.9	83-521
MUNIX EDB[a]	147.0	75.5	50-247
MUNIX MEGASCORE[a]	1094.6	161.2	917-1348
CMAP APB	8.9	2.2	5.3-14.4
CMAP ADM	11.7	1.8	8.4-15.4
CMAP BB	7.4	2.9	3.2-13.6
CMAP TA	7.8	1.1	5.9-10.2
CMAP AH	18.0	6.6	5.9-31
CMAP EDB[a]	9.5	4.8	3.4-15.5
CMAP MEGASCORE[a]	59.6	5.0	49.9-66.1

a - EDB, CMAP MEGASCORE and MUNIX MEGASCORE: n = 2 subjects

In table 7 are listed the mean values and standard deviations of CMAP and MUNIX of all measurements (four measurements per muscle), in the group with older patients.

Table 7: Mean values of MUNIX and CMAP in older controls

n = 6	**Mean**	**SD**	**Range**
MUNIX APB	109.2	19.6	82-157
MUNIX ADM	146.5	34.8	86-223
MUNIX BB	108.3	31.2	58-189
MUNIX TA	135.3	32.5	86-196
MUNIX AH	119.6	34.9	37-174
MUNIX EDB	98.4	49.1	39-213
MUNIX MEGASCORE [a]	717.4	118.9	560-994
CMAP APB	7.1	1.2	5-10.5
CMAP ADM	9.6	1.0	7.7-12.3
CMAP BB	4.8	1.6	2.3-8.4
CMAP TA	6.4	1.3	3.9-8.5
CMAP AH	8.5	2.4	4.5-12.6
CMAP EDB	6.3	2.4	3.1-12.2
CMAP MEGASCORE	42.8	5.3	34.9-53

The MUNIX and CMAP mean values were all significantly lower in the group with the older subjects, as demonstrated by an independent sample t-test. EDB muscle and MUNIX and CMAP megascores were not assessed since the group with young subjects had 2 controls (8 measurements) only.

Table 8: Comparison of MUNIX and CMAP values between both group of controls

	< 60 (n=9)		> 60 (n=6)		t	df	Sig. (2-tailed)
	Mean	SD	Mean	SD			
MUNIX APB	165.7	46.4	109.2	19.6	6.485	50.7	**.000**
MUNIX ADM	183.6	45.7	146.5	34.8	3.377	58.0	**.001**
MUNIX BB	177.9	67.5	108.3	31.2	5.384	52.7	**.000**
MUNIX TA	158.1	30.6	135.3	32.5	2.753	58.0	**.008**
MUNIX AH	280.5	125.9	119.6	34.9	7.170	41.3	**.000**
CMAP APB	8.9	2.2	7.1	1.2	3.840	56.9	**.000**
CMAP ADM	11.7	1.8	9.6	1	5.702	56.9	**.000**
CMAP BB	7.4	2.9	4.8	1.6	4.503	56.8	**.000**
CMAP TA	7.8	1.1	6.4	1.3	4.267	58.0	**.000**
CMAP AH	18	6.6	8.5	2.4	7.908	47.8	**.000**

Since the CMAP has importance in the MUNIX calculations, a correlation between CMAP and MUNIX values is to be expected. This is demonstrated by a Spearman correlation, where we can see that CMAP and MUNIX are highly correlated in all muscles analyzed.

Table 9: Correlation between MUNIX and CMAP

n = 60	APB	ADM	BB	TA	AH	EDB[a]	Megascores[a]
cc	0.681	0.708	.974	.777	.907	.910	.950
p	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**

a - EDB and Megascores: n = 32

5.3 Variability analysis

The first step was to analyze the ICC of the total variability (inter-rater and intra-rater) of all four measurements per muscle.

Table 10: Overall ICC values

Muscle	***ICC***
MUNIX APB	0.701
MUNIX ADM	0.677
MUNIX BB	0.567
MUNIX TA	0.618
MUNIX AH	0.782
MUNIX EDB	0.749
MUNIX	0.740
CMAP APB	0.627
CMAP ADM	0.578
CMAP BB	0.502
CMAP TA	0.709
CMAP AH	0.938
CMAP EDB	0.851
CMAP	0.855

The muscles with higher ICC were the small muscles in the hand (APB and ADM) and the small muscles of the foot (AH and EDB). The ICC of the MUNIX megascore (MUNIX) and the CMAP megascore (CMAP), given by the sum of the measurements of all muscles in one assessment, is 0.740 and 0.855, respectively.

The ICC of the mean inter-rater variability are listed in table 11.

Table 11: Inter-rater ICC values

Muscle	*ICC*
MUNIX APB	0.689
MUNIX ADM	0.652
MUNIX BB	0.520
MUNIX TA	0.666
MUNIX AH	0.782
MUNIX EDB	0.737
MUNIX	0.673
CMAP APB	0.636
CMAP ADM	0.454
CMAP BB	0.435
CMAP TA	0.718
CMAP AH	0.925
CMAP EDB	0.891
CMAP	0.853

MUNIX measurements in all muscles, apart from biceps brachii, showed ICC values above 0.6, with AH muscle exhibiting the highest ICC (0.782). The ICC of the MUNIX megascore was 0.673 and the CMAP megascore 0.853.

The ICC of the mean intra-rater variability are listed in table 12.

Table 12: Intra-rater ICC values

Muscle	*ICC*
MUNIX APB	0.709
MUNIX ADM	0.796
MUNIX BB	0.700
MUNIX TA	0.568
MUNIX AH	0.868
MUNIX EDB	0.749
MUNIX	0.827
CMAP APB	0.613
CMAP ADM	0.802
CMAP BB	0.666
CMAP TA	0.725
CMAP AH	0.974
CMAP EDB	0.839
CMAP	0.890

MUNIX measurements in all muscles, apart from tibialis anterior, showed ICC values above 0.7, with AH muscle exhibiting the highest ICC (0.868). The ICC of the MUNIX megascore was 0.827 and the CMAP megascore 0.890.

5.4 Clinical group - ALS patients

Our clinical sample of ALS patients comprised 11 patients, 10 males and 1 female, with mean age 66.45 ± 10.18 years (range 51-86; median age 63). The mean duration of symptoms at baseline assessment was 12.88 ± 4.59 months (range 5-18; median 13.6). Regarding the type of onset of the disease, 3 patients had bulbar onset and 8 patients had limb onset.

Table 13: Age and disease duration of the ALS population

	Onset					
	Bulbar			Limb		
	N	Mean	Standard Deviation	N	Mean	Standard Deviation
Age	3	64.21	12.89	8	67.34	8.93
Disease_duration		12.17	5.55		13.17	4.20

The mean and standard deviation values, at baseline assessment, of the several variables studied, are reported in table 14.

Table 14: Descriptive statistics of the studied variables at entry

	N	Minimum	Maximum	Mean	Std. Deviation	Median
MUNIX MEGASCORE	11	207	737	494.4	156.7	390
MUNE ADM	11	21	150	79.6	41.5	66
CMAP MEGASCORE	11	14.9	47.5	33.3	9.6	30.4
ALSFRS	11	37	45	41.4	2.6	36
MRC	11	124	160	149.8	11.3	143

We compared the values of MUNIX and CMAP megascores of ALS subjects at baseline to the values of the control group with a t-test for independent samples. The values of ALS subjects were significantly lower than the control group.

Table 15: Comparison of MUNIX and CMAP megascores between ALS patients and controls

	Controls		ALS subjects		t	df	Sig. (2-tailed)
	Mean	SD	Mean	SD			
MUNIX MEGASCORE	811.7	209.5	494.4	156.7	4.586	41	**.000**
CMAP MEGASCORE	47.0	9.0	33.3	9.6	4.266	41	**.000**

Before starting the analysis of these variables over the time, we looked for a correlation between them. Since all of them, each in its own way, can reflect disease status, a correlation between them should be expected. A Spearman's rho was used to assess these correlations. In fact, all of them showed a significant, positive and moderate to strong correlations between them.

Table 16: Correlation between all studied variables

		MUNIX	MUNE	CMAP	ALSFRS	MRC
MUNIX MEGASCORE	Correlation Coefficient	1.000	.629	.897	.860	.608
	Sig. (2-tailed)	.	.000	.000	.000	.000
MUNE ADM	Correlation Coefficient	.629	1.000	.696	.507	.746
	Sig. (2-tailed)	.000	.	.000	.000	.000
CMAP MEGASCORE	Correlation Coefficient	.897	.696	1.000	.716	.659
	Sig. (2-tailed)	.000	.000	.	.000	.000
ALSFRS	Correlation Coefficient	.860	.507	.716	1.000	.598
	Sig. (2-tailed)	.000	.000	.000	.	.000
MRC	Correlation Coefficient	.608	.746	.659	.598	1.000
	Sig. (2-tailed)	.000	.000	.000	.000	.

5.5 Analysis of progression

Since ALS is a neurodegenerative disease, and the purpose of this work was to analyze the suitability of a new marker of disease progression, we assessed these patients for 9 or 12 months since baseline visit. The assessments were made approximately every 3 months (3.3 ± 0.5 months; range 2.3 - 4.4). 6 patients had 3 follow-up visits and 5 patients had 4 follow-up assessments (9 and 12 months, respectively).

