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Assessing Side-Differences in the Organization of Biceps Brachii Motor Units in Healthy Subjects and Stroke Patients

An Evaluation from Surface EMG and Incremental Electrical Stimulation

By

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Declaration

I hereby declare that, the contents and organization of this dissertation constitute my own original work and does not compromise in any way the rights of third parties, including those relating to the security of personal data.

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2017

* This dissertation is presented in partial fulfillment of the requirements for **Ph.D. degree** in the Graduate School of Politecnico di Torino (ScuDo).

I would like to dedicate this thesis to my beloved Fabio, who has been always beside me, supporting and loving me unconditionally

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Abstract

Studies have suggested a degeneration of lower motoneurons in muscles affected after stroke, with a possible collateral reinnervation from the surviving motoneurons to the denervated muscle fibers. If this assumption holds, each surviving motoneuron would innervate a greater amount of muscle fibers following stroke, i.e., motor units' size would increase in muscles affected after stroke. By combining neuromuscular electrical stimulation with surface electromyography, the present PhD thesis aimed at investigating whether muscle reinnervation following stroke leads to greater variations in the amplitude of M waves elicited in muscles of the affected side of stroke patients, with respect to the contralateral, unaffected side. This issue was verified by applying current pulses at progressively greater intensities in the motoneurons that supply the biceps brachii muscle. Then, the size of increases in the amplitude of M waves elicited consecutively, hereafter defined as *increments*, was considered to evaluate structural adaptations in biceps brachii motor units following stroke. Changes in the amplitude of M waves evoked in a muscle is usually assumed to reflect changes in the number of motoneurons and, consequently, of muscle fibers activated. Hence, we hypothesized that for similar, relative increases in current intensity, greater *increments* in the M-waves amplitude would be observed in muscles of the affected than unaffected side of stroke patients. Before verifying this hypothesis, however, we investigated whether the size of *increments* in biceps brachii M waves differ between arms of healthy subjects. This question was motivated by the fact that, usually, humans tend to control more finely the muscle force production in dominant than non-dominant upper limbs. Once it is well established the recruitment of motor units is a key mechanism for which muscle force is controlled, we hypothesized that a relatively smaller number of motor units maybe recruited in muscles of dominant than non-dominant limbs, for any given increase in synaptic input. Hence, we expected to observe smaller *increments* in the amplitude of M waves evoked in biceps brachii of dominant than non-dominant arms. This PhD thesis was, therefore, based on two

main researches, entitled: (1) "*Does the biceps brachii muscle respond similarly in both limbs during staircase, electrically elicited contractions?*" and (2) "*Assessing structural adaptation of biceps brachii motor units after stroke*". Both studies were investigated with the same methodological approach mentioned above. Our main findings showed that: (1) *increments* were significantly smaller in biceps brachii of dominant than non-dominant arms. These results suggest there was a more gradual motor units' recruitment and, therefore, a broader spectrum of motor units' recruitment thresholds in muscles of dominant than non-dominant arms, which may contribute for a finer regulation of force production; (2) there was a clear trend towards greater *increments* in the amplitude of M waves elicited in biceps brachii of the affected than unaffected arms of most of the stroke patients evaluated. Although for few of these patients it was not clear whether side-differences in the *increments* magnitude were an outcome of dominance or stroke, the results found corroborate with the notion that collateral reinnervation takes place after stroke, increasing the number of muscle fibers per unit and, therefore, the magnitude of the muscle responses. Overall, the findings of this PhD thesis strengthen the idea that the organization of the neuromuscular system may contribute to accounting for upper limb dominance and that stroke may lead to structural adaptations in motor units of affected muscles.

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Chapter 1

Stroke effects on the neuromuscular system

1.1 What is the stroke?

The cerebrovascular accident, also referred to as stroke, is defined as a disruption of the blood supply to the brain leading to a sudden death of brain cells due to a deprivation of oxygen and nutrients (National Stroke Association; World Health Organization). The stroke is one of the types of cardiovascular diseases that most affects the world population, mainly aging people. Every year, about six million people worldwide die following stroke, and another five million remain disable. Indeed, according to the Word Heart Federation, the stroke is considered the second leading cause of disability in the world. Depending on the area affected in the brain, the stroke may cause a complete or partial paralysis of one side of the body (hemiplegia and hemiparesis, respectively), loss of vision and/or speech, confusion and many other problems.

The disruption of the blood supply to the brain may be caused either by a blockage or by a rupture of a blood vessel. In the first case, the cerebrovascular accident is defined as *ischemic stroke* and, in the second one, it is defined as *hemorrhagic stroke*. The *ischemic stroke* is the most common type of stroke, where the blood vessel in the brain is blocked by a blood clot generated either within the brain (*thrombotic stroke*) or in somewhere else in the body and traveled through the

circulation until lodge in the brain (*embolic stroke*). In the *hemorrhagic stroke*, the blood vessel in the brain may rupture when a brain aneurysm bursts or when a weakened blood vessel leaks (National Stroke Association). Regardless the type of stroke and the way it happens, the death of brain cells seems to be inevitable, leading to many other consequences in the body of stroke survivors.

1.2 Motoneurons degeneration in stroke survivors

Studies have suggested that after stroke there is a degeneration of the lower motoneurons that innervate muscles in the affected side of stroke survivors (McComas et al. 1973; Hara et al. 2004; Lukács 2005; Kouzi et al. 2014). This degeneration has been associated with a process called "anterograde transneural degeneration" or “trans-synaptic degeneration”, which according to Cowan (1970), denotes the death of neurons due to the interruption of the transmission of afferents synaptic impulses (deafferentation). Briefly, the terms “transneural degeneration” refer to the death of a second order neuron, i.e. there is a cell loss of postsynaptic neurons associated with presynaptic neurons which was damaged, and the term “anterograde” refers to the direction of the degenerative change, which in this case means that degeneration is secondary to deafferentation (Cowan 1970; pages 217-218).

Interesting, Terao et al. (1997) reported that lesions in the corticospinal tract due to stroke did not result in "anterograde transneural degeneration" of spinal anterior horn cells. The authors performed autopsies in the fourth lumbar segment of the spinal cord of four subjects who had severe spastic hemiplegia. The time elapsed since the stroke onset varied among subjects in a range from one to eight years. Although the authors have observed, for all subjects, an extensive loss of axons in the lateral corticospinal tract in the left dorsolateral column on both spinal segments analyzed, they did not find differences between affected and unaffected sides related to the amount, the diameter and the size distribution patterns of neurons in the ventral horn. Their results do not necessarily indicate, however, that lower motoneurons degeneration do not occur after stroke onset. As stated by McComas et al. (1973), the evidence of axonal degeneration in the presence of an apparently intact soma may be explained by a process called “dying back”. In this case, there is a distal to proximal gradient of degeneration, i.e., neurons die back gradually from the periphery to the cell body (Turner and Kiernan 2015; page 413).

Motoneurons degeneration in stroke survivors has been mainly suggested by studies which investigated the number of motor units in different muscles affected after

stroke (McComas et al. 1973; Hara et al. 2004; Li et al. 2011, 2014a; Kouzi et al. 2014), through techniques of "motor unit number estimation" (see chapter 2). Table 1 shows the specific techniques applied by each study, the respective muscle(s) assessed and the results found. In general, studies reported significant minor amount of motor units in muscles affected after stroke with respect to healthy muscles (i.e., muscles of healthy subjects or unaffected muscles of stroke survivors). Considering only the studies' results with significant difference between muscles ($P < 0.05$; cf. Table 1), the number of motor units was on average 23-57% smaller in affected than healthy or unaffected muscles. Li et al. (2014a), however, did not observe significant difference between the number of motor units in the affected and unaffected abductor digit minimi and abductor pollicis brevis muscles of 12 stroke patients.

Previous accounts have also suggested that degeneration mainly affects motoneurons of larger sizes. The first evidence of such statement seems to have been reported by McComas et al. (1973). By analyzing the contraction time of maximal isometric twitches electrically elicited in the extensor halluces brevis muscles, the authors observed a mean contraction time significantly slower in muscles of affected than unaffected sides of nine stroke patients, suggesting the surviving motoneurons in the affected side tended to innervate relatively slow twitch muscle fibers. Usually, the muscle fiber type is related to the size of motoneurons innervating the muscle fibers, where relatively small and large motoneurons innervate slow and fast twitch muscle fibers, respectively (McPhedran et al. 1965a, b). Thus, McComas et al. (1973) findings may indicate that there were motoneurons with relatively larger sizes in the unaffected with respect to the affected muscles of the stroke patients evaluated. Hence, their results may be evidence of a predominant degeneration of motoneurons with relatively larger sizes in muscles affected after stroke.

Table 1: Studies which compared the number of motor units between unaffected and affected muscles of stroke survivors.

Reference	Technique applied to estimate the number of motor units	Muscle(s) analyzed	Average number of motor units estimated
McComas et al. (1973)	MUNE (incremental method)	Extensor digitorum brevis	Unaffected: 216.7 ± 7.9 Affected: 93.7 ± 8.4 (N = 27; P < 0.001)
Hara et al. (2004)	MUNE (F-wave method)	Abductor pollicis brevis	Unaffected: 316 ± 43 Affected: 237 ± 50 (N = 14; P < 0.05)
Li et al. (2011)	MUNIX	First dorsal interosseous	Unaffected: 153 ± 38 Affected: 109 ± 53 (N = 9; P < 0.01)
Kouzi et al. (2014)	MUNE (multiple point method)	Abductor digit minimi	Unaffected: 363 ± 135 Affected: 227 ± 88 (N = 46; P < 0.001)
Li et al. (2014a)	MUNIX	First dorsal interosseous	Unaffected: 191 ± 17 Affected: 147 ± 21 (N = 12; P < 0.05)
		Abductor pollicis brevis	Unaffected: 135 ± 14 Affected: 112 ± 10 (N = 12; P > 0.2)
		Abductor digit minimi	Unaffected: 138 ± 10 Affected: 140 ± 21 (N = 12; P > 0.9)

The assumption above has been reinforced by more recent studies, which analyzed motor units' responses at time and frequency domain during voluntary isometric contractions. Lukács et al. (2008), for instance, recorded macro-electromyographic (macro-EMG) signals from the abductor digit minimi of 45 stroke patients and 40 healthy subjects (control group), during isometric contractions at two different force levels: 10 and 50 % of the maximal voluntary contraction (MVC). At the lower force level, median amplitude of action potentials was similar among muscles of the control group and of unaffected and affect limbs of stroke patients. Nevertheless, at the higher force level, the median amplitude of potentials recorded in affected muscles was smaller than muscles of unaffected and control groups and did not differ from the amplitude recorded at the low force level. According to the Henneman's size principle (Henneman 1957; Henneman et al. 1965a), the authors presumed that at low and high force levels would be activated, respectively, small and large motor units in the muscles evaluated. Consequently, the amplitude of macro motor unit potential should be larger at the high force levels. From that, Lukács et al. (2008) suggested the degeneration of large motoneurons as one possible reason for the results found in the abductor digit minimi muscles of affected limbs of the stroke patients, i.e., no changes in the amplitude of macro motor unit potentials for increase in the force level. Additionally, through the surface EMG (see chapter 2), Kallenberg and Hermens (2009) and Li et al. (2014b) detected EMG signals from biceps brachii and first dorsal interosseous muscles, respectively, in both affected and unaffected sides of stroke patients. By evaluating the power spectrum of the EMG signals detected in these muscles during isometric contractions at different force levels [5 to 50% of the muscle's MVC, with steps of 5% (Kallenberg and Hermens 2009); 30 to 70% of muscles' MVC, with steps of 10% (Li et al. 2014b)], both studies reported significantly lower mean power frequency in affected than unaffected muscles. Considering that a lower mean power frequency is associated with a lower muscle fiber conduction velocity, the authors of both studies suggested the muscles affected after stroke had a greater predominance of type I motor units, which are generally characterized by innervating slow twitch muscle fibers, with lower conduction velocity. Furthermore, since type I motor units have lower thresholds and smaller sizes, the authors also suggested a predominant degeneration of large motoneurons in muscles affected after stroke.

1.2.1 How long does motoneuron degeneration last in stroke survivors?

Results related to when motoneurons degeneration begins and ends after the stroke onset are controversial in the literature. Through the incremental MUNE method (see chapter 2), McComas et al. (1973) observed the number of motor units in extensor digitorum brevis muscles seems to have not been affected in stroke patients with less than two months since the stroke onset, while for patients who stroke happened longer than two months, there was a significantly reduction in the number of motor units in affected with respect to the unaffected muscles. After six months from the stroke onset, however, the authors did not observe a correlation between the time elapsed and the number of surviving motor units, suggesting the loss of motor unit occurred until six months from the stroke onset. Hara et al. (2004), however, reported that the motor unit loss begins no later than nine days after the stroke onset. By investigating the number of motor units in abductor pollicis brevis muscles of 14 stroke patients, through the F-wave MUNE method, the authors observed amounts of motor units significantly smaller in affected than unaffected muscles of stroke patients who the time elapse since the stroke onset ranged from 9 to 28 days. Additionally, to investigate the progress of the loss of motor units, Hara et al. (2004) reevaluated nine patients at three/four months and one year after their stroke onset. In both periods of reevaluation, the number of motor units remained significantly smaller in affected than unaffected muscles, however, no statistical difference were observed when comparing the number of motor units between the periods of reevaluation. The authors suggest therefore there was no progress in the loss of motor units after three/four months from the stroke onset.