We started by analyzing the progression of each variable with a Repeated measures ANOVA. The first thing is to assess the Sphericity of each group of variables. The Mauchly's test is one of the most used for this purpose. As we can see in table 17, only CMAP MEGASCORE and ALSFRS do not fulfill the sphericity assumption for an $\alpha = 0.05$.

Table 17: Mauchly's test of Sphericity

	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
MUNIX MEGASCORE	.473	6.539	5	.260	.749	.976	.333
MUNE ADM	.811	1.831	5	.873	.869	1.000	.333
CMAP MEGASCORE	.246	12.240	5	**.033**	.541	.630	.333
ALSFRS	.204	13.851	5	**.017**	.563	.665	.333
MRC	.564	4.992	5	.419	.777	1.000	.333

We then proceed to the Repeated measures ANOVA, using the Greenhouse-Geisser correction factor when the sphericity assumption is violated. Since only 5 of the 11 patients had 4 follow-up assessments, we only analyzed the data up to the 3rd return visit (9 months). Table 18 resumes these results.

Table 18: Repeated measures ANOVA results for the studied variables

	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
MUNIX MEGASCORE	.957	3	.319	27.345	**.000**	.732	82.036	1.000
MUNE ADM	1.459	3	.486	8.583	**.000**	.462	25.748	.987
CMAP MEGASCORE[a]	.725	1.622	.447	17.033	**.000**	.630	27.632	.996
ALSFRS[a]	.330	1.689	.195	17.495	**.000**	.636	29.551	.997
MRC	.072	3	.024	15.149	**.000**	.602	45.446	1.000

a - Analysis with Greenhouse-Geisser correction factor

From this table we can conclude that all variables have significant differences between any two assessments. Looking at the graphic displayed in Figure 13, we can see that all variables decline over the time. Merging the Repeated measures ANOVA results with the data observed from the graphic, we can conclude that all variables decline with time in a significant way.

The *post-hoc* tests for each variable that allow the identification of the significant differences in each pair of assessments can be found in the annexes (Annex A, tables 31-35).

Knowing that all variables decline significantly with time, we wanted to see if there were significant differences in each assessment. For that purpose, we applied the Friedman test. The statistical analysis revealed significant differences between any two variables in all assessments (table 19) – 3 Months ($\chi_F^2\,(4) = 14.036;\ p = 0.005;\ N = 11$); 6 Months ($\chi_F^2\,(4) = 13.567;\ p = 0.006;\ N = 11$); 9 Months ($\chi_F^2\,(4) = 14.312;\ p = 0.004;\ N = 11$).

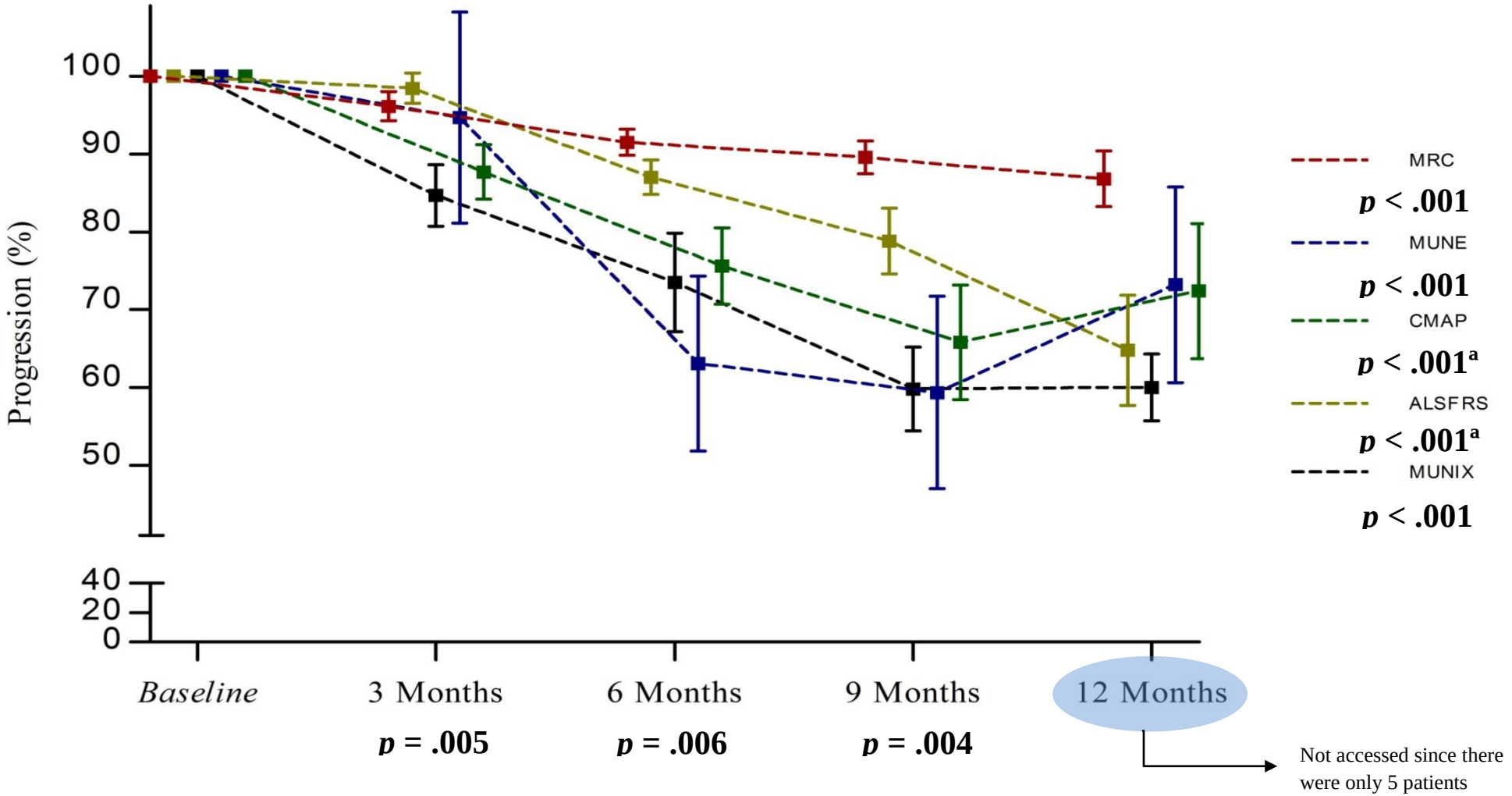


Figure 13: Progression of the five studied variables. Values represented are mean ± 1 standard error of mean (SEM).
p values on the right side are from repeated measures ANOVA for each variable from baseline to 9 months. (a - Analysis with Greenhouse-Geisser correction factor)
p values on the bottom are from Friedman tests comparing all variables in each assessment.

Table 19: Friedman test results for each assessment

	3 Months	6 Months	9 Months
N	11	11	11
Chi-square	14.036	13.567	14.312
df	4	4	4
Asymp. Sig.	.007	.009	.006
Exact Sig.	**.005**	**.006**	**.004**
Point Probability	.000	.000	.000

We then proceed to investigate which pairs had significant differences in each evaluation time. For that purpose, we applied the Wilcoxon test for each pair within an assessment. Since this analysis requires multiple comparisons, we only consider significant p values ≤ 0.01. The significant differences are listed in table 20.

Table 20: Wilcoxon test results

	ALSFRS_3m - MUNIX_3m	ALSFRS_3m - CMAP_3m	MRC_6m - CMAP_6m	ALSFRS_9m - MUNIX_9m	MRC_9m - MUNIX_9m	MRC_9m - CMAP_9m
Z	-2.852	-2.581	-2.58	-2.536	-2.934	-2.584
Asymp. Sig. (2-tailed)	0.004	0.01	0.01	0.011	0.003	0.01
Exact Sig. (2-tailed)	**0.002**	**0.006**	**0.007**	**0.009**	**0.001**	**0.007**
Exact Sig. (1-tailed)	0.001	0.003	0.003	0.004	0	0.003
Point Probability	0	0	0.001	0.002	0	0.001

Again, adding these results to the data observed from the graphic, we can conclude that MUNIX MEGASCORE and CMAP MEGASCORE show significant lower values, thus more progression, than ALSFRS and MRC.

Despite these first results have shown statistical significance, they do not quantify the progression over time. In order to do that, we calculated the normalized AUC of all variables, according to the trapezoid rule. Although some assumptions are made with this method, it is a suitable form of measure progression. After calculating

the AUC for each variable (table 21), we compared them using the Friedman test ($\chi_F^2\,(4) = 11.855;\ p = 0.015;\ N = 11$). Figure 14 displays a graphic representing the mean ± 1 SEM of the normalized AUC of each variable.