1.2.2 Motoneurons degeneration and severity of stroke

A higher degree of motor impairment following stroke seems to lead to further lower motoneurons degeneration in stroke survivors. Hara et al. (2004), for instance, observed the greater was the hemiparetic severity in abductor pollicis brevis muscles of 14 stroke patients, evaluated through the Stroke Impairment Assessment Set (SIAS), the lower was the number of motor units estimated with F-wave MUNE method in affected than unaffected muscles. The authors suggested, therefore, further motor unit loss in patients with severe hemiparesis. Combining supramaximal electrical stimulation with surface EMG, Lukács (2005) investigated the maximal M-wave (see chapter 2) amplitude recorded in the abductor digiti

minimi muscles of 48 stroke patients. The author reported that for patients in the acute phase, smaller M-wave amplitude were more explicit in the cases with higher severity of the hemiparesis, evaluated through the Scandinavian Stroke Scale. Lukács (2005) suggested therefore that the inactivity or degeneration of lower motoneurons was more evident for higher stroke severity. By using a different approach to investigate changes in motor units following stroke, Lukács et al. (2008) evaluated the amplitude of macro motor unit potentials (macro-MUP) in the abductor digit minimi of 45 stroke patients, at a low (10% MVC) and a high (50% MVC) force level of voluntary isometric contractions. The authors correlated the ratio between the macro-MUP amplitudes at high and low force levels with the degree of stroke severity assessed with the Scandinavian Stroke Scale. The greater was the degree of stroke severity in the patients evaluated, more similar were the macro-MUP amplitudes measured in the affected muscles between the force levels. Considering the degeneration of large motoneurons as a possible reason for no changes in the macro-MUP amplitudes for increase in the force level, the authors suggested the higher degree of stroke severity seems to lead a greater loss of motoneurons in muscles affected after stroke. Kallenberg and Hermens (2009) found similar results by measuring the root mean square (RMS) amplitude of MUAPs detected on biceps brachii muscles of 15 stroke patients, during isometric voluntary contractions performed at ten different force levels (5 to 50% of the muscle's MVC, with steps of 5%). The authors observed a significant positive correlation between the degree of motor recovery, evaluated through the Fugl-Meyer scale, and the ratio of RMS amplitudes measured on affected divided by unaffected muscles. In other words, the higher was the stroke severity of the patients evaluated, the lower was the amplitude of MUAPs detected in muscles of affected with respect to unaffected sides. Although the authors have not discussed about this, the lower MUAP amplitude observed in the affected muscles of five out of 15 patients, for the different force levels, may indicate a loss of large motoneurons in these muscles. Interesting, these subjects were those with the lower Fugl-Meyer score and, therefore, the degeneration of motoneurons seems to be influenced by the degree of stroke severity.

1.2.3 What comes after the motoneuron degeneration in stroke survivors?

It is still uncertain what happens after the degeneration of motoneurons in muscles affected following the stroke. There is some evidence that a reinnervation process takes place after stroke, through collateral reinnervation by the surviving

motoneurons into the muscle fibers denervated due to the "anterograde transneural degeneration" of the affected motoneurons. The amplitude of motor units' potentials, evaluated either in voluntary or in electrically elicited contractions, have been the main indicator of the occurrence of collateral reinnervation in muscles affected after stroke. McComas et al. (1973), for instance, showed the amplitude of M waves, elicited in extensor digitorum brevis muscles of stroke patients, remain similar between affected and unaffected legs of patients who the time elapsed since the stroke onset ranged between six and 19 months. However, for patients who the length of the illness was above 19 months, M-waves amplitude was greater in affected than unaffected muscles, suggesting, according to the authors, either a reinnervation of denervated fibers or a muscle fiber hypertrophy. During voluntary isometric contractions performed at different force levels, Kallenberg and Hermens (2009) reported higher RMS amplitudes of MUAPs detected on affected with respect to unaffected biceps brachii muscles of seven out of 15 stroke patients. Although there was no statistical difference between subjects' limbs, considering that larger RMS amplitudes indicate the presence of larger motor units in the muscle, the authors suggested that muscle fibers of affected muscles were reinnervated by collateral sprouting and branching, leading to an increase in the MUAPs' amplitude. Additionally, Lukács et al. (2009) evaluated the fiber density of abductor digiti minimi muscles of 42 healthy subjects and 59 stroke patients, and observed higher fiber density in muscles affected after stroke with respect to those of healthy subjects. Although the method used by the authors likely allowed the evaluation of a relatively small portion of the entire pool of motor units in the muscle, Lukács et al. (2009) believed the greater number of muscle fibers innervate by single motor unit in a given area of the affected muscles (i.e., greater fiber density) may be explain by a collateral reinnervation originating from the survivors motoneurons. Finally, Li et al. (2013) analyzed surface EMG signals from the first dorsal interosseous muscle while 14 stroke patients performed isometric contractions at different force levels (20-80% MVC with 10% of step). The authors found that, for all force levels evaluated, the amplitude distribution of the EMG signals detected shift toward larger amplitude peaks over the affected than unaffected muscles of nine out of 14 subjects. Although there was no significant difference between sides, Li et al. (2013) interpreted these results as a possible occurrence of muscle fiber reinnervation due to spinal motoneuron degeneration in muscles affected after stroke.

If a reinnervation process really happens after stroke, each surviving motoneuron may innervate a greater amount of muscle fibers in the affected limb of stroke patients, i.e. the number of muscle fibers per motor unit (innervation ratio) of the

affected muscle would increase after stroke. Such alleged change in the innervation ratio of muscles affected after stroke likely accounts for the inability of stroke survivors in finely controlling movements with the affected limb. The force generated by single motor units in relation to the total muscle force reflects the quantal increment in muscle force, thereby indicating how finely force maybe regulated. In addition, the amount of force produced by individual units is proportional to the number of muscle fibers they supply. Hence, given similar synaptic inputs, a muscle with relatively low innervation ratio (i.e. few muscle fibers per motor unit) would be able to produce finer increments in force, with respect to muscles with higher innervation ratios. This implies that, if the innervation ratio of muscles affected after stroke increases due to a collateral reinnervation, the muscles affected would be expected to do not produce increments in twitch force as finely as healthy muscles. Indeed, a recent systematic review of Kang and Cauraugh (2015) reported evidence of impaired accuracy of force production in muscles of stroke survivors.

Chapter 2

Incremental electrical stimulation and surface EMG for indirectly assessing neuromuscular adaptations in stroke

2.1 Surface EMG

The functional unit of the neuromuscular system is the motor unit, which consist of two components: a single motoneuron, comprising it dendrites and axon, and the muscle fibers innervated by its axon, also referred to as the muscle unit. When a motoneuron fires, it triggers, through the neuromuscular junction, a depolarization in the sarcolemma of the muscle fibers innervated by its axon, that propagates in opposite directions towards the two ends of the muscle fibers. Such depolarization generates an electric potential, called action potential, which can be recorded through electrodes placed on the skin over the muscle; technique known as surface electromyography. The signal detected by surface electrodes (electromyogram; EMG) reflect the summation of multiple motor unit action potentials (MUAPs) generated by the depolarization of the sarcolemma of their muscle fibers (Heckman and Enoka 2012; Merletti and Farina 2016).

Typically, a single pair of surface electrodes is used for the sampling of EMGs from individual muscles. This type of EMG recording is known as a bipolar surface EMG. Briefly, the bipolar EMG corresponds to the difference between the voltage measured through the couple of electrodes and that measured by the reference electrode (presumably zero), often located at bony regions on the skin. Due to the short inter-electrode distance usually adopted, bipolar EMG reflects muscle activity locally. More recent detection systems enable the sampling of surface EMGs from different regions of a single muscle. *High-density* surface EMG is the general terminology used to indicate the sampling of muscle activity with multiple (more than two) closely spaced electrodes from an individual muscle. Two electrode configurations are usually available to collect multiple surface EMGs; monopolar or bipolar configuration. Briefly, in the monopolar configuration, the EMG signal correspond to the difference between the electrical potential measured by one surface electrode and that measured by the reference electrode (Merletti and Farina 2016). Even though monopolar configuration ensures the recording of the actual electrical potential, interferences from outside sources (e.g., power line interference due to parasitic capacitance) and/or the activity of muscles located closer to the targeted muscle (crosstalk) might be recorded from this configuration (Vieira and Garcia 2011). Given that the bipolar or differential EMG consists in the difference between two monopolar EMGs, the common-mode embedded in both monopolar signals, due to crosstalk or to power line interference, is fairly attenuated in the differential EMG. However, the contribution of deep motor units might be reduced in bipolar EMGs, since the motor unit action potentials generated by deep units normally appear with equal amplitudes in the monopolar EMGs (Roeleveld et al. 1997a; Rodriguez-Falces and Place 2016). Then, depending on the type of electrode recording, the contribution of deep motor units within the muscle or the contribution of interference sources can be attenuated to the surface EMGs.

2.2 Electrical stimulation of the peripheral nervous system

Neuromuscular electrical stimulation consists in the application of electrical stimuli to superficial skeletal muscles, with the main purpose of generate visible muscle contractions by activating the motoneuron axons or the intramuscular nerve branches (Hultman et al. 1983). Differently from voluntary contractions, the neuromuscular electrical stimulation enables to control the discharge frequency and the number of motor units activated in a muscle, through the frequency and the amplitude of the stimulation pulses, respectively. Hence, as a research tool, the neuromuscular electrical stimulation allows the in vivo evaluation of the

neuromuscular function, inducing muscle contractions in a more standardized way with respect to voluntary contractions.

Muscle contractions may be electrically induced by positioning superficial stimulation electrodes either in the nerve trunk or in the muscle motor point [i.e., the location where the motor branches of a nerve enter the muscle belly (Botter et al. 2011; Merletti and Farina 2016)], hereafter named as nerve and muscle stimulation, respectively. Usually, at the level of the nerve trunk, axons beneath the stimulation electrodes are located in close proximity to one another and, therefore, nerve stimulation allows to delivery electrical charges to most, if not all, of the motor fibers innervating a muscle. At the level of the muscle, however, axon terminal branches may be distinctly distributed within muscles. Recent findings of Rodriguez-Falces and colleagues (Rodriguez-Falces et al. 2013a; Rodriguez-Falces and Place 2013), for instance, suggested the motor nerve endings of the vastus medialis muscle are distributed over a small portion of the muscle cross-section, while motor branches of the vastus lateralis muscle would be more spread out within the muscle. The authors suggested, therefore, that muscle stimulation depends on the spatial organization of the axonal terminal branches within the muscle.

Regardless of where electrical stimulation is applied, over the nerve trunk or the muscle motor point, the activation of the motor axons innervating a muscle depends on the amount of electrical charges required to reach their excitability thresholds (i.e., to depolarized the axon membrane). Specifically, large-diameter axons have a lower threshold of electrical excitability than small-diameter axons and, therefore, are more easily excited by imposed electrical fields (Stephens et al. 1978). This is explained by the inverse relation between axon diameter and axial resistance, which allows the flow of current across the axon to occur at a lower transmembrane current for axons with large-diameter (Enoka 2002). Based on that, one could think that motor units are electrically elicited in a reverse order as the one observed for voluntary contractions [Henneman's size principle (Henneman 1957; Henneman et al. 1965a)], i.e. that motor units are activated in order of decreasing size when electrically stimulated. However, during electrical stimulation of increasing intensity, the recruitment order of motor units within a muscle depends not only on the diameter of the axon, but also on its distance from the stimulation electrode and its orientation with respect to the current field. Contradictory results have been reported with respect to the motor unit recruitment order during electrical stimulation of increasing intensity. The recruitment order according to the

Henneman's size principle was proposed by studies which stimulated either the nerve trunk (Rodriguez-Falces and Place 2013) or the muscle (Knaflitz et al. 1990; Farina et al. 2004; Rodriguez-Falces and Place 2013). Other studies, however, suggested a reverse recruitment order during nerve (Stephens et al. 1978; Heyters et al. 1994; Hennings et al. 2007) or muscle (Heyters et al. 1994) stimulation. And finally, some studies observed a nonselective or random motor unit recruitment when stimulating at the level of the muscle (Gregory and Bickel 2005; Jubeau et al. 2007; Rodriguez-Falces and Place 2013). Since motor fibers are usually more spatially localized (i.e., located in close proximity to one another) in the nerve trunk with respect to the muscle motor point, during nerve stimulation motor units seem to be mainly recruited according to their electrical excitability threshold, while for the muscle stimulation recruitment seems to be determined by the geometrical distribution of axonal branches.

2.2.1 Stimulation techniques

Usually, two distinct stimulation techniques, defined as *monopolar* and *bipolar stimulation*, are used for the neuromuscular electrical stimulation. One of the differences between these techniques is with respect to the size of the stimulation electrodes and their relative position over the skin. In *monopolar* configuration two electrodes of different sizes are used; the electrode of smaller dimension (also known as "negative" or "cathode" or "reference" or "return" electrode) may be positioned near the nerve trunk or above a muscle motor point, and it is defined as the active stimulation electrode since electrical stimulation occurs in its vicinity. The other electrode (also known as "positive" or "anode" electrode) has a larger size with respect the active electrode and it is usually positioned on the opposite side of the active electrode. In *bipolar* configuration, however, two electrodes with similar size are positioned over the tissue to be electrically stimulated (Figure 1). The second difference between these techniques is related to the current distribution. In *monopolar* configuration, current density is greater in the proximity of the active electrode and smaller near the return electrode. In this way, excitation threshold of motor fibers located close to the active electrode is exceed, while for motor fibers located near the return electrode the current density is probably not enough to reach their excitation threshold. In *bipolar* configuration, however, the current distribution is more confined in the area where stimulation electrodes are applied and the current density is more uniform in the current field (Merletti et al. 1992; Botter et al. 2011).

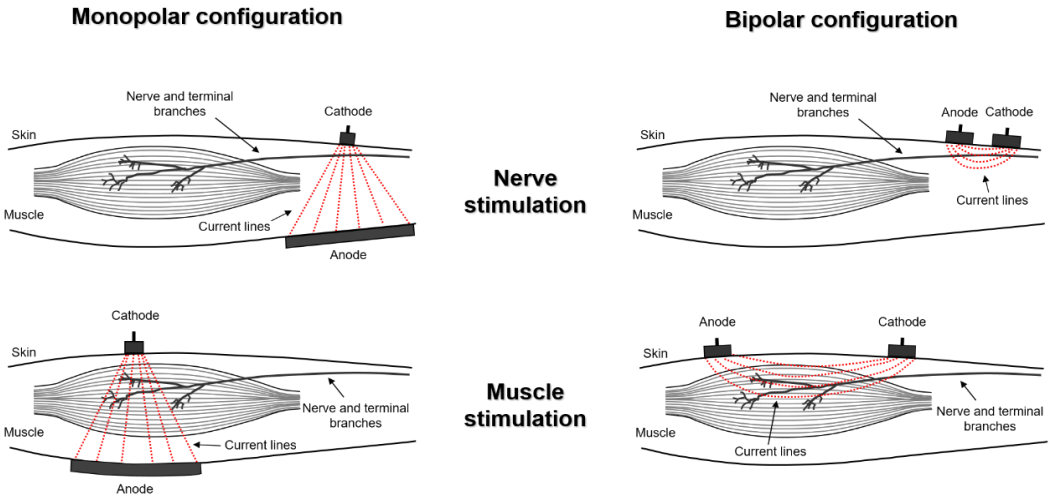

Figure 1: Schematic representation of monopolar and bipolar stimulation electrode configuration applied during nerve and muscle stimulations.

2.2.2 M wave

Differently from voluntary contractions, during the electrical stimulation of the axons of motoneurons or of their terminal branches, MUAPs are synchronously evoked by the external stimuli, generating an electrophysiological response defined as compound muscle action potentials (CMAPs) or massed action potentials (M waves) which is recorded from the surface electromyography. In order words, the M wave is the summation of action potentials of the synchronously activated muscle fibers in a muscle (Merletti et al. 1992). Therefore, the maximum M wave evoked in a muscle through the supramaximal electrical stimulation represent the recruitment of all motor units corresponding to the pool of motoneurons activated.

2.3 Physiological and clinical information obtained by combining incremental electrical stimulation and surface EMG

2.3.1 Incremental stimulation

The incremental stimulation technique consists in gradually increase the stimulus intensity applied to a peripheral nerve in order to recruit successive motor units (McComas et al. 1971). Since the motoneurons that innervate a muscle have different diameter sizes and, therefore, different excitation threshold (McPhedran et al. 1965a, b), as the stimulation intensity increases additional motor units are recruited and M-waves amplitude grows. If no variations in the M-wave amplitude are observed when stimulation intensity is increased, this indicates that a maximum M-wave response was evoked in the muscle, i.e., motor units corresponding to the pool of motoneurons stimulated in the peripheral nerve were fully recruited (Figure 2). Hence, variations in the M-wave amplitude is usually assumed to reflect a change in the number of motor units activated (McComas et al. 1971). Based on that, different methods involving the incremental stimulation, such as the "*motor unit number estimation* – MUNE" (McComas et al. 1971) and the "*electrophysiological muscle scan*" (Blok et al. 2007), have been proposed to indirectly assess structural adaptations on the neuromuscular system.

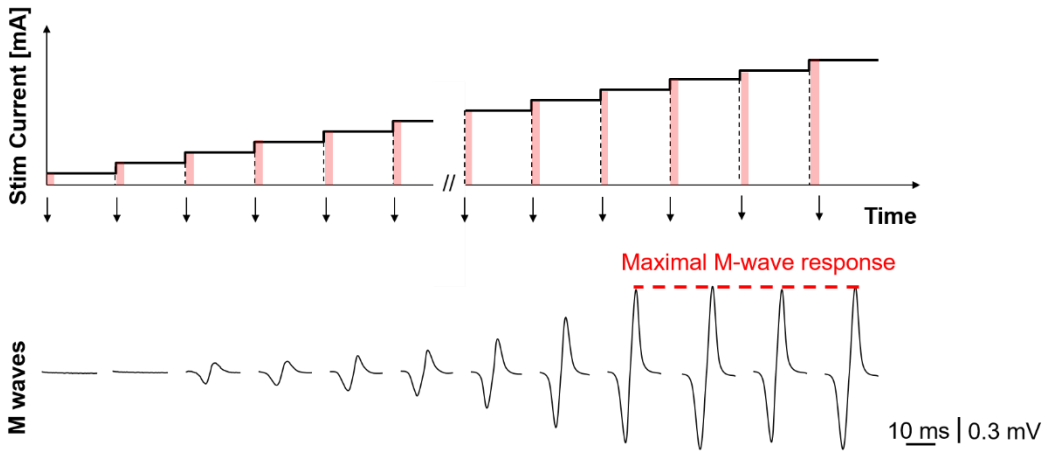

Figure 2: Schematic representation of M-waves responses during an incremental electrical stimulation.

2.3.2 MUNE

The incremental stimulation seemed to have been firstly proposed by McComas et al. (1971), with the aim of give an estimate of the number of motor units within a human muscle. Briefly, this pioneer method, known as incremental MUNE, consists in applying electrical stimuli over a peripheral nerve and gradually increased the intensity, from a subthreshold value until ten or more increments in the M-wave peak-to-peak amplitude are obtained. An increment refers to an increase in the M-wave amplitude and it is assumed that each increment reflects the activation of an additional motor unit. Then, the amplitude of the M wave evoked with all the increments is divided by the number of increments, resulting in a mean motor unit potential, also defined as single motor unit potential (SMUP). The number of motor units in the muscle is therefore defined dividing the M-wave amplitude obtained during a supramaximal stimulation of the muscle by the SMUP. The limitation of this method proposed by McComas et al. (1971) is that it samples a small population of motor units within the muscle, which may not represent the whole pools of motor units in this muscle. In other words, the estimation of the number of motor units is only based on the responses of the first units elicited in the muscle and on the assumption that such responses would represent those from the other motor units not recruited in this muscle, which may not be true. Therefore, other MUNE methods have been proposed with the aim of increase the representation of motor units in the sample, by evaluating SMUPs at different muscle contraction levels (Gooch et al. 2014). In addition, the MUNE methods have been extensively used as a research tool to investigate axonal loss caused by a number of disorders, such as amyotrophic lateral sclerosis, spinal muscle atrophy, stroke, among others (McComas et al. 1973; Hara et al. 2004; Bromberg 2007; Arasaki et al. 2009; Nandedkar et al. 2010; Li et al. 2011; Kouzi et al. 2014). Concerning the stroke, specifically, studies have mainly used the methods which sample small populations of motor units, such as the traditional incremental MUNE (McComas et al. 1973), the F-wave MUNE (Hara et al. 2004) and the multiple point stimulation MUNE (Arasaki et al. 2009; Kouzi et al. 2014), to investigate motor unit loss in muscles of stroke survivors.

2.3.3 Electrophysiological muscle scan

In 2007, Blok et al. proposed a method denominated "*electrophysiological muscle scan*" as an alternative evaluation tool to extract clinically relevant information regarding the effects of a variety of neurogenic disorders on the neuromuscular system, such as motor unit loss and muscle reinnervation. This method is also based on the incremental stimulation technique and it consists in evaluate the stimulus-response curve of a muscle, by plotting the response size against the stimulus intensity. Briefly, the initially proposed *muscle scan* consists in applying over a peripheral nerve 300 electrical stimuli, equidistant in intensity, from the threshold of the lowest-threshold motor unit until the lowest intensity at which a maximal M wave is evoked. Then, the amplitude of the M wave recorded for each stimulation intensity is plotted against the stimulus number. By analyzing several parameters from the scan (e.g. steps, maximum response, variability, decrements, stimulus intensity), the following clinical information may be obtained according to Blok et al. (2007): motor unit number, size and stability; neuromuscular transmission; and axonal excitability. The parameter *steps*, specifically, has been used as an indicator of motor unit loss and muscle reinnervation. *Steps* are calculated as follow: after performing the full muscle scan, M waves are sorted according to their size, and consecutive differences between their amplitudes are calculated. Then, *steps* are defined as consecutive differences exceeding a subject-dependent threshold, adjusted by the operator to agree with visual estimates of step number and location. *Steps* are therefore quantified by their size and their summed size as a percentage of the maximum M wave. They represent discontinuities in the stimulus-response curve that, in the case of impaired muscles, may reflect motor unit loss and muscle reinnervation. Thus, Blok et al. (2007) suggest that the number of *steps* observed in the *muscle scan* provides a good indication of the number of remaining motor units in an impaired muscle. Unlike the MUNE methods, the *muscle scan* seems to provide a more global evaluation of the motor units constituting a muscle. As far as we know, *muscle scan* assessment in stroke survivors seems to not be reported in the scientific literature.

Chapter 3

Does the biceps brachii muscle respond similarly in both limbs during staircase, electrically elicited contractions? [1]

3.1 Introduction

Regardless of age and sex, people use preferentially their left or right upper limb in daily activities, likely because they tend to perform better with either limb. Differences in the ability to use both limbs, hereafter termed dominance, are more clearly appreciated in tasks requiring the fine control of movements. Triggs et al. (2000), for instance, observed that healthy subjects, right- and left-handers, performed better manual tasks requiring a fine motor control with their dominant than non-dominant hands. Specifically, the authors evaluated how many times subjects performed correctly a *finger tapping task* (tapping a specific key of a computer with the index finger as rapidly as possible) and a *pegboard dexterity task* (removing small metal pegs from a well and placing them into a vertically oriented

[1] Scientific paper to be submitted to an international journal. Authors: Pinto TP, Gazzoni M, Botter A, Vieira, TM.

row of holes). Right- and left-handers showed scores significantly higher when performing the tasks with their right and left hands, respectively.

Interesting, asymmetries in the use of the upper limbs are apparently manifesting since the early stages of gestation. By analyzing the number of right- and left-arm movements of fetuses at different weeks gestation through ultrasound recordings, Hepper et al. (1998) and Hepper (2013) observed that fetuses performed significantly greater number of movements with their right than left arms. Such upper limbs asymmetry seems to have its origin within the organization of the nervous system. Anatomical studies reported indeed left-right differences in brain morphologies. Through magnetic resonance morphometry, Amunts et al. (1996) observed that the brain area where hand representation occurs is deeper in the left and right hemispheres of right- and left-handers, respectively. By means of a cytoarchitecture analysis, the authors also found a larger volume of cells occupied by dendrites, axons, and synapses in the left than right primary motor cortex of each subject evaluated. Additionally, through the magnetoencephalography technique, Volkmann et al. (1998) evaluated the hand area size activated while subjects performed different movements with the hands and fingers. The authors observed the brain area size where occurs hand representation was larger in the primary motor cortex opposite to the preferred hand side of the left- and right-handed subjects evaluated. These pieces of evidence suggest the upper limb dominance may be accounted for by asymmetries in the central structures of the nervous system.

The organization of the neuromuscular system may contribute as well to upper limb dominance. Through needle electromyographic (EMG) recordings during isometric voluntary contractions at 30% of maximal voluntary contraction (MVC), Adam et al. (1998) observed the recruitment and firing rate of motor units in the first dorsal interosseous muscle of both limbs. Motor units in the dominant hand showed lower average firing rates and lower recruitment thresholds than those in the non-dominant hand. Since slow-twitch muscle fibers are usually innervated by small motoneurons, with low recruitment thresholds (McPhedran et al. 1965a, b), these results may indicate a higher percentage of slow-twitch fibers in muscles in dominant than non-dominant limbs. Evidence from ten cadavers further revealed larger perikaryions in the right side of the C8-Th1 spinal cord segment, where motoneurons parenting muscles in the hands and arms are located (Melsbach et al. 1996). Collectively, these findings suggest differences may exist in the mechanisms underpinning motor units' recruitment between dominant and non-dominant limbs.

While it is well established the recruitment of motor units is a key mechanism for which muscle force is controlled (Clamann 1993; Kernell 2003), reports on the systematic association between motor units' recruitment and dominance are uncommon. Given dominance is related to fine motor control, it is therefore reasonable to expect muscle force to be more finely regulated in dominant than non-dominant arms. Such finer regulation may be related to a more gradual motor units' recruitment in muscles of dominant than non-dominant arm (the smaller the relative number of motor units recruited for a given increase in synaptic input, the smaller the muscle force production), implying a larger spectrum of recruitment thresholds in motoneurons serving muscles of dominant than non-dominant arms. To our knowledge, the association between dominance and variations in the relative number of motor units recruited remains an unexplored issue.

In this study we use incremental, neuromuscular electrical stimulation (Botter et al. 2009; Botter and Merletti 2016) to indirectly assess differences in motor unit recruitment between limbs. We specifically ask: does the amplitude of massed action potentials (M waves) and force twitches increase similarly when current pulses at progressively greater intensities are delivered to the biceps brachii muscle of both arms? If there is a broader spectrum of motor unit recruitment thresholds in the dominant arm then, for relative similar increases in current intensity, the amplitude of M waves and force twitches is expected to increase more gradually in the dominant than non-dominant arms; that is, a relatively smaller number of motor units is expected to be elicited for similar, relative increases in current intensity. We address our question for biceps brachii muscle because, differently from the small hand muscles for which dominance is typically assessed (Kamen et al. 1992; Adam et al. 1998; Li et al. 2015), the biceps brachii may be electrically elicited and assessed selectively from the skin. Furthermore, biceps brachii has a quite contribution for most of the humans' daily activities, including those requiring fine motor skills. Finally, understanding whether the control mechanisms underpinning the motor units' recruitment are distinct between limbs, may assist the evaluation and treatment of biceps muscles compromised by neuromuscular injuries and disorders, such as brachial plexus injury and stroke.

3.2 Methods

3.2.1 Participants

Twenty healthy subjects (14 men; range values; age: 19÷35 years; body mass: 50÷82 kg; height: 1.57÷1.87 m) participated in this study after providing written informed consent. The protocol and consent procedures were conducted in accordance with the Declaration of Helsinki and were approved by the Local Ethic Committee. Criteria for participation included no history of orthopedic or neurological injury that could affect upper-extremity muscle function.

3.2.2 Dominance evaluation

Arm preference was evaluated for each individual through the *laterality quotient* from the Edinburgh Handedness Inventory (Oldfield 1971); the *laterality quotient* may range between -100 (fully left-handed) and +100 (fully right-handed) and it is calculated based on the side preference of the subject when using hands in ten different activities. Specifically, subjects should indicate their preference by putting a + in the column referring the limb side used to perform the task, right or left. If subject's preference was very strong that he would never try to use the other hand to perform the task unless forced, then he should put two + (i.e., ++) in the respective column. If he was indifferent about which hand prefer to use to perform the task, he should put one + in both columns. Finally, the number of + on each column were considered to calculate the *laterality quotient* as follow:

$$Laterality\ quotient = \frac{\sum_{i=1}^{10} X(i,R) \ - \ \sum_{i=1}^{10} X(i,L)}{\sum_{i=1}^{10} X(i,R) \ + \ \sum_{i=1}^{10} X(i,L)} \times 100$$

where *X(I,R)* and *X(I,L)* are the numbers of + for the *i*th task in the right and left columns, respectively.