Table 21: Descriptive statistics of the variables AUC

	N	Mean	Std. Deviation	Std. Error of Mean	Median
MUNIX MEGASCORE	11	22,1	11,4	3,4	25,6
MUNE ADM	11	21,3	26,9	8,1	9,8
CMAP MEGASCORE	11	18,4	12,3	3,7	14,6
ALSFRS	11	10,8	5,3	1,6	10,8
MRC	11	6,3	4,0	1,2	4,7

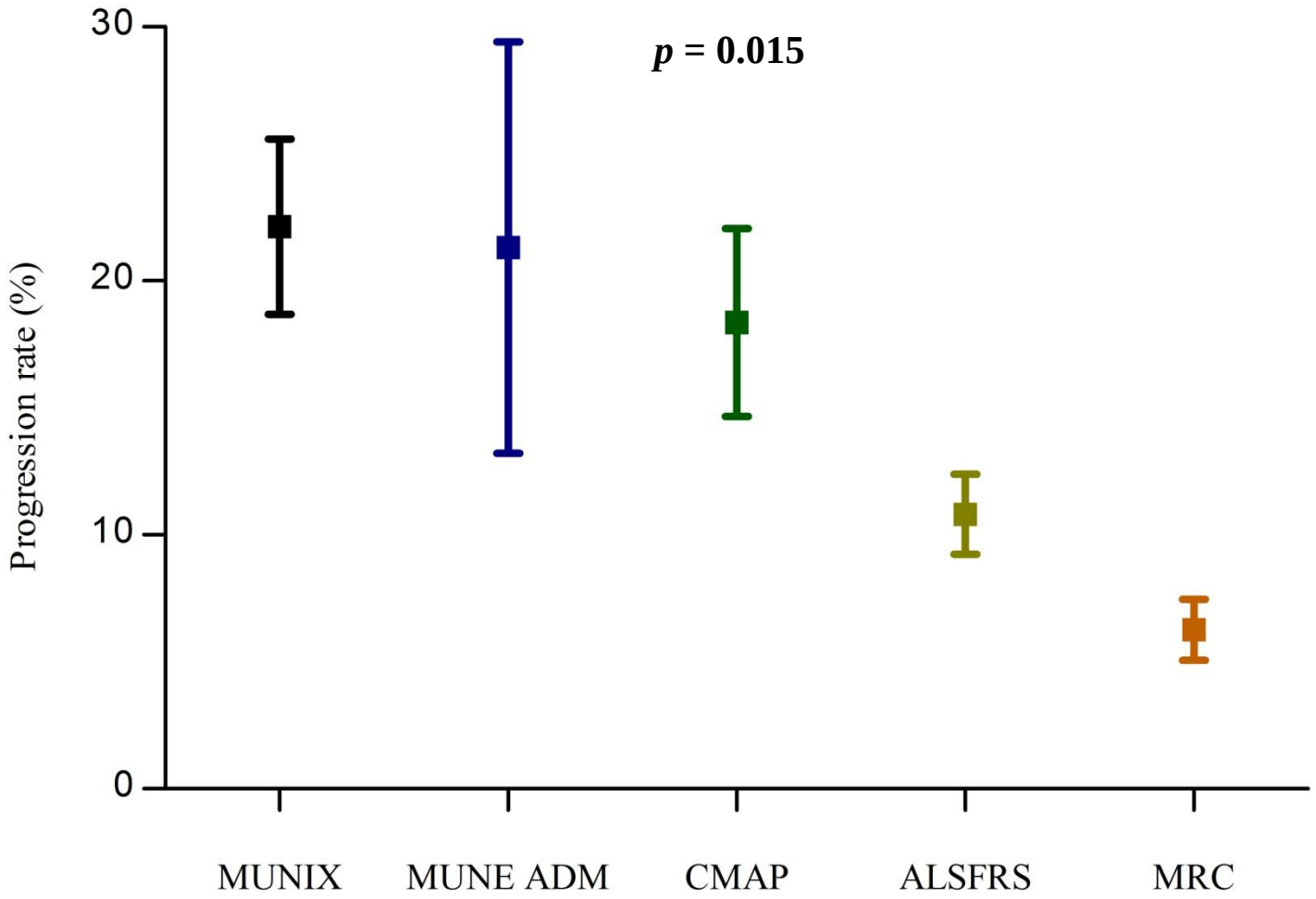


Figure 14: Graphic representation of each variable AUC distribution. Values represented are mean ± 1 standard error of mean (SEM).
p value on the top is from the Friedman test comparing the AUC of all variables.

Given the significant result of the Friedman test, we went on to search which pairs had significant differences. We applied the Wilcoxon test to every pair of variables, considering only *p* values ≤ 0.01 as significant, since we were dealing with multiple comparisons.

Table 22: Wilcoxon test results

	ALSFRS – MUNIX MEGASCORE	MRC – MUNIX MEGASCORE	MRC - CMAP MEGASCORE
Z	-2.667	-2.669	-2.49
Asymp. Sig. (2-tailed)	0.008	0.008	0.013
Exact Sig. (2-tailed)	**0.005**	**0.005**	**0.01**
Exact Sig. (1-tailed)	0.002	0.002	0.005
Point Probability	0.001	0.001	0.001

MUNIX MEGASCORE had a significantly larger progression rate than ALSFRS and MRC, whereas CMAP MEGASCORE had only had a larger progression rate than MRC.

Bulbar onset vs. Limb onset – an exploratory analysis.

It is well known that patients may have different progression rates, according to the type of onset of the disease. Also, bulbar onset patients may go further without limb compromise, hindering the ability of these methods to measure disease progression. In order to see if these different methods show significant differences between limb onset patients and bulbar onset patients, we applied a Mann-Whitney test to the AUC of those variables.

Table 23: Mann-Whitney test results

	MUNIX MEGASCORE	MUNE ADM	CMAP MEGASCORE	ALSFRS	MRC
Mann-Whitney U	11.000	8.000	10.000	8.500	12.000
Wilcoxon W	17.000	14.000	46.000	44.500	48.000
Asymp. Sig.	.838	.414	.683	.474	1.000
Exact Sig.	**.921**	**.497**	**.776**	**.515**	**1.000**
Point Probability	.073	.061	.073	.024	.079

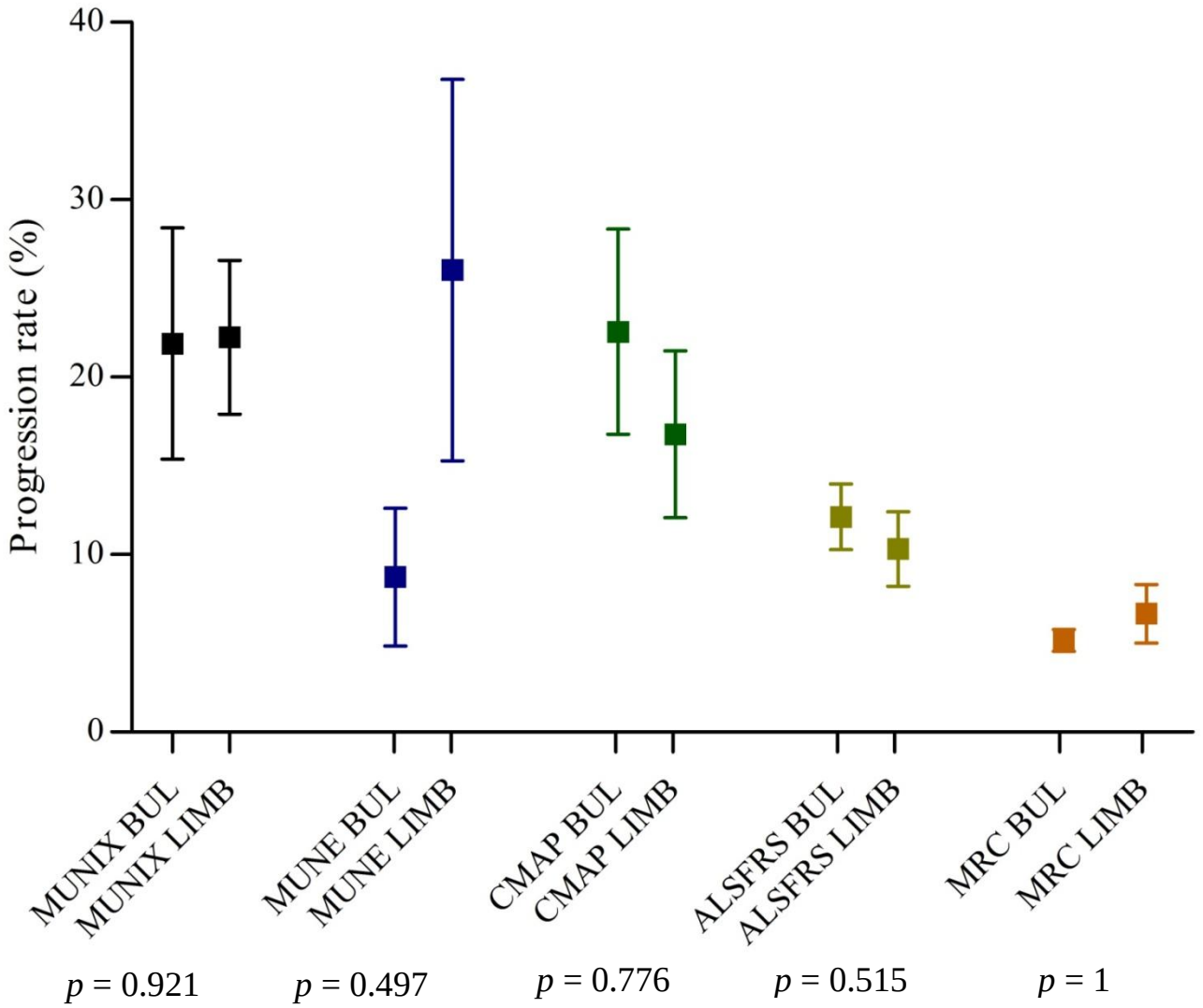


Figure 15: Distribution of AUC of all variables, divided by type of onset. Values represented are mean ± 1 standard error of mean (SEM).

p value on the bottom are from Mann-Whitney tests.

Although no statistically significant difference was found between bulbar onset and limb onset patients, for any of the variables, these results have to be seen as an exploratory analysis, since we had only 3 bulbar onset patients on this group.

5.6 MUNIX progression in different muscles

6 muscles have been chosen for calculating the MUNIX megascore, 3 from the upper limb and 3 from the lower limb, 2 distal and 1 proximal in each limb. The MUNIX absolute values from each muscle at baseline are reported in table 24.

Table 24: Descriptive statistics of MUNIX for each muscle

	N	Mean	Std. Deviation	Median	Minimum	Maximum
APB	11	74.2	41.3	74	23	171
ADM	11	96.5	55.6	111	32	215
BB	11	102.8	39.1	98	40	162
TA	11	78.5	35.7	78	20	135
AH	11	99.3	55.6	105	11	193
EDB	11	43	28.3	47	5	82

We went on to see if there were significant differences between these muscles in MUNIX measurements.

The first step was to see if all muscles progress significantly with time. For that purpose we applied a Repeated measures ANOVA. The Mauchly's test (table 25) showed that only AH muscle did not fulfill the sphericity assumption.

Table 25: Mauchly's test of Sphericity

	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
APB	.403	7.929	5	.162	.706	.900	.333
ADM	.535	5.457	5	.365	.761	.998	.333
BB	0.541	5.362	5	.376	0.745	0.969	0.333
TA	.505	5.955	5	.313	.717	.919	.333
AH	.233	12.713	5	**.027**	.608	.737	.333
EDB	0.739	2.634	5	.757	0.848	1	0.333

We proceeded to the Repeated measures ANOVA, using the Greenhouse-Geisser correction factor when the sphericity assumption is violated. Because only 5 of the 11 patients had 4 follow-up assessments, we only analyzed the data up to the 3rd return visit. Table 26 resumes these results.

Table 26: Repeated measures ANOVA results for all muscles

	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power
APB	1.659	3	.553	7.016	**.001**	.412	21.049	.963
ADM	1.825	3	.608	17.133	**.000**	.631	51.398	1.000
BB	.712	3	.237	8.930	**.000**	.472	26.790	.990
TA	.817	3	.272	6.080	**.002**	.378	18.239	.934
AH[a]	0.539	1.825	0.295	1.842	0.189	0.156	3.362	0.321
EDB	1.066	3	.355	3.287	**.034**	.247	9.861	.692

a - Analysis with Greenhouse-Geisser correction factor

From this table we can conclude that all muscles, except AH, have significant differences between any two assessments. Looking at the graphic displayed in Figure 16, we can see that all muscles decline with time. Adding the Repeated measures ANOVA results with the data observed from the graphic, we can conclude that all muscles, except AH, decline with time in a significant way.

The *post-hoc* tests for each muscle that allow the identification of the significant differences in each pair of assessments can be found in the annexes (Annex A, tables 36-40).

Knowing that all muscles decline significantly with time, we looked for significant differences in each assessment. We applied a Friedman test for this purpose. The statistical analysis revealed no significant differences between any two muscles in any assessment (table 27) – 3 Months ($\chi_F^2\,(5) = 8.992;\ p = 0.109; N = 11$); 6 Months ($\chi_F^2\,(5) = 7.130;\ p = 0.211;\ N = 11$); 9 Months ($\chi_F^2\,(5) = 3.887;\ p = 0.566;\ N = 11$).

Table 27: Friedman test results for each assessment

	3 Months	6 Months	9 Months
N	11	11	11
Chi-square	8.992	7.130	3.887
df	5	5	5
Asymp. Sig.	.109	.211	.566

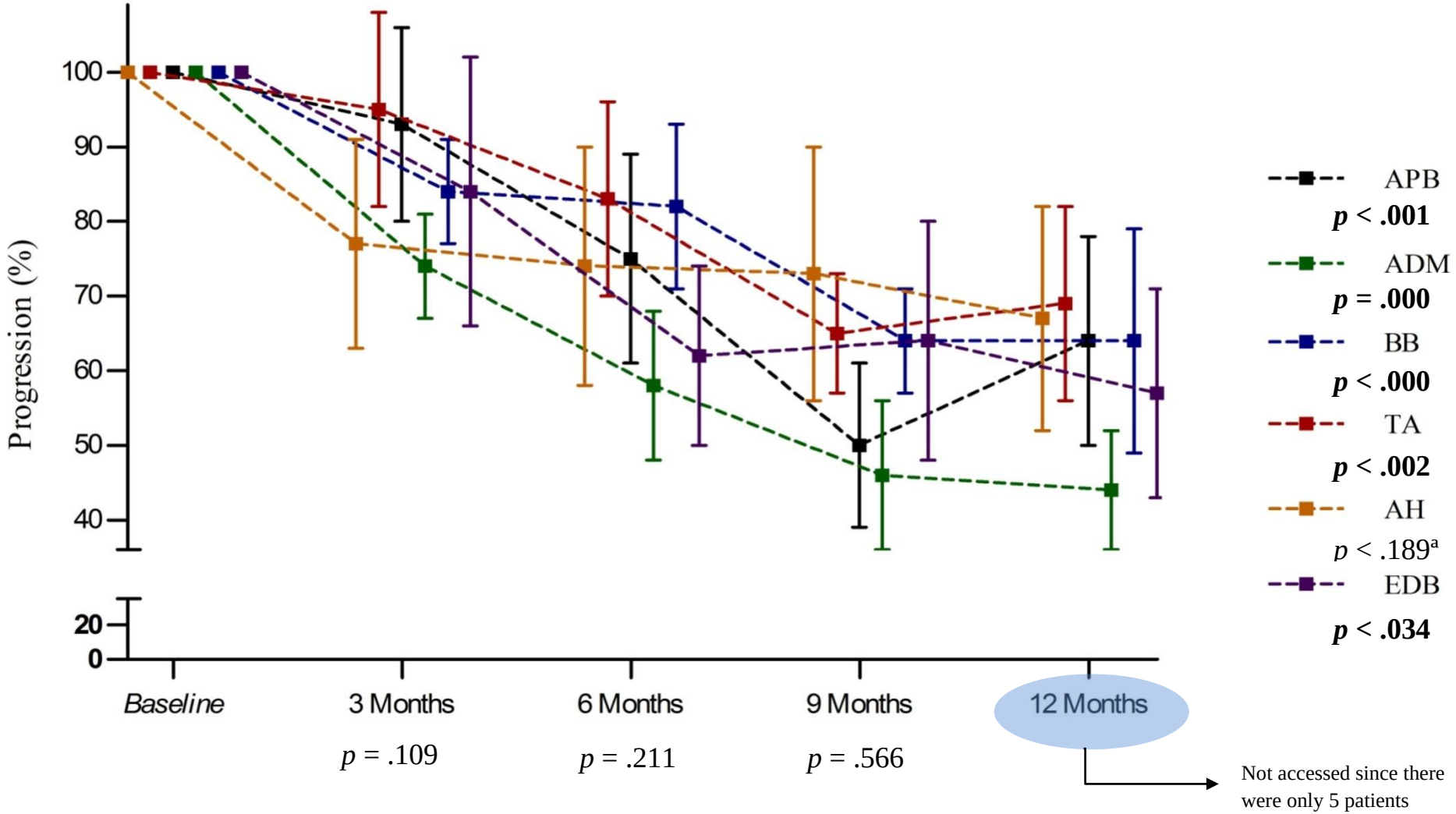


Figure 16: Progression of the studied muscles. Values represented are mean ± 1 standard error of mean (SEM).
p values on the right side are from repeated measures ANOVA for each muscle from baseline to 9 months. (a - Analysis with Greenhouse-Geisser correction factor)
p values on the bottom are from Friedman tests comparing all variables in each assessment.

We also quantified the progression of each muscle by calculating their respective AUC. After this calculation, we compared them using the Friedman test ($\chi_F^2(5) = 5.237;\ p = 0.388;\ N = 11$). Figure 17 displays a graphic representing the mean ± 1 SEM of the AUC of each variable.