3.2.3 Stimulation protocol

Participants were comfortably seated with both upper limbs secured to an isometric brace (Figure 3A), each at a time. The forearm of the limb under investigation was held in neutral position, the elbow joint flexed at 110˚ (180˚ being full extension)

and the shoulder joint slightly abducted. Shoulder abduction ranged from 10˚ to 25˚ across subjects.

A staircase, stimulation profile was considered to deliver current pulses to biceps brachii. Starting from the minimal current delivered by the stimulator (2 mA; Rehastim Science Mode, Hasomed, Germany), current intensity was increased automatically gradually up to the maximal intensity tolerated by each subject, at the smallest possible current step (2 mA) allowed by the stimulation device. For each stimulation intensity, four biphasic, rectangular current pulses (100 μs per phase) were applied at 1 pps. The experimental protocol was conducted consecutively for each arm, at random order and with at least 5 min interval in-between. The duration of the stimulation protocol depended on the maximal current intensity tolerated by each subject; it lasted 3.4 minutes at most.

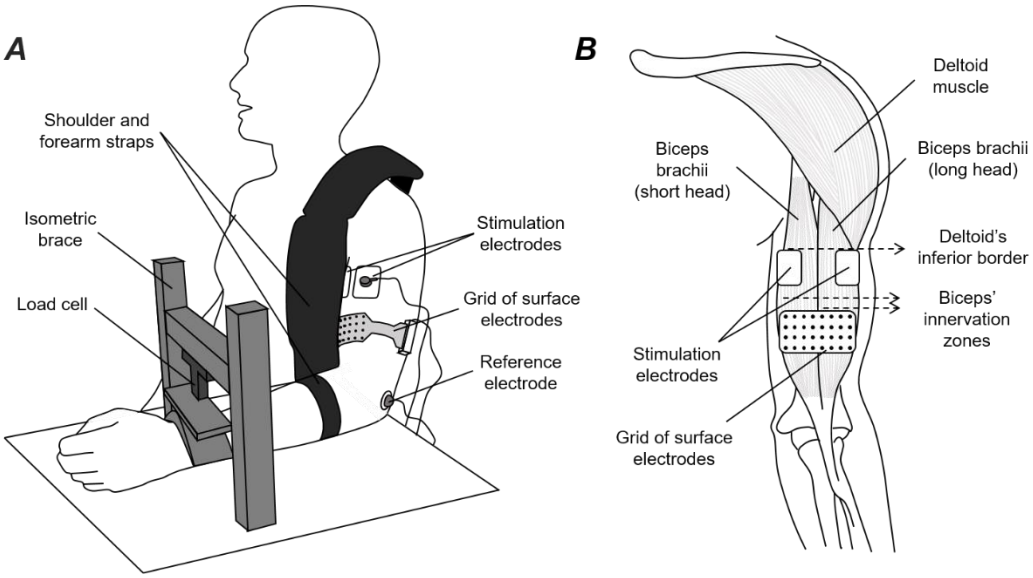

Figure 3: Schematic illustration showing the experimental setup (A) and the position of detection and stimulation electrodes in relation to the biceps brachii muscle (B). M waves were sampled with a grid of 32 surface electrodes, positioned below the most distal innervation zone identified and with the junction between the muscle short and long heads located in-between the fourth and fifth columns of electrodes. Stimulation electrodes positioned as proximal as possible over biceps brachii, with care to do not cover the deltoid muscle (see text). Force signals were acquired with a biaxial load cell positioned over the subject's wrist.

3.2.4 Positioning of detection electrodes

The innervation zone positions were identified for both short and long head of the biceps brachii using an array of 16 dry electrodes (5 mm inter-electrode distance). The innervation zone was identified as the location corresponding to the pair of electrodes where phase opposition could be observed for consecutive rows of single differential EMGs detected during moderate level, isometric contractions (Merletti et al. 2003). Given the location of innervation zones in relation to electrodes change with the elbow and shoulder joints' angle (Martin and MacIsaac 2006), innervation zones were identified with subjects positioned as shown in Figure 3A. Ultrasound imaging (Echo Blaster 128, Telemed Ltd., Vilnius, Lithuania) was used to identify the junction between the biceps short and long heads. A grid of 32 surface electrodes (4x8 arrangement; 3 mm diameter; 10 mm inter-electrode distance; Figure 3) was used to detect EMGs from both heads of biceps brachii thus including as much muscle fibers as possible into the electrodes' pick-up volume. Columns of electrodes were aligned parallel to the muscle longitudinal axis, with the most proximal row positioned, whenever possible, just distal to the most distal innervation zone identified for both heads (Figure 3B).

3.2.5 Positioning of stimulation electrodes

A pair of stimulation electrodes (size 35x45mm) was positioned proximally and arranged orthogonally to the muscle longitudinal axis (Figure 3B) as described in the following. First, with the participant's arm secured to the isometric brace, the lateral and medial borders of biceps brachii were identified with ultrasound imaging (Echo Blaster 128, Telemed Ltd., Vilnius, Lithuania) and marked on the skin. Similarly, the deltoid contour was identified and marked. Then, in relation to the biceps longitudinal axis, the external edges of the cathode and anode electrodes were positioned at or just internally to the borders of the biceps short and long heads, respectively. The superior edge of both electrodes was positioned at or just distally to the deltoid inferior border (Figure 3B). EMGs and stimulation electrodes were positioned after cleaning the skin with abrasive paste (Nuprep, Weaver and company, USA).

3.2.6 Signal recordings

Surface EMGs were recorded in monopolar derivation (192 V/V gain; 10–750 Hz bandwidth amplifier; W-EMG LISiN-Politecnico di Torino, Turin, Italy). EMGs were digitized at 2441.4 Hz with a 24 bits A/D converter. Elbow flexion and

forearm supination forces ($F_{flexion}$ and $F_{supination}$, respectively) were measured with a biaxial load cell (50 Kg full-scale; 2.596 mV/V sensitivity; CCT transducers, Italy) positioned over the subject's wrist (Figure 3A). Force signals were amplified either 500 or 1.000 times, whichever provided the highest signal-to-noise ratio without saturation (0–33 Hz bandwidth amplifier; Forza; OT-Bioelettronica, Turin, Italy) and acquired as auxiliary signals with the W-EMG amplifier. An output digital signal from the stimulator synchronous with the stimulation pulses was recorded as an auxiliary signal with the W-EMG amplifier and used for the identification of M waves and force twitches.

3.2.7 Data analysis

The quality of EMGs and force signals was first evaluated through visual analysis. Low quality monopolar EMG signals likely associated with electrode-skin contact problems were occasionally observed. In cases where low-quality signals were isolated, they were replaced using a linear interpolation of the neighboring channels (4 point connectivity), otherwise they were excluded from analysis. After visual inspection, monopolar EMGs and force signals were band-pass (10-400 Hz cutoff; bidirectional filter) and low-pass filtered (10 Hz cutoff; bidirectional filter) with a fourth order Butterworth filter, respectively. Data of three subjects were excluded from analysis because stimulation artifact could not be removed by offline blanking (Botter et al. 2009; 4 to 5 ms from the stimulation onset). For the remaining 34 muscles, the channels where stimulation artifact greatly overlapped M waves were not considered for analysis. Figure 4 shows an example of M waves and stimulation artifacts recorded from one muscle evaluated.

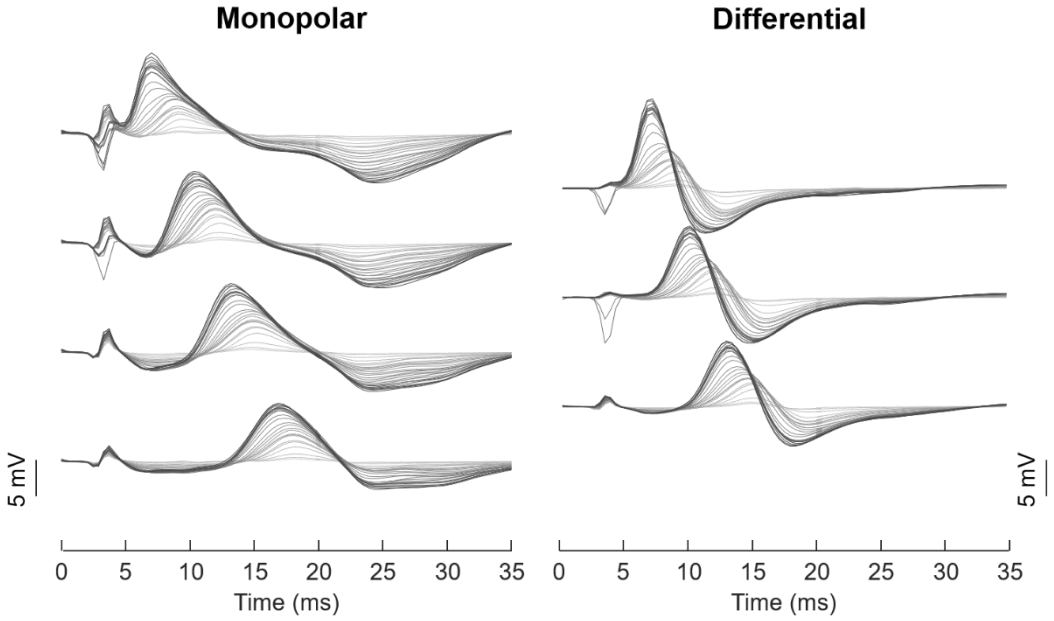

Figure 4: Representative example of monopolar (left panel) and differential (right panel) M waves recorded in the fourth column of a matrix applied to one biceps brachii evaluated, at increasing stimulation intensities from 22 to 70 mA (with steps of 2 mA). Note that stimulation artifact overlapped the first samples of monopolar M waves recorded in the first row of the column.

M-wave amplitude

Single-differential EMGs were calculated by differentiating monopolar signals along consecutive rows. M waves for each stimulation intensity were obtained by averaging EMGs across the four stimulation pulses using an epoch 30 ms long. Then, the M-wave peak-to-peak amplitude was computed for each channel and for each stimulation intensity (Figure 5C). Notwithstanding the careful procedure we considered for electrodes' positioning, innervation zone of at least one biceps head could not be avoided for 12 out of 34 muscles. In all these instances, innervation zone was located either in the first or between the first and second rows (Figure 5B). Thus, to avoid the influence of the innervation zone on the M-waves amplitude (Merletti et al. 2003) and considering previous account which reported variations in the amplitude of bipolar motor units potentials in the biceps' region between innervation zone and tendons (Rodriguez-Falces et al. 2013b), the greatest peak-to-peak amplitude obtained for each column in the grid was considered for analysis. Then, amplitude values were averaged across columns, producing a single,

representative response per stimulation intensity. With the grid of electrodes we were therefore able to obtain representative responses of most motor units of the whole biceps brachii muscle, while minimizing the influence of sustained confounding factors.

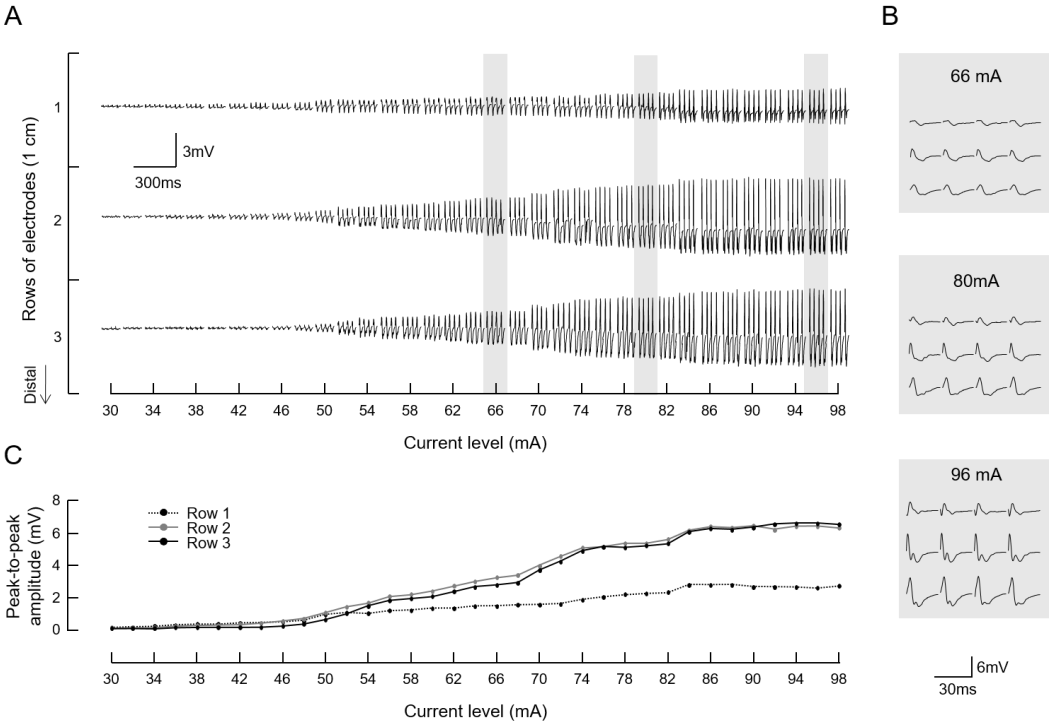

Figure 5: (A) Example of single-differential M waves provided by one column (7) of channels, elicited for current intensities ranging from 30 to 98 mA in 2 mA steps. Four M waves were evoked per current intensity. (B) Expanded view of M waves within the shaded rectangles shown in (A). Note the phase opposition between M waves in the first and second rows, indicating the presence of an innervation zone between these rows. (C) Peak-to-peak amplitude averaged across the four M waves shown in A, separately for each current intensity.

Force twitch

Twitch forces were obtained by triggering (300 ms epochs) the force signals identified as $F_{flexion}$ and $F_{supination}$. Twitches were averaged across stimulation pulses, separately for each current intensity and, then, peak-to-peak amplitudes of

the average twitches from $F_{flexion}$ and $F_{supination}$ were computed. A resultant force was obtained for each current intensity as follow:

$$Resultant\ force(i) = \sqrt{Amp\_Fflexion(i)^2 + Amp\_Fsupination(i)^2}$$

where *I* corresponds to the *i*th current intensity and $Amp\_F_{flexion}$ and $Amp\_F_{supination}$ correspond to the amplitude of the average twitch calculated from $F_{flexion}$ and $F_{supination,}$ respectively.