Table 28: Descriptive statistics of the muscles AUC

	N	Mean	Std. Deviation	Std. Error of Mean	Median
APB	11	21.4	23.1	6.9	21.3
ADM	11	33.2	20.0	6.0	30.8
BB	11	18.3	19.0	5.7	17.3
TA	11	14.8	27.1	8.2	15.7
AH	11	19.1	37.8	11.4	13.3
EDB	11	22.8	40.6	12.2	21.7

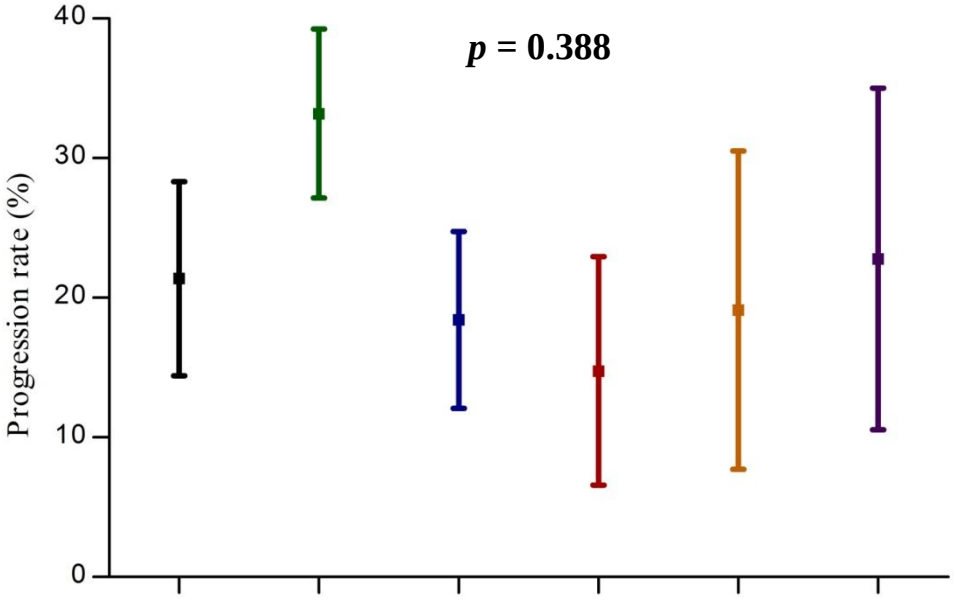


Figure 17: Graphic representation of each muscle AUC distribution. Values represented are mean ± 1 standard error of mean (SEM).
p value on the top is from the Friedman test comparing the AUC of all muscles.

Although no significant differences have been found between the progression rates of the 6 studied muscles, we can notice that MUNIX in ADM muscle seems to have a slightly higher progression rate than other muscles.

5.7 MUNIX vs. MUNE in ADM muscle

An interesting analysis that we could do was to compare different techniques of motor unit number estimation. We compared the MUNIX to Multiple point stimulation MUNE in the ADM muscle. Figure 18 displays a graphic representation of the progression of these two variables with time.

The repeated measures ANOVA for these variables has been previously calculated: MUNIX ADM ($F(3,30) = 17.133; p < 0.001; Observed\ power = 1.000$); MUNE ADM ($F(3,30) = 8.583; p < 0.001; Observed\ power = 0.987$)

We applied a Wilcoxon test to look for significant differences in each assessment (table 29).

Table 29: Wilcoxon test results

	3 Months	6 Months	9 Months
Z	-1.718	-1.224	-1.734
Asymp. Sig. (2-tailed)	.086	.221	.083
Exact Sig. (2-tailed)	.098	.240	.090
Exact Sig. (1-tailed)	.049	.120	.045
Point Probability	.012	.008	.006

No statistical significant difference has been found between MUNIX and MUNE in each assessment.

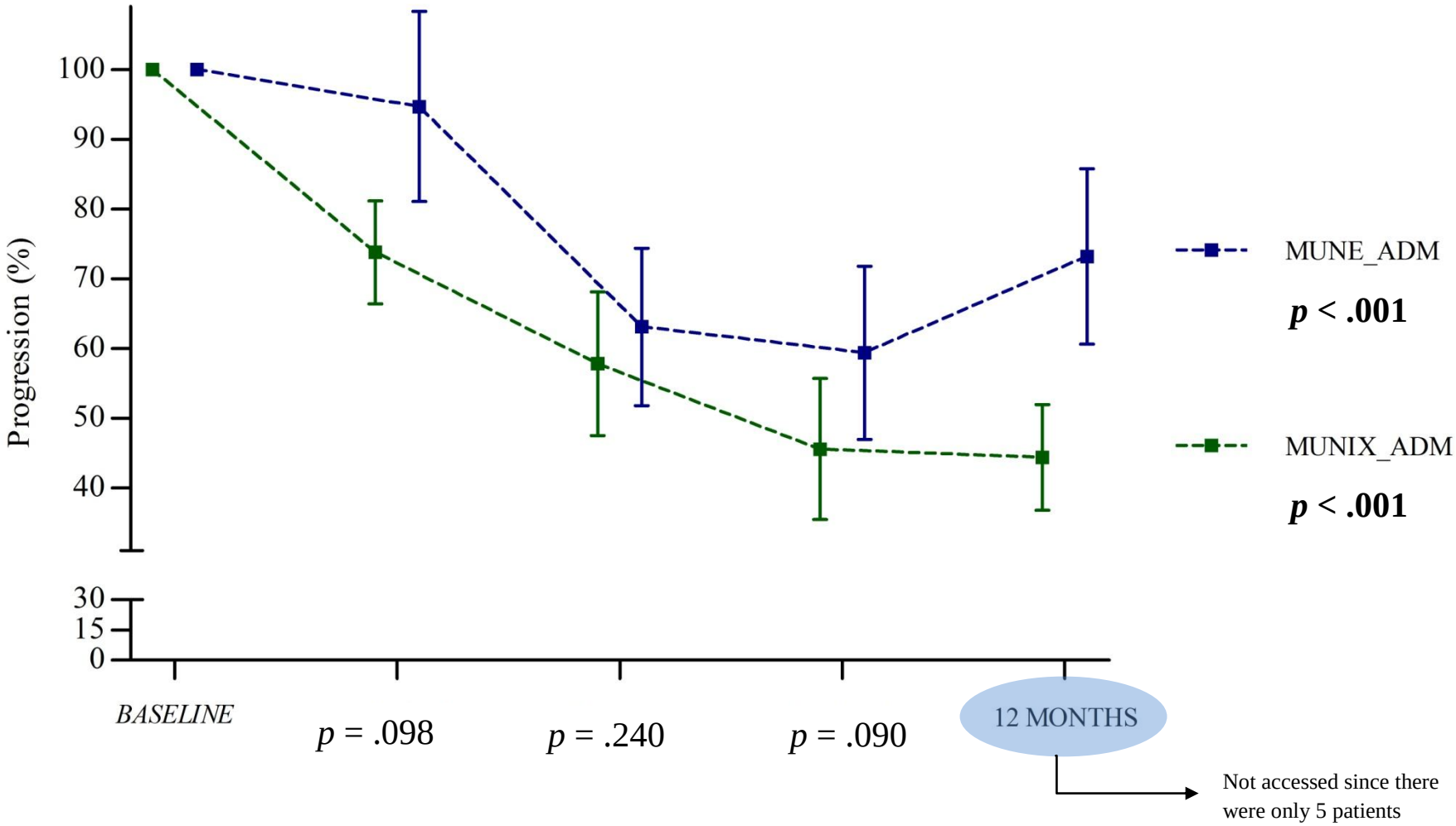


Figure 18: Progression of MUNIX and MUNE in the ADM muscle. Values represented are mean ± 1 standard error of mean (SEM).
p values on the right side are from repeated measures ANOVA for each method from baseline to 9 months.
p values on the bottom are from Wilcoxon test comparing the two methods.

Despite no significant differences between MUNE and MUNIX in any assessment, when we look at the graphic we can notice that MUNIX has a steepest and linear decline than MUNE. Given that, we went to compare the AUC of the two techniques (table 30; Figure 19).

Table 30: Descriptive statistics of ADM MUNIX and MUNE AUC

	N	Mean	Std. Deviation	Std. Error of Mean	Median
MUNIX	11	33.2	20.0	6.0	30.8
MUNE	11	21.3	26.9	8.1	9.8

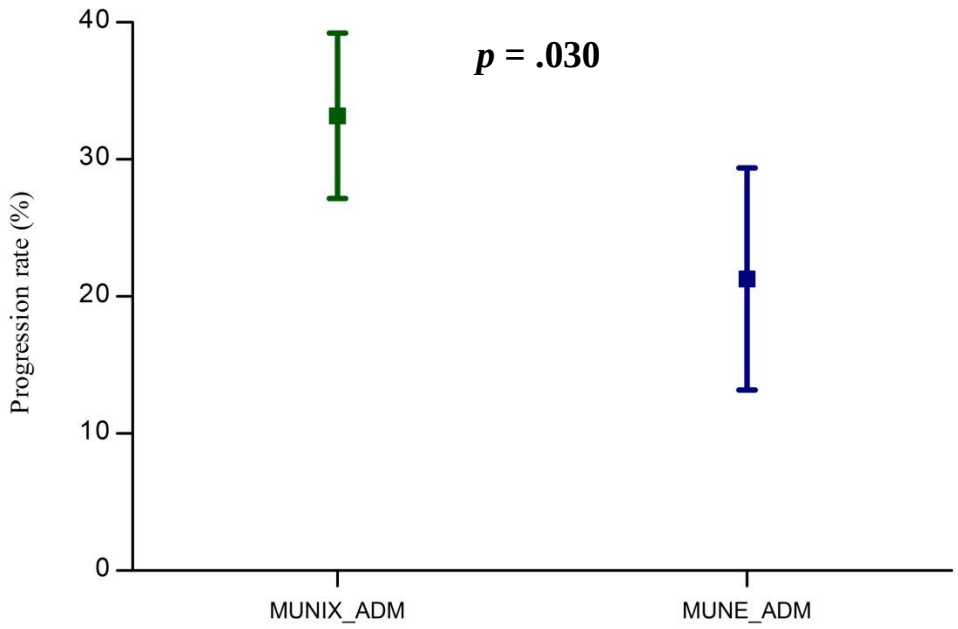


Figure 19: Graphic representation of each method AUC distribution. Values represented are mean ± 1 standard error of mean (SEM).
p value on the top is from the Wicoxon test comparing the AUC of the two methods.

We applied a Wilcoxon test to compare the two methods ($\bar{S}+= 4.5; \bar{S}-= 6.33; Z = -2.135; p = 0.030; N = 11$). This test showed a significant difference between the two variables, with MUNIX having a higher progression rate than MUNE in the ADM muscle.

6. DISCUSSION

The objectives of this study were to evaluate the intra- and inter-rater reliability of MUNIX, a new method of MUNE described in 2004 (Nandedkar et al., 2004), as well as its suitability as a biomarker of disease progression in ALS patients. The technique is quick to perform (20 to 30 minutes for the 6 muscles), non-invasive and overall well tolerated by healthy volunteers and ALS patients. When compared to other well established MUNE techniques, MUNIX has some obvious advantages has it studies more muscles in the same time frame, requiring less electrical stimuli (Bromberg, 2007).

Part of this work is included on a multicentre study designed to assess MUNIX reproducibility, from which some papers have already been published (Neuwirth et al., 2011; Neuwirth et al., 2011a).

6.1 MUNIX absolute values

In normal subjects the absolute values of MUNIX were in line with the few studies published to date (Nandedkar et al., 2004; Ahn et al., 2010; Nandedkar et al., 2010; Neuwirth et al., 2011; Neuwirth et al., 2011a), giving the first indication of the reproducibility of the method across centers.

The comparison of the values obtained by the MUNIX method with values reported using other MUNE techniques (Daube 1988; Doherty and Brown, 1993; Wang and Delwaide, 1995; Shefner, 2004; Boe, 2007; Boe 2009), showed a high agreement for some of the muscles, indicating that MUNIX is at least as good as other techniques for the estimation of the number of motor units.

In ALS patients, MUNIX values at baseline were, as expected, significantly lower than controls, reflecting the loss of motoneurons at the time of diagnosis (Swash and Ingram, 1988). Comparison of these values with other MUNIX studies in ALS patients is somewhat difficult given the differences in disease duration or type of onset. Of the few studies with this method, the majority of them only study the hypothenar muscle (Nandedkar et al., 2004; Ahn et al., 2010; Nandedkar et al., 2010), one studies

hypothenar and thenar muscle (Nandedkar et al., 2011) and one reports the MUNIX megascore from 8 muscles – APB, ADM, AH and EDB bilaterally (Neuwirth et al., 2010). Regardless, the baseline values from our work in ALS patients are similar to the ones reported in the aforementioned studies.

We should emphasize that MUNIX is an index related to the number of functioning motoneurons that might change depending on the parameters chosen, such as the area selected for the MUNIX calculation. Taking this into account, the comparison of MUNIX values with other techniques as to be taken cautiously.

6.2 Variability analysis

All muscles assessed showed a reasonably good ICC. There are no defined values for a bad/weak or good/strong ICC, only that the closest to 1, the highest the correlation. Its interpretation depends on the situation in analysis. In our particular case, taking into account that there was no previously experience with this method, the ICC values obtained can be considered rather good. It is expected that with training in the technique, these values can improve further.

The muscles that showed the lowest test-retest-reliability were the BB and the TA. Since MUNIX depends highly on CMAP amplitude, it is expected that higher variability in CMAP measurements influence negatively the MUNIX reproducibility. The CMAP amplitude is highly dependent of the position of the active electrode (Bromberg and Spiegelberg, 1997), achieving the maximal response over the motor point of the muscle. In large muscles such as BB and TA, this can present a significant challenge. This difficulty in obtaining a maximal CMAP can lead to a higher variability in CMAP responses thus hindering the reproducibility of MUNIX.

Another possible explanation for these lower reproducibility values is the increased difficulty in discerning levels of force in the contraction of larger muscles. In larger muscles, where fine motor control in unnecessary, a given motoneuron can supply more than 200 muscle fibers, making it difficult to distinguish slight increments on the contraction force. This can lead to erroneous SIP measurements, which in turn can hamper the reproducibility of the method in these muscles.

Our test-retest-reliability results are in line with other studies on MUNIX reproducibility (Nandedkar et al., 2004; Ahn et al., 2010; Nandedkar et al., 2010; Neuwirth et al., 2010; Neuwirth et al., 2011;).

6.3 Progression analysis

In ALS patients, MUNIX megascore had a progression of 22,1% from baseline to 9/12 months. MUNIX megascore showed a significantly higher relative drop from baseline than ALSFRS-R and MRC. Regarding CMAP and ADM MUNE, MUNIX showed a higher relative drop, although without statistical significance. Of note is that MUNIX showed a lower standard deviation than CMAP and ADM MUNE, as well as a steepest and steadier decline.

A recent work with MUNIX method (Boekestein et al., 2012), also found significant higher decline of MUNIX in APB, comparing with ALSFRS and MRC. This study also reported a significant difference between MUNIX and CMAP, which we did not found.

ALSFRS-R has been used as a primary or secondary outcome measure in ALS clinical trials (Shefner et al., 2004; Scelsa et al., 2005), has high reproducibility and linearity (Kaufmann et al., 2007) and it is easily applicable in non-specialized centers. Nevertheless, a marker of disease progression should track the underlying pathology of the disease besides being a functional measure.

MRC has also been used a primary outcome measure in ALS clinical trials (Miller et al., 2001; Cudkowicz et al., 2003). However, MRC may lack the sensitivity needed to detect small but meaningful changes in deterioration and therapeutic efficacy.

CMAP also has been previously used as an outcome measure in clinical trials (Brooke et al., 1986; Kaji et al., 1998). Still, CMAP may not succeed in detecting motor unit loss due to successful collateral reinnervation, thus failing to understand the potential effect of a tested drug in a clinical trial.

Several MUNE techniques have been studied in ALS clinical trials (Shefner et al., 2004; Shefner et al., 2007; Bromberg and Brownell, 2008). However, we are still not sure if MUNE methods are equivalent or even better markers than other established

measures like ALSFR-R or forced vital capacity. Moreover, the value of MUNE techniques as a sensitive drug point for ALS drug studies has not been fully established (Bromberg, 2007), since it lacks the ability to predict clinical outcome (Bryan, 2003).

About two thirds of ALS patients present with limb onset and the remaining with bulbar onset (Wijesekera and Leigh, 2009). Depending on the type of onset, the clinical features of ALS progression varies, with bulbar onset patients developing limb weakness later than limb onset patients. MUNE techniques are not usually applied in bulbar muscles, and MUNIX in this study also was not. This may lead to an underestimation of disease progression in bulbar ALS patients. In our preliminary analysis, MUNIX did not show differences in assessing disease progression in bulbar and limb onset patients, suggesting that it is a suitably technique independently of the disease onset type. However, more research is needed on this subject, since we had only three bulbar onset patients, thus limiting the conclusions that can be drawn.

Given these considerations and the performance of MUNIX in this study, this novel method for estimating the number of motor units shows a high potential for a future role as a surrogate marker of disease progression in ALS clinical trials.

6.4 Comparison of MUNIX progression in individual muscles

Since ALS patients may present with a variety of clinical presentation, starting with focal weakness in upper or lower limb or bulbar muscles and then spreading to other areas (Ravits and La Spada, 2009), we opted to study several muscles, instead of one "index" muscle. However, the choice of the muscles included has to be carefully weighted.

Due to its intrinsic technical characteristics, MUNIX offers the possibility of studying virtually every muscle where a maximal CMAP by electrical stimulation can be obtained. In this study we included six muscles, three for upper limb and three from lower limb, one proximal and two distal in each member.

Our analysis showed that only AH did not progress significantly with time in ALS patients. The most likely explanation for this is that the electrical activity either in CMAP or SIP recordings is not only generated by the AH muscle, but also by volume-

conducted activity from other muscles innervated by the tibial nerve (Nandedkar and Barkhaus, 2007). Also, from our experience, in AH muscle there is a particular difficulty in controlling the force of contraction for SIP recordings. This is special true in patients with lower motor unit numbers. This may lead to increased difficulty in complying with the technique specifications, thus hindering the method's ability to detect disease progression in this muscle.

There were no significant differences in the rate of progression between all muscles, suggesting that MUNIX can be applied in a wide variety of muscles.

Despite these results, the ADM muscles showed a slightly increased rate of progression, in comparison to the other five muscles, with a steepest and steadier decline, suggesting that it can be useful in settings where only one muscle is chosen, or for the comparison with other MUNE techniques.

6.5 MUNIX vs. MUNE in ADM muscle

This is the first study to compare MUNIX to another widely used MUNE technique (Multiple point stimulation MUNE). There was a significant correlation between MUNIX and MUNE in ALS patients at the baseline assessment. The two methods declined significantly with time, with MUNIX showing less variability than MUNE. In our results, MUNIX had a significantly lager rate of progression (33,2% vs. 21,3%; $p = 0{,}30$) than MUNE.