3.2.8 Assessment of variations in muscle responses of dominant and non-dominant arms

When analyzing the stimulus-response curves for M-waves amplitudes and force twitch, we observed that, for 22 out of 34 arms, force amplitude continued to increase after M-waves amplitude have reached a plateau in a given current intensity (Figure 6). This suggests the biceps brachii may not have been the only muscle contributing to force signals. Such findings were already expected, since the arm region where stimulation was applied may have nerve fibers which supply other muscles besides biceps brachii, such as the brachialis and the supinator muscles. Therefore, in order to obtain selective results about the recruitment of biceps brachii motor units, we decided to disregard the analysis of force signals from the study.

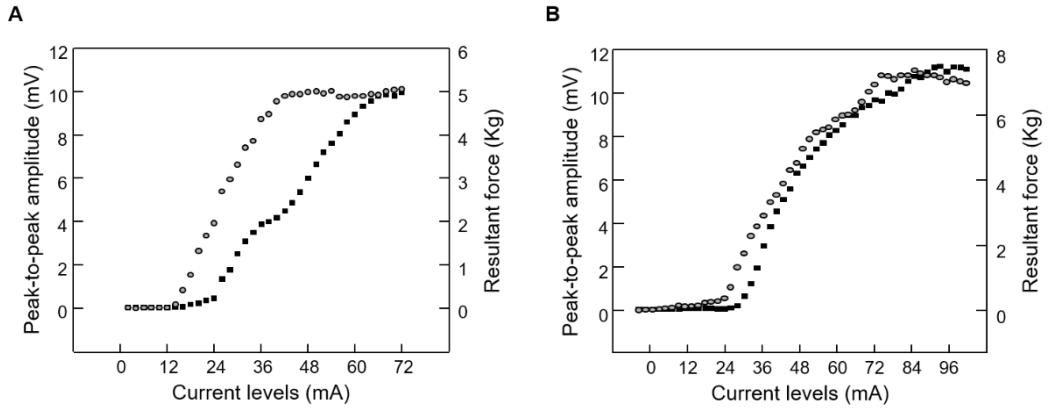

Figure 6: Stimulus-response curves obtained from EMG and force signals of one of the arms of two subjects evaluated (panels A and B). Circles correspond to M-waves amplitudes averaged across stimulation pulses and channels. Squares correspond to the amplitudes of resultant forces obtained from the twitches identified in $F_{flexion}$ and $F_{supination}$ and averaged across stimulation pulses. For the example in panel (A), M-wave amplitude reached a plateau at about 38 mA, while force amplitude increased up to the current tolerated by the subjects (72 mA). For the example in panel (B), both M-wave and force amplitudes reached a plateau, but at different current intensities: 72 mA and 90 mA, respectively.

Differences in the mean peak-to-peak amplitude were considered to evaluate how gradually biceps brachii responded to stimulation increments in both arms. First, the range of current intensities leading to the smallest and greatest M waves was identified as follow: the distribution of peak-to-peak amplitudes obtained for all stimulation intensities was computed (Figure 7). This distribution was clearly bimodal for 27 out of 34 muscles tested, with the first and second modes respectively indicating the absence of M waves and the maximal muscle response (Figure 7A). When the distribution of peak-to-peak values was multimodal (five out of 34 muscles; Figure 7B), only the first and the last modes were considered for analysis. Data from one participant was discarded from analysis because M-waves' amplitude increased indefinitely with stimulation intensity, producing a unimodal distribution of peak-to-peak values (Figure 7C). Therefore, 16 subjects (32 muscles) remained for analysis. The highest current below which the peak-to-peak amplitude was smaller or equal than the first amplitude mode was then identified and defined as the *motor threshold*. Similarly, the smallest current over which peak-to-peak amplitude equaled or exceeded the second or last amplitude mode was identified. These values define the *current range* (cf. shaded rectangles in Figure

8A and Figure 8D) within which increases in stimulation intensity resulted in increased muscle response; i.e., increased M-wave amplitude. The number of stimulation steps within the *current range* can be determined by dividing the *current range* by the current step (2 mA) and subtracting the result by one. There are other methods used by previous studies to determine the *motor threshold* and the *current range* (Blok et al. 2007; Rodriguez-Falces et al. 2013a).

Only two of the 16 participants retained for analysis showed the same *current range* for both arms, indicating the 2 mA step represented different, relative stimulation intensity between arms for most subjects. Therefore, to ensure like-with-like comparisons, the amplitude of M waves obtained from the side with the smaller *current range* was linearly interpolated considering a fixed, stimulation step smaller than 2 mA. This new stimulation step was computed as the value of the smaller *current range* divided by the number of stimulation levels comprised within the greater *current range*. As outlined in Figure 8, this procedure provided an equal number of peak-to-peak values for the biceps brachi muscle in both sides. Afterward, we computed the difference between peak-to-peak values obtained for consecutive stimulation levels, hereafter referred to as *increment*. *Increments* were normalized in relation to the difference between the maximal and minimal peak-to-peak values for each muscle (Figure 8C and Figure 8F). Then, the median value of the *increments* was calculated producing a single, representative value per muscle. Median *increments* values were therefore considered to assess side-differences in electrically elicited, motor unit recruitment.

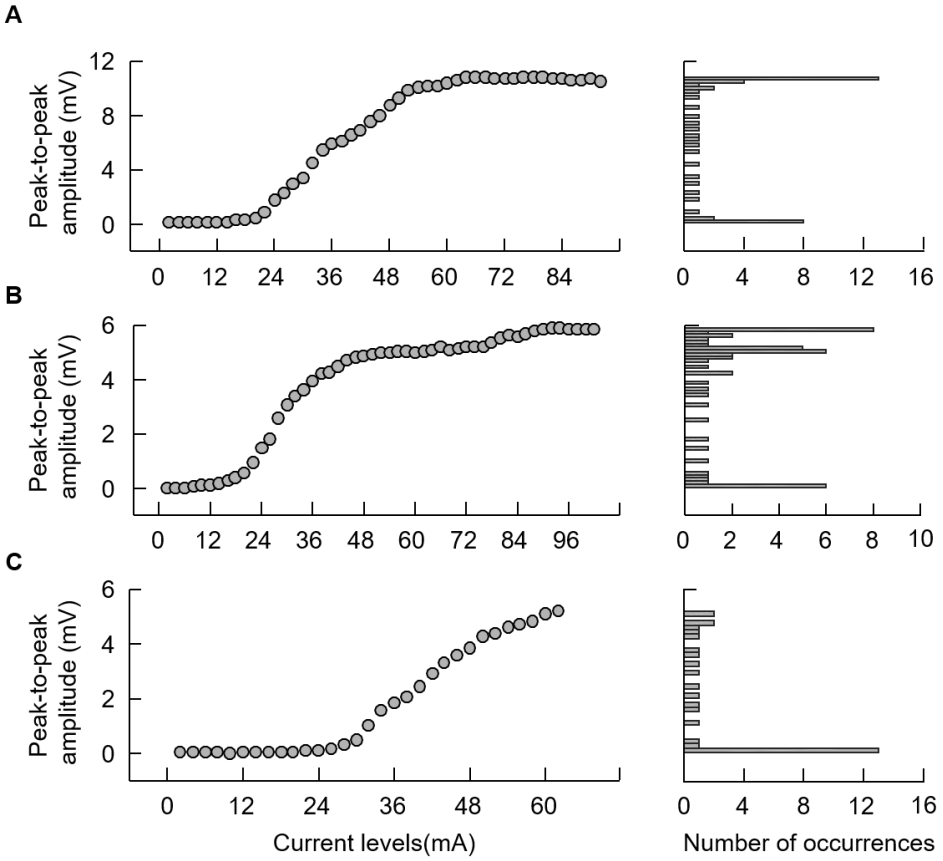

Figure 7: Example of different distribution profiles of peak-to-peak amplitudes obtained for three distinct muscles evaluated (panels A, B and C). Circles correspond to amplitude values averaged across stimulation pulses and channels. Panel (A) shows a bimodal profile, in panel (B) the distribution shows more than two modes and in panel (C) a single mode is observed.

3.2.9 Statistics

Given the data distribution was not Gaussian (Shapiro-Wilk's W test, $p < 0.05$) for part of the parameters evaluated (i.e., *current range* and median *increment* of dominant and non-dominant arms), both parametric and non-parametric statistics were used to test our hypothesis. The paired Wilcoxon test and the Student's t-test for dependent samples were applied to evaluate side differences in the median *increments* and *current ranges*, respectively. The level of statistical significance was set at 5 % and data were reported using both parametric and non-parametric descriptors.

3.3 Results

All 16 participants considered for analysis had the right arm as the dominant one, according to the median, *laterality quotient* score of 86.2 % (interquartile interval: 62.1-100 %). Subjects whose *laterality quotient* was < 50 or > -50 would have been excluded from analysis. We defined these thresholds based on previous results reported by Triggs et al. (2000), where subjects with *laterality quotients* within these ranges (< 50 or > -50) showed significantly greater ability to perform tasks requiring a finer control of movements with dominant than non-dominant hands. Stimulation intensity values corresponding to the *motor threshold* and the maximal muscle response for the dominant and non-dominant arms are described in Table 2.

Table 2: Stimulation intensities corresponding to the *motor threshold* and maximal muscle response for biceps brachii of dominant and non-dominant limbs.

	Stimulation intensity (mA)	
	Motor threshold	Maximal muscle response
Dominant arm	18 (12-22)	67 (61.5-86)
Non-dominant arm	17 (14-20)	62 (54-75)

Values are *median (interquartile interval)*.

3.3.1 Side differences in electrically elicited, muscle responses

Regardless of whether testing the dominant or non-dominant side, a clear sigmoidal profile was observed for most of the subjects when plotting M-wave amplitude vs. current intensity. As illustrated for a representative participant in Figure 8, M-wave amplitude remained equally low for both arms when current intensity increased up to the *motor threshold*. M-wave amplitude increased monotonically from the *motor threshold*, reaching a plateau respectively at 64 and 52 mA for the dominant and non-dominant arms (Figure 8A and Figure 8D). For this participant, the *current range* and therefore the number of the stimulation steps between the *motor threshold* and the maximal M wave was smaller in the non-dominant (19 stimulation levels) than in the dominant arm (26 levels; cf. shaded rectangles in Figure 8A and Figure 8D).

When considering all participants, side differences were observed in the magnitude of muscle response for similar, relative increases in the current intensity. The paired Wilcoxon test revealed significant, dominance effect on the median *increment* values calculated for biceps brachii of both arms (P = 0.017, N=32; 16 subjects x 2 arms; Figure 9A). Despite the inter-individual variability, smaller *increments* were observed in muscles of dominant than non-dominant arms for most of the subjects (11 out of 16), whose difference ranged from 0.1 to 2.5 %. For the other five subjects, one showed similar *increment* values (3.4 %) between arms and the others had smaller median *increments* in non-dominant than dominant arms, differing from 0.2 to 0.6 %. Additionally, the Student's t-test revealed no significance difference between *current range* of dominant and non-dominant arms (P = 0.31, N=32; 16 subjects x 2 arms; Figure 9B). For half of subjects (eight out of 16), c*urrent range* was greater in biceps brachii of the dominant arm, with differences ranging from 2 to 42 mA. While for six out of 16 subjects *current range* was greater in non-dominant arms, differing from 2 to 28 mA.

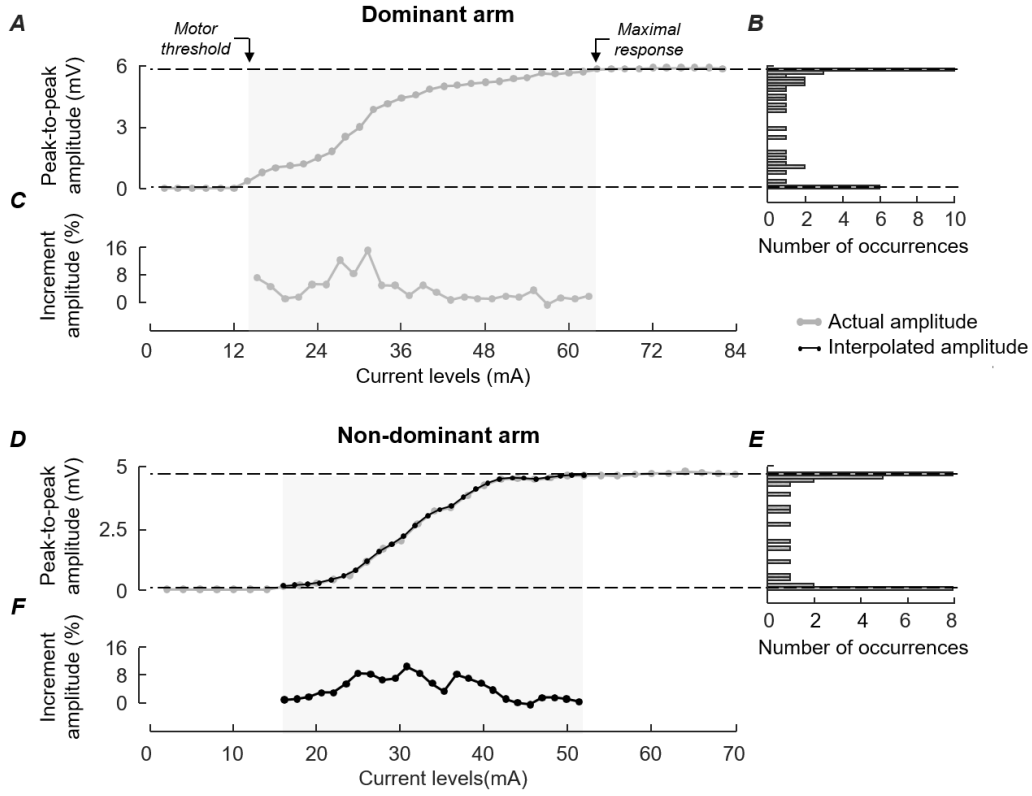

Figure 8: Variations in the M-waves amplitude for increases in current intensity are shown for the dominant (A) and non-dominant (D) arm of a single, representative participant. Grey circles correspond to amplitude values averaged across stimulation pulses and channels. Black circles correspond to the interpolated amplitude values within the *current range* (shaded rectangle) of the non-dominant arm. After linear interpolation, the number of *increments* within the *current range* of the non-dominant arm increases from 19 to 26, to match the number of stimulation levels of the dominant arm. (B) and (E) show the first and second modes of the distribution of amplitude values obtained for each limb (cf. dashed lines). The current intensities below and over which the amplitude values were respectively smaller or equal and greater or equal than the first and second amplitude modes were identified; these current values define the *current range* within which increases in stimulation intensity elicited *increments* in M-wave amplitude (shaded rectangles). (C) and (F) show the amplitude of *increments* between consecutive stimulation levels for the dominant and non-dominant arms, respectively. *Increments*' amplitude is normalized w.r.t the M-wave amplitude corresponding to the muscle's motor threshold and maximal response.

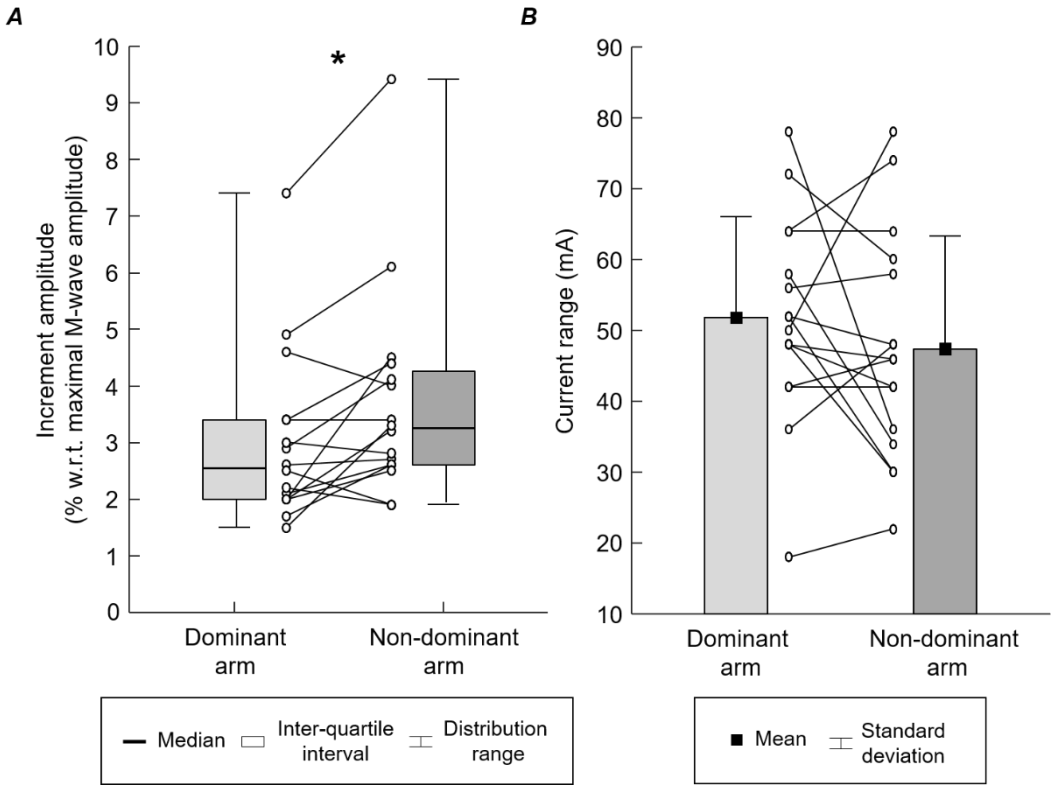

Figure 9: Boxplots in panel (A) show the median *increment* amplitude calculated from the *increments* between consecutive M waves within the *current range* of dominant and non-dominant arms of subjects. Bars in panel (B) show the *current range* within which the amplitude of M waves increased from the smallest to the greatest value. Circles connected through a line correspond to *increments* and *current ranges* for the dominant and non-dominant arm of the same subject. Light and dark grey boxes respectively indicate values for the dominant and non-dominant arm. Asterisks indicate significant differences between arms ($p < 0.05$).

3.4 Discussion

The present study investigated whether the amplitude of M waves increase similarly in the dominant and non-dominant arms when current pulses at progressively greater intensities are delivered to the biceps brachii muscle of healthy, young subjects. Surface EMGs were collected from the biceps brachii while current pulses were applied gradually increasing the current intensity up to the maximal intensity tolerated by each subject. Our main results suggested that M-waves amplitude increased more gradually in muscles of dominant arms, likely due to a broader

spectrum of recruitment thresholds in biceps brachii motor units of dominant than non-dominant arms.

3.4.1 Methodological considerations

The experimental protocol applied here aimed at obtaining representative and selective myoelectric responses of biceps brachii motor units. Methodological issues related to the configuration of stimulation and sEMG electrodes were taken into account in this study. Specifically, the following procedures were performed:

(i) the stimulation electrodes were positioned in the most proximal portion of biceps brachii, where the nerve trunk and primary motor branches that supply this muscle are localized (Pacha Vicente et al. 2005; Lee et al. 2010; Cambon-Binder and Leclercq 2015). This electrodes' positioning probably ensured the electrical current crossed most of axons that innervate biceps brachii since they are more clustered in this region;

(ii) bipolar stimulation technique was used with stimulation electrodes positioned in the transverse direction of the muscle, maximizing their distance over the biceps. As compared to monopolar technique, in bipolar stimulation the current crosses muscle tissue with similar density (Botter and Merletti 2016), thus reducing the effects of geometrical factors (e.g. relative electrode-axon position) on MU recruitment;

(iii) a grid of 32 electrodes was used to acquire biceps brachii responses. Since previous evidence have shown spatial variations in the EMG activity of biceps brachii during isometric contractions (Rodriguez-Falces et al. 2013b; Staudenmann et al. 2013), the acquisition of action potentials from a wide muscle region provides representative M waves of biceps muscles. High-density surface EMG also assisted us to avoid unreliable amplitude estimations due to the occasional presence of the innervation zone (Merletti et al. 2003; see methods section). Additionally, the analysis of differential EMG signals recorded with a relative small inter-electrode distance likely attenuated a possible contribution of potentials coming from brachialis motor units that may have been activated during the stimulation protocol (Roeleveld et al. 1997b; Rodriguez-Falces et al. 2013b). Thus, we believe the results found in this study are mainly related to responses of biceps brachii motor units.

Finally, we would like to stress why we decided to evaluate the biceps brachii muscle instead of small hand muscles for which dominance is typically assessed (Kamen et al. 1992; Adam et al. 1998; Li et al. 2015). First of all, the biceps brachii may be electrically elicited and assessed selectively from the skin. Due to its relatively great size, it is possible to stimulate the motor branches that innervate the biceps brachii and acquire the EMG responses through surface electrodes positioned over the skin. Hand muscles, on the other hand, are usually electrically elicited by stimulating the nerve which innervates them. Since several hand muscles are usually innervated by the same nerve, it is difficult to obtain selective EMG responses from a single hand muscle through superficial detection electrodes. Indeed, previous studies used invasive techniques to investigate hand muscle responses during electrically elicited contractions through nerve stimulation (Kamen et al. 1992; Adam et al. 1998). A second reason for which we did not evaluate a hand muscle was because the smallest possible current step delivered by our stimulator is 2 mA. In a pilot experiment on one subject, we observed that such current step was relatively great to gradually elicited motor units of a hand muscle.

3.4.2 M waves were elicited more gradually in biceps brachii of dominant arms

Before interpreting our results, two considerations regarding the data analysis applied in this study are necessary. First, we would like to point out how differences between the motor units' recruitment in biceps brachii of dominant and non-dominant arms may be identified through the analysis of *increments* in M-waves amplitude. As the M wave represents the summation of the motor units' action potentials synchronously evoked by an external electric stimuli (Merletti et al. 1992), changes in the M-wave amplitude is usually assumed to reflect a change in the number of motor units activated (McComas et al. 1971). Therefore, variations in the magnitude of the *increments* in the M-waves amplitude analyzed in this study reflect variations in the relative number of additional motor units elicited in the muscle, for increases in current intensity. The smaller the *increment* in the M-waves amplitude, the smaller the relative amount of motor units activated for increases in the current intensity applied to the muscle and, hence, more gradual is the recruitment of its motor units. M-waves amplitude were normalized with respect to the minimal and maximal muscle responses (i.e., amplitudes corresponding to the *motor threshold* and the maximal M-wave amplitude,

respectively) to compensate the effect of anatomical differences between arms on the surface EMGs (Farina et al. 2002).

The second consideration we would like to underline is related to our decision of equal the number of *increments* within the *current range* between arms, through the linear interpolation (see methods). Previous studies have investigated motor units' recruitment during involuntary elicited contractions through staircase stimulation protocols where the number of stimulation levels, between the muscle's motor threshold and the maximal elicited M wave, was similar among muscles (McComas et al. 1971; Blok et al. 2007). In our study, however, we could not apply similar, relative increases in current intensity between subjects' arms because the smallest possible current step delivered by our stimulator is 2 mA. Occasionally, *current ranges* were similar between arms of two subjects (cf. Figure 9B), which means that the number of stimulation levels and therefore of *increments* was similar between subjects' arms. Nevertheless, for the other 14 subjects evaluated *current range* differed between arms in a range of 2 to 42 mA. Although statistical group analysis showed the *current range* was not significantly different when comparing arms, distinct number of *increments* between dominant and non-dominant arms could lead to biased interpretations of muscles' responses. Indeed, in previous analysis performed before we applied the linear interpolation, we observed that subjects whose *current range* was much greater in one arm than the other, usually the dominant arm, tended to have median *increments* much smaller in the arm with the larger *current range*, likely due to a higher number of *increments*. Thus, the linear interpolation was applied to ensure like-with-like comparisons between dominant and non-dominant arms and to ensure that our findings (Figure 9A) were not influenced by methodological issues, such as distinct number of *increments* between limbs. Additionally, when applying the linear interpolation in the stimulus-response curve of the subjects' arm with the smaller *current range*, we assumed that motor units were recruited between consecutive current levels. In the case this assumption was not true, i.e., that there was no motor unit recruitment between consecutive stimulation levels, median *increments* were probably underestimated in muscles which stimulus-response curve was interpolated. Although there was no consistency with respect to the subjects' arm, dominant or non-dominant, which interpolation was performed, larger *current ranges* were mostly observed in dominant arms. Therefore, the linear interpolation may have mainly affected median *increments* of non-dominant arms, i.e. median *increments* may have been mainly underestimated in biceps brachii of non-dominant arms.

Our findings showed that biceps brachii motor units seem to be recruited more gradually in dominant with respect to non-dominant arms of healthy, young subjects. Indeed, for similar, relative increases in the current intensity, we observed *increments* in the M-waves amplitude significantly smaller in muscles of dominant than non-dominant arms (Figure 9A). In other words, increases in M-waves amplitude were progressively larger in biceps brachii of non-dominant than dominant arms. The average difference between *increments* of arms was about 0.7%, however, such difference may have been even greater since median *increments* may have been mostly underestimated in non-dominant arms, as commented above. Taking in consideration the methodological issues (i) and (ii) discussed in the previous subsection, we assumed that all biceps' axons received similar electrical inputs (i.e. the same amount of electric charges) for a specific stimulation intensity. Based on this assumption, we believe the activation of biceps' axons likely depended on their diameter, where large-diameter axons would have been depolarized before small-diameter axons due to a lower threshold of electrical excitability (Stephens et al. 1978). If motoneurons were more gradually elicited in dominant muscles, it suggests there was a greater variability among their thresholds of electrical excitability and therefore among their axons' diameters, with respect to the motoneurons of non-dominant muscles. Since previous evidence showed that the susceptibility of motoneurons to discharge during voluntary contractions (i.e. the motoneuron activation threshold) is a function of the size of their cell bodies and, consequently, of the diameter of their axons (Henneman 1957; Henneman et al. 1965b), a more gradual motor unit's recruitment may indicate therefore a broader spectrum of activation thresholds among motoneurons of dominant than non-dominant arms. Differences between limbs in the motoneurons recruitment threshold were also observed by Adam et al. (1998). Through needle EMG, the authors investigated asymmetries in the recruitment of motor units in the first dorsal interosseous muscle of both limbs, during isometric voluntary contractions at 30% of maximal voluntary contraction (MVC). They found a highest percentage of motor units recruited at lower force levels in dominant hands, while motor units from non-dominant hands revealed a more spread-out recruitment pattern with a progressive increase in force. Although Adam et al. (1998) have analyzed only part of motor units of the first dorsal interosseous muscle (i.e., those activated until 30% of MVC), their findings point out to differences between limbs in the mechanisms underpinning the motor units' recruitment.

3.4.3 Physiological and practical implications

Distinct motor units' recruitment strategy observed between biceps brachii of dominant and non-dominant arms may lead to differences in the ability of these muscles to regulate the force production. Briefly, it is well established that variations in the number of motor units activated in a muscle is a key mechanism for the modulations of muscle force, with muscle force increasing progressively for increases in the number of motor units recruited (Clamann 1993; Kernell 2003). Therefore, the more gradual recruitment of biceps brachii motor units in the dominant arm, observed using incremental stimulation, is likely associated with the finer regulation of muscle force production in the dominant arm with respect to the non-dominant arm. The median *increments* differed on averaged 0.7% between limbs (cf. Figure 9A). Such a value can be considered as indicative of the greater amount of additional motor units elicited in the non-dominant muscle with respect to the dominant muscle for similar, relative increases in the stimulation intensity. In this hypothesis, if we translate this side-difference in terms of incremental force, biceps brachii of dominant arms would produce smaller relative force *increments* with respect to non-dominant arms. Moreover, considering the *increment* results separately for each subject (cf. Figure 9A), side differences in force production may be even greater. Therefore, our findings point out to asymmetries in the organization of the neuromuscular system that may contribute to a more accurate muscle force control in biceps brachii of dominant than non-dominant arms.

Our methodological approach could have relevant implications for clinical research. For instance, neurodegenerative diseases, such as the stroke, seem to impact potentially the organization of the neuromuscular system. Previous accounts have suggested the occurrence of collateral reinnervation in muscles compromised after stroke due to the degeneration of their motoneurons (McComas et al. 1973; Lukács 2005; Lukács et al. 2009; Li et al. 2011, 2013). If this assumption holds, it is reasonable to expect changes in the mechanisms underpinning the motor units' recruitment in muscles affected by stroke, which could be somewhat investigated through the experimental protocol proposed in this study.