MUNIX does not rely on a calculation of the mean SMUP, as the majority of MUNE techniques (Bromberg, 2007), thus surpassing some of the physiological limitations intrinsic of these methods. Our results suggest that this may be a more suitably method to monitor disease progression in ALS patients than multiple point stimulation MUNE.

A recent study comparing MUNIX to High-density MUNE in the thenar muscle (Boekestein et al., 2012) did not find any difference in the progression rate of these two methods. However HD-MUNE requires specific software and adapted electrodes for the high-density surface EMG that may not be easily available. Also, the detection of SMUPs in this method is very time consuming, thus limiting its use in clinical practice.

In the future it would be interesting to compare the progression of MUNIX with other MUNE techniques, such as the statistical MUNE, to observe if our results are replicated with other methods.

7. CONCLUSIONS AND STUDY LIMITATIONS

Our work has achieved the objectives that we proposed. MUNIX seems to have a good test-retest-reliability in healthy subjects thus stressing its applicability in clinical practice. Regarding the method suitability to monitor disease progression in ALS patients, MUNIX has performed as well or even better than current used methods, such as ALSFRS-R, multiple point stimulation MUNE, CMAP or MRC.

Throughout our work we encountered several problems and limitations.

The first and more important limitation is the reduced number of ALS patients included in this work. Eleven patients is not at all an unacceptable number, particularly in a work with a novel method, but it is not enough to draw solid conclusions. Another important drawback is the follow-up time used in this study. All patients had nine months of follow-up, and only five had one year. Although some conclusions can be taken, as we demonstrated throughout this work, a longer follow-up time would be desirable in order to study the late phases of disease progression in ALS.

Although, ALS is characterized by rapid clinical progression, and many of our patients could not be further investigated due to severe weakness, the previously mentioned problems could be approached by including more patients. Unfortunately, deadlines were imposed for the delivery of this work.

Another question that was not addressed in this study was the test-retest-reliability in ALS patients. Although this was in important point in studying MUNIX suitability, we did not had the opportunity to study each patient twice.

Overall, our results support MUNIX as probably the most convenient and effective MUNE method to investigate progression in ALS. However, its limitation in very spastic limbs and in patient with poor cognitive function deserve further consideration in future studies.

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ATTACHMENT A

Table 31: *Post hoc* test comparing MUNIX MEGASCORE in each assessment

(I) MUNIX MEGASCORE	(J) MUNIX MEGASCORE	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,153	,032	**,005**	,047	,258
	6 Months	,265	,057	**,006**	,077	,452
	9 Months	,402	,054	**,000**	,225	,578
3 Months	Baseline	-,153	,032	**,005**	-,258	-,047
	6 Months	,112	,039	,096	-,015	,238
	9 Months	,249	,043	**,001**	,107	,392
6 Months	Baseline	-,265	,057	**,006**	-,452	-,077
	3 Months	-,112	,039	,096	-,238	,015
	9 Months	,137	,046	,086	-,015	,289
9 Months	Baseline	-,402	,054	**,000**	-,578	-,225
	3 Months	-,249	,043	**,001**	-,392	-,107
	6 Months	-,137	,046	,086	-,289	,015

a - Adjustment for multiple comparison: Bonferroni

Table 32: *Post hoc* test comparing MUNE ADM in each assessment

(I) MUNE ADM	J) MUNE ADM	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,053	,110	1,000	-,307	,412
	6 Months	,369	,102	**,028**	,036	,703
	9 Months	,406	,124	**,049**	,001	,812
3 Months	Baseline	-,053	,110	1,000	-,412	,307
	6 Months	,316	,085	**,024**	,038	,595
	9 Months	,354	,094	**,023**	,045	,662
6 Months	Baseline	-,369	,102	**,028**	-,703	-,036
	3 Months	-,316	,085	**,024**	-,595	-,038
	9 Months	,037	,090	1,000	-,256	,331
9 Months	Baseline	-,406	,124	**,049**	-,812	-,001
	3 Months	-,354	,094	**,023**	-,662	-,045
	6 Months	-,037	,090	1,000	-,331	,256

a - Adjustment for multiple comparison: Bonferroni

Table 33: *Post hoc* test comparing CMAP MEGASCORE in each assessment

(I) CMAP MEGASCORE	(J) CMAP MEGASCORE	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,123	,028	**,009**	,030	,215
	6 Months	,244	,044	**,002**	,099	,388
	9 Months	,342	,073	**,005**	,101	,583
3 Months	Baseline	-,123	,028	**,009**	-,215	-,030
	6 Months	,121	,035	**,038**	,005	,236
	9 Months	,219	,059	**,025**	,025	,414
6 Months	Baseline	-,244	,044	**,002**	-,388	-,099
	3 Months	-,121	,035	**,038**	-,236	-,005
	9 Months	,098	,051	,488	-,068	,264
9 Months	Baseline	-,342	,073	**,005**	-,583	-,101
	3 Months	-,219	,059	**,025**	-,414	-,025
	6 Months	-,098	,051	,488	-,264	,068

a - Adjustment for multiple comparison: Bonferroni

Table 34: *Post hoc* test comparing ALSFRS-R in each assessment

(I) ALSFRS-R	(J) ALSFRS-R	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,016	,016	1,000	-,034	,067
	6 Months	,130	,020	**,000**	,064	,196
	9 Months	,212	,042	**,003**	,074	,350
3 Months	Baseline	-,016	,016	1,000	-,067	,034
	6 Months	,114	,027	**,011**	,025	,203
	9 Months	,195	,041	**,004**	,061	,329
6 Months	Baseline	-,130	,020	**,000**	-,196	-,064
	3 Months	-,114	,027	**,011**	-,203	-,025
	9 Months	,082	,045	,594	-,066	,229
9 Months	Baseline	-,212	,042	**,003**	-,350	-,074
	3 Months	-,195	,041	**,004**	-,329	-,061
	6 Months	-,082	,045	,594	-,229	,066

a - Adjustment for multiple comparison: Bonferroni

Table 35: *Post hoc* test comparing MRC in each assessment

(I) MRC	(J) MRC	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,039	,015	,162	-,010	,089
	6 Months	,085	,015	**,001**	,035	,134
	9 Months	,105	,021	**,003**	,036	,173
3 Months	Baseline	-,039	,015	,162	-,089	,010
	6 Months	,045	,018	,174	-,013	,104
	9 Months	,065	,018	**,030**	,005	,125
6 Months	Baseline	-,085	,015	**,001**	-,134	-,035
	3 Months	-,045	,018	,174	-,104	,013
	9 Months	,020	,014	1,000	-,025	,065
9 Months	Baseline	-,105	,021	**,003**	-,173	-,036
	3 Months	-,065	,018	**,030**	-,125	-,005
	6 Months	-,020	,014	1,000	-,065	,025

a - Adjustment for multiple comparison: Bonferroni

Table 36: *Post hoc* test comparing APB muscle in each assessment

(I) APB	(J) APB	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,067	,104	1,000	-,272	,407
	6 Months	,253	,122	,394	-,148	,654
	9 Months	,501	,106	**,005**	,153	,849
3 Months	Baseline	-,067	,104	1,000	-,407	,272
	6 Months	,185	,125	1,000	-,225	,596
	9 Months	,434	,156	,118	-,079	,946
6 Months	Baseline	-,253	,122	,394	-,654	,148
	3 Months	-,185	,125	1,000	-,596	,225
	9 Months	,248	,094	,149	-,060	,557
9 Months	Baseline	-,501	,106	**,005**	-,849	-,153
	3 Months	-,434	,156	,118	-,946	,079
	6 Months	-,248	,094	,149	-,557	,060

a - Adjustment for multiple comparison: Bonferroni

Table 37: *Post hoc* test comparing ADM muscle in each assessment

(I) ADM	(J) ADM	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,262	,060	**,008**	,066	,458
	6 Months	,422	,093	**,006**	,117	,726
	9 Months	,545	,101	**,002**	,213	,877
3 Months	Baseline	-,262	,060	**,008**	-,458	-,066
	6 Months	,160	,065	,196	-,052	,372
	9 Months	,283	,074	**,021**	,040	,526
6 Months	Baseline	-,422	,093	**,006**	-,726	-,117
	3 Months	-,160	,065	,196	-,372	,052
	9 Months	,123	,081	,969	-,143	,389
9 Months	Baseline	-,545	,101	**,002**	-,877	-,213
	3 Months	-,283	,074	**,021**	-,526	-,040
	6 Months	-,123	,081	,969	-,389	,143

a - Adjustment for multiple comparison: Bonferroni

Table 38: *Post hoc* test comparing BB muscle in each assessment

(I) BB	(J) BB	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,160	,060	,139	-,036	,356
	6 Months	,180	,090	,433	-,113	,473
	9 Months	,359	,061	**,001**	,160	,559
3 Months	Baseline	-,160	,060	,139	-,356	,036
	6 Months	,020	,058	1,000	-,169	,209
	9 Months	,199	,067	,084	-,021	,419
6 Months	Baseline	-,180	,090	,433	-,473	,113
	3 Months	-,020	,058	1,000	-,209	,169
	9 Months	,179	,077	,252	-,073	,431
9 Months	Baseline	-,359	,061	**,001**	-,559	-,160
	3 Months	-,199	,067	,084	-,419	,021
	6 Months	-,179	,077	,252	-,431	,073