Chapter 4

Assessing structural adaptation of biceps brachii motor units after stroke

4.1 Introduction

Neurophysiological studies (McComas et al. 1973; Hara et al. 2004; Lukács 2005; Kouzi et al. 2014) have suggested the occurrence of motoneurons degeneration following stroke is associated with a process called "trans-synaptic or transneuronal degeneration", which represents the death of neurons due to the interruption of the transmission of electrical impulses between neighboring neurons (Cowan 1970). The main evidence of such degeneration process came from studies which investigated the number of motor units in muscles of stroke survivors, through techniques of motor unit number estimation (MUNE) (McComas et al. 1973; Hara et al. 2004; Li et al. 2011, 2014a; Kouzi et al. 2014). Findings from these studies indicate a significant decrease of about 20-60 % in the number of motor units of muscles affected after stroke with respect to healthy muscles (usually the contralateral, unaffected muscles). As a consequence, such motor units' loss may lead to changes in the organization of the neuromuscular system following stroke.

There is evidence that a reinnervation process takes place after stroke, through collateral reinnervation by the surviving motoneurons into the muscle fibers denervated (Martínez et al. 1982; Kallenberg and Hermens 2009; Lukács et al. 2009; Li et al. 2013). In such case, each surviving motoneuron may innervate a greater amount of muscle fibers after the stroke, i.e. the number of muscle fibers per motor unit (innervation ratio) would increase in muscles affected after stroke. Indeed, through an invasive analysis of single-fiber electromyography (EMG), Martínez et al. (1982) and Lukács et al. (2009) reported a significantly higher motor unit fiber density in hand muscles in the affected side of stroke patients, with respect to healthy muscles (either of the unaffected side of stroke patients or of a control group). Since fiber density has been considered an indicator of muscle's innervation ratio (Stålberg 1990), their results suggest an increase of the innervation ratio in muscles affected after stroke, likely due to a collateral reinnervation process. Such structural change of motor units following stroke was also suggested by studies which investigated alterations in muscle responses due to stroke, through surface EMGs during voluntary contractions. Kallenberg and Hermens (2009) and Li et al. (2013), for instance, observed larger amplitudes of EMG signals detected in muscles affected after stroke with respect to unaffected muscles, during voluntary isometric contractions performed at different force levels. The authors suggested the muscle fiber reinnervation to explain the higher muscle responses observed in affected muscles, even if their results were not consistent among the stroke patients evaluated.

Besides the fiber density analysis performed by previous studies through the single-fiber EMG (Martínez et al. 1982; Lukács et al. 2009), changes in the size of motor units (i.e. in the muscle's innervation ratio) following stroke may also be evaluated indirectly by combining surface EMG with the incremental, neuromuscular electrical stimulation (Blok et al. 2007). Since the motoneurons that innervate a muscle have different diameter sizes and, therefore, different activation threshold (McPhedran et al. 1965a, b), it is possible to recruit them gradually by increasing gradually the stimulation intensity applied to their nerve fibers (McComas et al. 1971). As the stimulation intensity increases, more motoneurons are activated and, consequently, more muscle fibers are recruited. Such progressive motor units recruitment can be observed through the increase in the M-waves amplitude for each increase in the stimulation intensity (McComas et al. 1971; Blok et al. 2007). The greater the number of muscle fibers activated for increases in the stimulation intensity, the greater the increment in the M-waves amplitude. Therefore, if collateral reinnervation takes place in muscles affected after stroke, increasing their innervation ratio, it is reasonable to expect for similar, relative increases in current

intensity, greater variations in the M-waves amplitude of muscles affected after stroke than healthy muscles.

Enlargements of muscles' innervation ratio following stroke, however, likely accounts for the inability of stroke survivors in finely controlling movements with the affected limb. The force generated by single motor units with respect to total muscle force reflects the quantal increment in muscle force, thereby indicating how finely force maybe regulated. In addition, the amount of force produced by individual units is proportional to the number of muscle fibers they supply (Heckman and Enoka 2012), i.e. the smaller the number of muscle fibers innervated by a single motoneuron, the smaller is the force produced per motor unit. Therefore, a muscle with relatively low innervation ratio would be able to produce finer increments in force than muscles with higher innervation ratios, for similar synaptic inputs. This implies that, if the innervation ratio of muscles affected after stroke increases due to a collateral reinnervation, the muscles affected would be expected to do not produce increments in twitch force as finely as healthy muscles.

The current study aim, therefore, at verifying whether an enlargement of the motor units' size following stroke leads to greater variations in the amplitude of M waves elicited in affected rather than unaffected muscles of stroke patients, for similar, relative increases in the stimulation intensity. We addressed this question for the biceps brachii, a muscle usually affected in stroke survivors. Once confirmed our hypothesis, the analysis proposed in this study, combining surface EMG with incremental electrical stimulation, could be useful for clinicians, therapists and researchers to indirectly assess structural adaptations on the neuromuscular system of stroke survivors.

4.2 Methods

4.2.1 Participants

Twenty stroke patients (14 men; range values; age: 42÷84 years; body mass: 52÷102 kg; height: 1.52÷1.85 m) were recruited to participate in this study after providing written informed consent according to the Declaration of Helsinki. The experimental protocol was approved by the Ethical Committee of the S.Camillo Hospital (Lido di Venezia, Italy). The stroke patients eligible for this study were identified according to the seventh edition of the Italian guideline for prevention, care and rehabilitation of stroke (Inzitari and Carlucci 2006). The patient was classified according to the Oxford Community Stroke Project (OCSP) criteria

(Bamford et al. 1991) and the diagnosis confirmed by computed tomography or magnetic resonance imaging examinations. Both ischemic and hemorrhagic stroke were screened for this study and all the patients meeting the following inclusion criteria were considered eligible for enrolment: the stroke has affected a brain area involved in the motor system; no associated traumatic brain injury; no history of orthopedic or neurological injury that might affect upper-extremity muscle function. The demographic information of the 20 stroke patients recruited is presented in Table **3**.

4.2.2 Motor function evaluation and dominance evaluation

The Fugl-Meyer Assessment scale (Fugl-Meyer et al. 1975) was used to assess the motor function of the biceps brachii muscles of the patients recruited in this study and, therefore, ensure that these muscles were somewhat affected after stroke. Briefly, this scale provides a subjective evaluation of the degree of sensorimotor impairment in subjects who have had stroke. It assesses the motor function of upper and lower limbs, balance, sensory function, range of motion of joints and joint pain. Nevertheless, for the present study, we considered for analysis only the items related to the motor function of biceps brachii. Such items were:

- Reflex activity of biceps brachii;
- Flexor synergy - Elbow flexion;
- Flexor synergy - Forearm supination;
- Shoulder flexion 0˚ - 90˚ with elbow at 0˚ and pronation supination at 0˚;
- Pronation-supination with elbow at 90˚ and shoulder at 0˚;
- Pronation-supination with elbow at 0˚ and shoulder at 30˚- 90˚ of flexion.

There were three possible points for each item evaluated: 0 (the task cannot be performed), 1 (the task is performed partially) and 2 (the task is performed fully) points. For the evaluation of reflex activity, however, there were two possible points: 0 (absence of reflex) or 2 (presence of reflex). The maximum possible score by subject was 12 points (Table **3**). Patients whose Fugl-Meyer (biceps brachii) evaluation was above 90 % of the maximum score, i.e., above 10 points (six out of 20 patients; cf. Table **3**), were excluded from the study.

Since our previous study, reported in chapter 3, showed a significant dominance effect on the muscles responses evaluated in biceps brachii motor units of healthy young subjects, patients' dominance was also took into consideration for this study. Arm preference was evaluated through the Oldfield questionnaire (see chapter 3,

Table 3: Demographic information of the stroke patients recruited.

ID	Sex	Age (years)	Stroke type	Lesion location	Time elapsed after stroke (months)	Fugl-Meyer (biceps brachii)
1	M	63	Hemorrhagic	Internal capsule and caudate nucleus (left hemisphere)	8	2/12
2	F	57	Ischemic	Parietal lobe (right hemisphere)	5	10/12
3	M	48	Hemorrhagic	Basal nuclei	19	5/12
4	F	68	Ischemic	Internal capsule (left hemisphere)	10	4/12
5	M	70	Hemorrhagic	Parietal lobe (right hemisphere)	2	12/12
6	F	42	Hemorrhagic	Frontal, temporal and parietal lobes (left hemisphere)	25	4/12
7	M	60	Hemorrhagic	Insular cortex (left hemisphere)	5	3/12
8	M	77	Ischemic	Frontal, temporal and parietal lobes (right hemisphere)	3	7/12
9	M	46	Ischemic	Frontal and parietal lobes (left hemisphere)	3	12/12
10	M	51	Ischemic	Territory of the internal carotid artery (left hemisphere)	3	10/12
11	M	61	Hemorrhagic	Internal capsule and caudate nucleus (right hemisphere)	39	10/12
12	F	71	Ischemic	Frontal and parietal lobes (left hemisphere)	1	3/12
13	M	73	Ischemic	Temporal and occipital lobes (right hemisphere)	1	12/12
14	M	68	Ischemic	Periventricular nucleus (bilateral)	8	11/12
15	M	59	Ischemic	Territory of the middle cerebral artery (left hemisphere)	2	2/12
16	M	56	Ischemic	Internal capsule and caudate nucleus (right hemisphere)	2	12/12
17	F	84	Ischemic	Internal capsule and caudate nucleus (left hemisphere)	24 days	12/12
18	F	77	Ischemic	Insular cortex, temporal and frontal lobes, putamen and caudate nucleus (right hemisphere)	7	9/12
19	M	70	Ischemic	Frontal and parietal lobes, internal capsule and caudate nucleus (right hemisphere)	43	6/12
20	M	71	Ischemic	Internal capsule and caudate nucleus (left hemisphere)	2	4/12

subsection 3.2.2, for more details), where patients were asked to answer the query according to the period before the stroke onset.

4.2.3 Electrodes positioning, stimulation protocol and signal recording

Patients were comfortably seated with the upper limb under investigation secured to an isometric brace (Figure 10). For both affected and unaffected limbs, the forearm was held in pronated position, the elbow joint was flexed and the shoulder joint was slightly abducted. The elbow and shoulder joint angles were measured with the subject's arm in the most comfortable position. For most of subjects (ten out of 14), joints' angles were similar between affected and unaffected sides, but varied among patients. Specifically, elbow flexion ranged from 100˚ to 120˚ (180˚ being full extension) and shoulder abduction ranged from 35˚ to 65˚ across subjects.

The positioning of EMG and stimulation electrodes, the stimulation protocol and the signal recordings were conducted as described in the study of chapter 3 (cf. subsection 3.2). The identification of biceps' innervation zones with a dry array of electrodes, however, was not performed in order to limit the duration of the experiment. The grid of surface EMG electrodes were, therefore, positioned as distal as possible from the stimulation electrodes, without covering the muscles' tendon zone. The possible presence of innervation zone(s) under the rows of the grid was investigated during signal visual analysis, in order to avoid unreliable muscles' responses (Merletti et al. 2003). The duration of the stimulation protocol depended on the maximal current intensity tolerated by each patient; it lasted 4.2 minutes at most. Total duration of experiment was about one hour and a half.

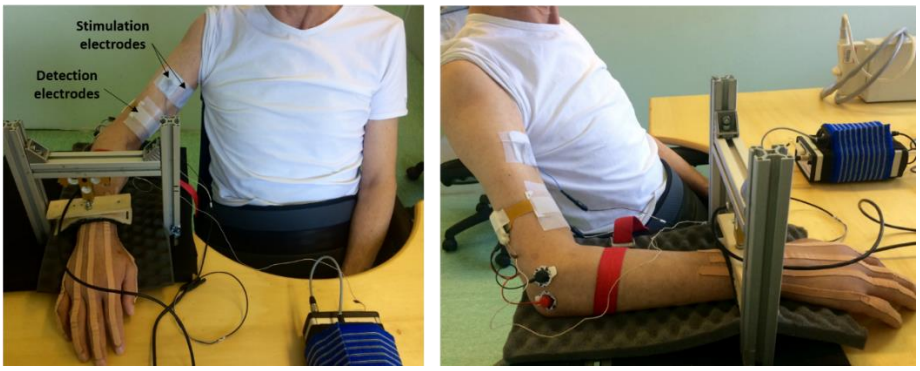

Figure 10: Frontal (left image) and lateral (right image) views of the experimental setup performed with the affected arm of a patient. Detection and stimulation electrodes were positioned in the distal and proximal portions of biceps brachii muscle, respectively.

4.2.4 Assessment of muscle responses in affected and unaffected arms

EMG signals' processing was performed as described in the study of chapter 3 (cf. subsection 3.2.7); the presence of innervation zones, either in the first or between the first and second rows of the grid, was also observed for some patients' muscles (9 out of 28 muscles). Therefore, the same procedure of consider the greatest peak-to-peak amplitude of each column in the grid and, then, calculate the average across them, was performed. Such representative muscle response, obtained for each current intensity, was used to assess variations in the size of M waves elicited in the biceps brachii muscles. Briefly, for each muscle evaluated, the *current range* between the muscle's *motor threshold* and maximal response was identified by analyzing the distribution of peak-to-peak amplitudes (Figure 11). Because *current range* was different between affected and unaffected sides, the increases of 2 mA steps in stimulation intensity represented different percentage of the *current range* for affected and unaffected muscles. Therefore, to ensure like-with-like comparisons, the amplitude of M waves obtained from the side with the smaller *current range* was linearly interpolated considering a fixed, stimulation step smaller than 2 mA (Figure 11D). This new stimulation step was computed as the value of the smaller *current range* divided by the number of stimulation levels comprised within the greater *current range*. Then, increases between the amplitude

of consecutive M waves *(increments*), normalized with respect to the difference between the M-waves amplitude corresponding to the *motor threshold* and the maximal muscle response (Figure 11C and Figure 11F), were calculated and their median value (median *increment*) was used to assess differences between biceps' responses of affected and unaffected patients' arms.