a - Adjustment for multiple comparison: Bonferroni

Table 39: *Post hoc* test comparing TA muscle in each assessment

(I) TA	(J) TA	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,049	,108	1,000	-,304	,402
	6 Months	,175	,117	1,000	-,210	,559
	9 Months	,353	,084	**,011**	,076	,630
3 Months	Baseline	-,049	,108	1,000	-,402	,304
	6 Months	,125	,079	,856	-,133	,384
	9 Months	,304	,077	**,017**	,051	,556
6 Months	Baseline	-,175	,117	1,000	-,559	,210
	3 Months	-,125	,079	,856	-,384	,133
	9 Months	,178	,065	,123	-,034	,391
9 Months	Baseline	-,353	,084	**,011**	-,630	-,076
	3 Months	-,304	,077	**,017**	-,556	-,051
	6 Months	-,178	,065	,123	-,391	,034

a - Adjustment for multiple comparison: Bonferroni

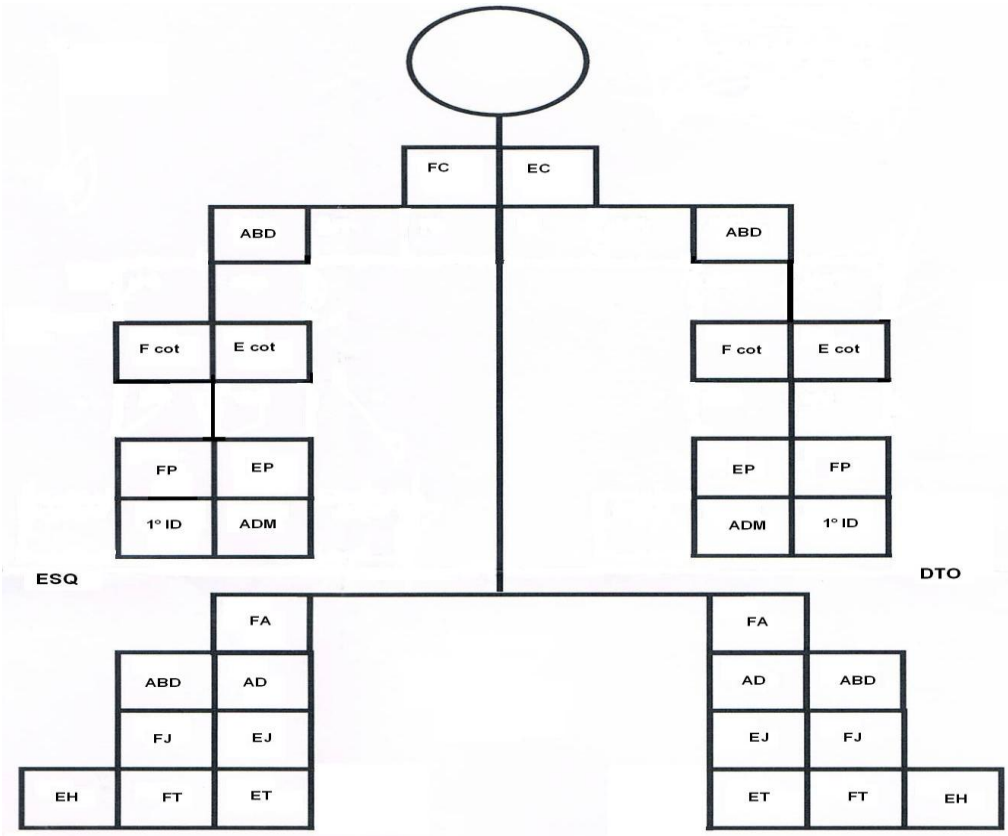
Table 40: *Post hoc* test comparing EDB muscle in each assessment

(I) EDB	(J) EDB	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
Baseline	3 Months	,163	,149	1,000	-,326	,651
	6 Months	,384	,107	**,030**	,033	,734
	9 Months	,356	,161	,306	-,170	,883
3 Months	Baseline	-,163	,149	1,000	-,651	,326
	6 Months	,221	,148	,993	-,263	,705
	9 Months	,194	,133	1,000	-,244	,631
6 Months	Baseline	-,384	,107	**,030**	-,734	-,033
	3 Months	-,221	,148	,993	-,705	,263
	9 Months	-,027	,137	1,000	-,477	,422
9 Months	Baseline	-,356	,161	,306	-,883	,170
	3 Months	-,194	,133	1,000	-,631	,244
	6 Months	,027	,137	1,000	-,422	,477

a - Adjustment for multiple comparison: Bonferroni

ATTACHMENT B

Muscles included in MRC measurements.



ATTACHMENT C

ALSFRS-R scale in Portuguese.

ESCALA FUNCIONAL DA ESCLEROSE LATERAL AMIOTRÓFICA

(ALSFRS)

1. Deve ser comparado o **estadio actual** do doente **com o estadio** em que se encontrava **antes do início da doença** (e não com o estadio da última consulta)

2. Na escala (de 4 a 0), deve ser registada a resposta à pergunta: **"Como é que está em relação a ….?"**

1. FALA

4	Discurso normal
3	Perturbações detectáveis no discurso
2	Inteligível com repetição
1	Discurso combinado com comunicação não verbal
0	Perda do discurso útil

2. SALIVAÇÃO

4	Normal
3	Ligeiro, mas com excesso de saliva na boca Talvez se babe durante a noite
2	Moderado excesso de saliva: um mínimo de baba
1	Marcado excesso de saliva com alguma baba
0	Marcado excesso de baba: requer o uso constante de um lenço

3. ENGOLIR

4	Hábitos alimentares normais
3	Problemas prematuros ao comer, com ocasional sufocamento
2	Alterações na consistência da comida
1	Necessita de sonda de alimentação suplementar
0	Não se alimenta pela boca (alimentado exclusivamente por via entérica ou parentérica)

4. ESCRITA

4	Normal
3	Lenta e irregular, todas as palavras são legíveis
2	Nem todas as palavras são legíveis
1	Consegue agarrar na caneta mas não é capaz de escrever
0	Não consegue agarrar na caneta

5a. CORTAR A COMIDA E MANEJAR OBJECTOS

(Doentes SEM gastrostomia)

4	Normal
3	Algo lento e desajeitado mas não precisa de ajuda
2	Pode cortar a maior parte da comida, embora lento e desajeitado necessita de alguma ajuda
1	A comida tem que ser cortada por alguém mas ainda se consegue alimentar lentamente
0	Necessita de ser alimentado

5b. CORTAR A COMIDA E MANEJAR OBJECTOS

(Escala alternativa para doentes COM gastrostomia)

4	Normal
3	Desajeitado mas capaz de desempenhar todas as actividades independentemente
2	Precisa de alguma ajuda para apertar e desapertar o botão de gastrostomia
1	Dá ajuda mínima à pessoa que cuida dele/dela
0	Completamente dependente

6. VESTIR E HIGIENE PESSOAL

4	Normal
3	Independente apesar da tarefa requerer esforço e ter eficácia diminuída
2	Ajuda intermitente ou substituição de métodos
1	Necessita de auxílio para o cuidado pessoal
0	Total dependência

7. VOLTAR-SE NA CAMA E AJUSTAR A ROUPA DA CAMA

4	Normal
3	Algo lento e desajeitado mas não necessita de ajuda
2	Pode voltar-se sozinho e ajustar os lençóis, mas com muita dificuldade
1	Pode iniciar mas não voltar-se ou ajustar os lençóis sozinho
0	Incapaz

8. ANDAR

4	Normal
3	Prematuras dificuldades ambulatórias
2	Caminha com ajuda
1	Apenas movimento funcional, não ambulatório
0	Sem movimentos úteis dos membros inferiores

9. SUBIR ESCADAS

4	Normal
3	Lento
2	Moderada instabilidade e fadiga
1	Necessita de assistência
0	Impossível

(10. RESPIRAR)

4	Normal
3	Falta de ar para ao mínimo esforço (ex. andar, falar)
2	Falta de ar em repouso
1	Assistência ventilatória intermitente (ex. nocturna)
0	Dependente do Ventilador

10. DISPNEIA

4	Normal
3	Ocorre na marcha
2	Ocorre num ou mais dos seguintes (comer, tomar banho, vestir-se – AVD)
1	Ocorre em repouso, dispneia quando sentado ou deitado
0	Dificuldade severa – considera-se uso de ventilação mecânica invasiva com entubação ou traqueostomia

11. ORTOPNEIA

4	Normal
3	Alguma dificuldade no sono nocturno por dispneia – usualmente não é necessário mais que 2 almofadas
2	Necessita de mais de 2 almofadas para dormir
1	Apenas consegue dormir sentado
0	Incapaz de dormir

12. INSUFICIÊNCIA RESPIRATÓRIA

4	Normal
3	Uso intermitente do BiPAP
2	Uso contínuo do BiPAP durante a noite
1	Uso contínuo do BiPAP durante o dia e a noite
0	Ventilação mecânica invasiva com entubação ou traqueostomia

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