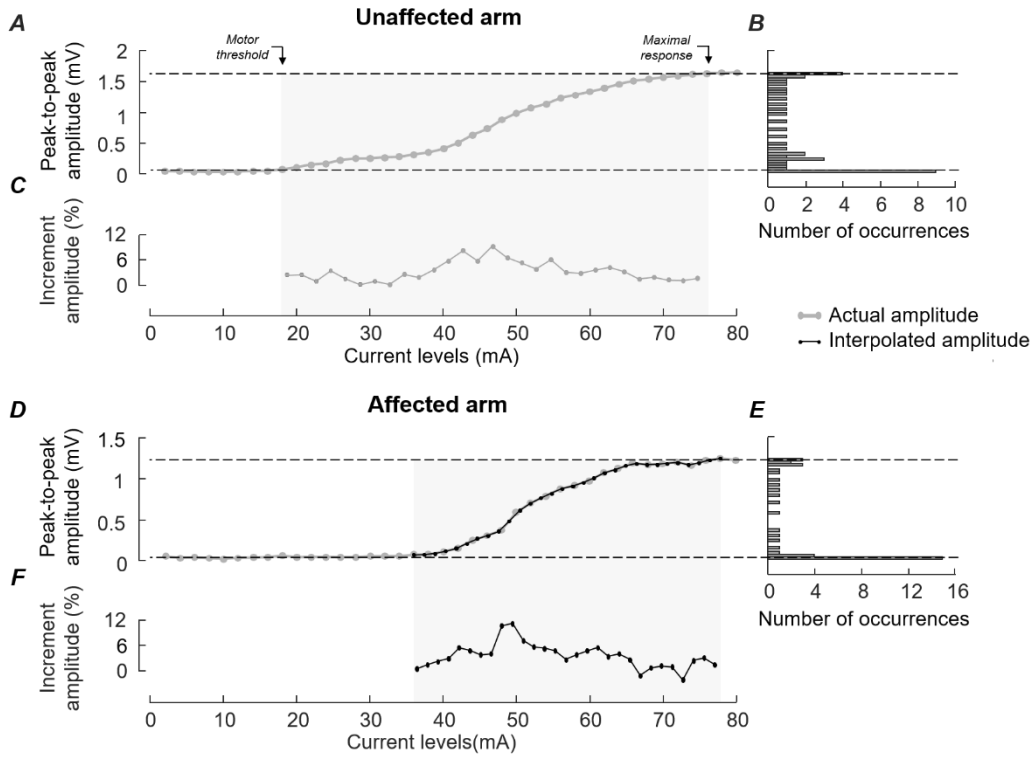

Figure 11: Variations in the M-waves amplitude for increases in current intensity are shown for the unaffected (A) and affected (D) arm of a single, representative patient. Grey circles correspond to amplitude values averaged across stimulation pulses and channels. Black circles correspond to the interpolated amplitude values within the *current range* (shaded rectangle) of the affected arm. After linear interpolation, the number of *increments* within the *current range* of the affected arm increases from 21 to 29, to match the number of stimulation levels of the unaffected arm. (B) and (E) show the first and second modes of the distribution of amplitude values obtained for each limb (cf. dashed lines). The current intensities below and over which the amplitude values were respectively smaller or equal and greater or equal than the first and second amplitude modes were identified; these current values define the *current range* within which increases in stimulation intensity elicited *increments* in M-wave amplitude (shaded rectangles). (C) and (F) show the amplitude of *increments* between consecutive stimulation levels for the unaffected and affected arms, respectively. *Increments*' amplitude is normalized w.r.t the M-wave amplitude corresponding to the muscle's motor threshold and maximal response.

4.2.5 Statistics

After verifying that the data distribution was not Gaussian (Shapiro-Wilk's W test, $p < 0.05$) for part of the parameters evaluated (i.e., *current range* and median *increment* of affected and unaffected arms), both parametric and non-parametric statistics were used to test our hypothesis. The Student's t-test for dependent samples and the paired Wilcoxon test were applied to evaluate differences in the median *increments* and *current ranges* between affected and unaffected muscles, respectively. In addition, Spearman's rank-order correlation was applied to verify the relationship of the ratio between the median *increment* of unaffected and affected muscles (ratio of median *increments*) with the time elapsed since the stroke onset of patients. The level of statistical significance was set at 5 % and data were reported using both parametric and non-parametric descriptors.

4.3 Results

Data of six out of the 14 patients evaluated were disregarded from analysis because the maximal muscle response was not reached in both subject's arms. The eight patients considered for analysis had the right arm as the dominant one, according to the median, *laterality quotient* score of 81 % (interquartile interval: 63-88 %). Stimulation intensity values corresponding to the *motor threshold* and the maximal muscle response for the unaffected and affected arms are described in Table 4.

Table 4: Stimulation intensities corresponding to the *motor threshold* and maximal muscle response for biceps brachii of unaffected and affected limbs.

	Stimulation intensity (mA)	
	Motor threshold	Maximal muscle response
Unaffected arm	21 (19.5-26.5)	74 (68-98)
Affected arm	24 (23-30)	83 (68-105.5)

Values are *median (interquartile interval)*.

4.3.1 Differences between muscle responses in affected and unaffected arms

Figure 12 shows the curves profiles when plotting the normalized M-waves amplitudes and the stimulation levels within the *current range* for both affected and unaffected muscles of the patients. A sigmoidal curve profile, as the one verified for most of the healthy, young subjects evaluated in the study of chapter 3 (c.f. subsection 3.3.1), can be observed in eight out of the 16 muscles analyzed: in both arms of patients 1, 3 and 12, in the affected arm of patient 11 and in the unaffected arm of patient 15. Differences between the stimulus-response curves of affected and unaffected patients' arms seemed to be more evident for patients 4, 11, 12, 15, 19 and 20. Specifically, for patients 11 and 20, the slope of the curve increased in the affected compared with the unaffected muscle from approximately half of the stimulation levels, while for patients 12 and 15 the slope was greater in the first half of the stimulation levels. For patient 4, slope seemed to mainly differ between arms in the first stimulation levels, up to level 8, and between stimulation levels 14 and 18. For patient 19, although the curve for the unaffected muscle have shown a higher slope in the first eight stimulation levels, the curve of the affected muscle seemed to have been higher for most of the stimulation levels.

Six out of the eight patients considered for analysis showed greater median *increment* values for affected than unaffected biceps brachii muscles, differing from 0.1 to 1.3 % (Figure 13A); four of these six patients had the affected arm as the dominant one while the other two had the unaffected arm as the dominant one. For the other two patients whose median *increment* values were smaller in affected than unaffected muscles, the affected arm was the dominant one and differences between arms ranged from 0.2 to 0.4 % (Figure 13A). When comparing median *increments* between affected and unaffected arms, the Student's t-test revealed a P value (P = 0.08, N = 16; 8 subjects x 2 arms) close to the one used to define the statistical significance (P = 0.05). Regarding the *current range*, half of the patients showed greater ranges in the unaffected than affected arm, differing from 2 to 16 mA (i.e., from one to eight stimulation levels). For three patients, *current range* was 2 to 18 mA (i.e., one to nine stimulation levels) greater in the affected arm, and for one patient it was similar between arms. The paired Wilcoxon test revealed no significance difference between *current range* (i.e. the number of the stimulation steps between the *motor threshold* and the maximal response) of unaffected and affected arms (P = 0.73, N = 16; 8 subjects x 2 arms; Figure 13B).

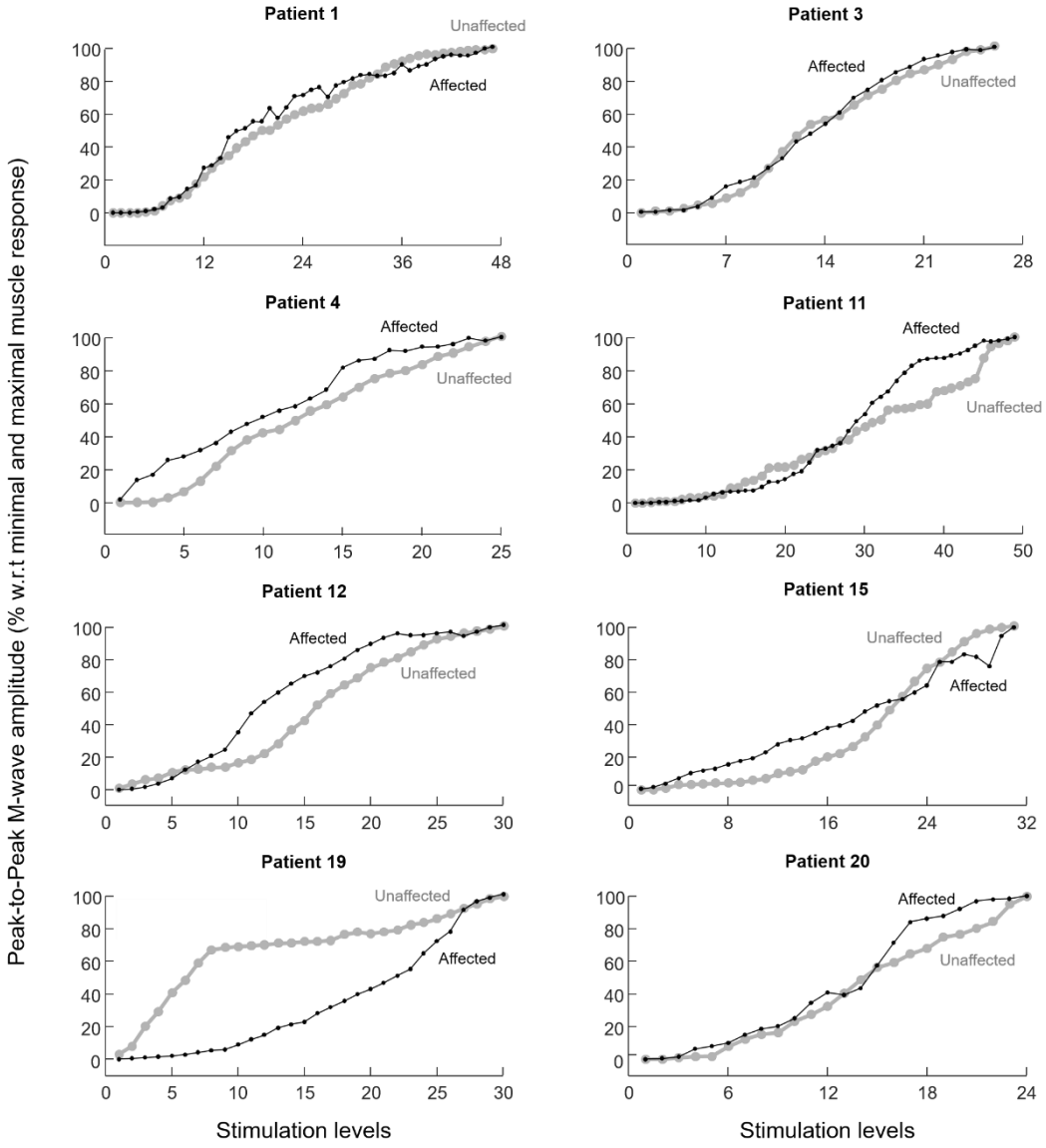

Figure 12: Variations in the normalized M-waves amplitude of both affected (fine black line) and unaffected (thick gray line) muscles of the eight patients evaluated, for relatively, similar increases in current intensity (stimulation levels).

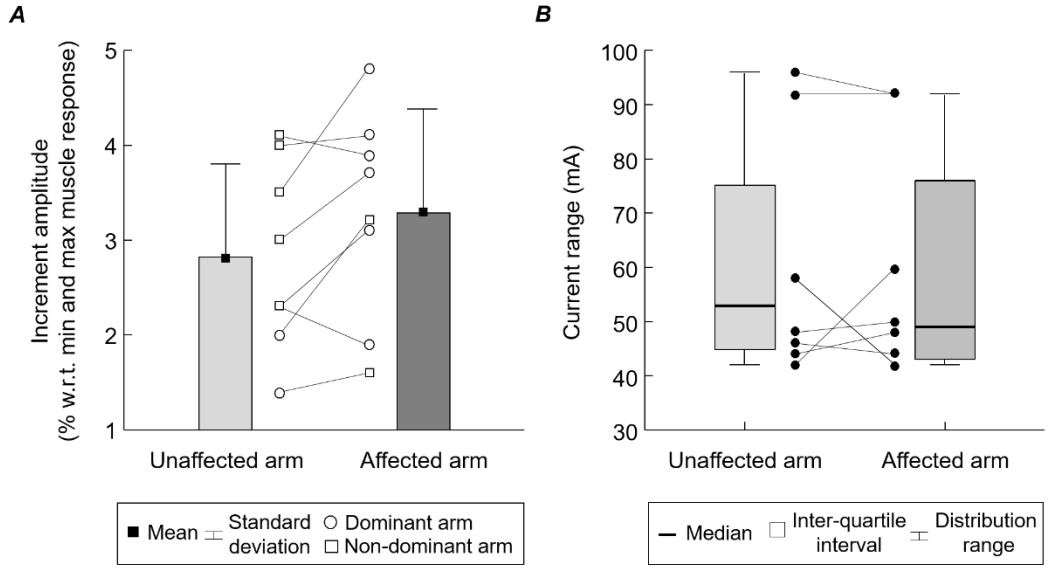

Figure 13: Bars in panel (A) show the median *increment* amplitude calculated from the *increments* between consecutive M waves within the *current range* of unaffected and affected arms of patients. Circles and squares connected through a line correspond to median *increments* for the unaffected and affected arm of the same subject. Circles and squares indicate the dominant and non-dominant arm of patient, respectively. Boxplots in panel (B) show the *current range* within which the amplitude of M waves increased from the smallest to the greatest value. Black circles connected through a line correspond to *current ranges* for the unaffected and affected arm of the same subject. Light and dark gray boxes respectively indicate values for the unaffected and affected arm.

4.3.2 Correlation between side-differences in muscle responses and the time elapsed since the stroke onset

No significant correlation was found between the ratio of median *increments* and the time elapsed since the stroke onset (R = -0.38; P = 0.36; N = 8; Figure 14). Figure 14 denotes that patients whose median *increment* was greater in affected than unaffected muscles (i.e., the ratio of median *increments* was bellow one), showed a great variability with respect to the time elapsed since the stroke onset, ranging from one to 43 months. For the patients whose median *increment* was smaller in affected than unaffected muscles (i.e., the ratio of median *increments* was above one), time since the onset of stroke varied from two to eight months.

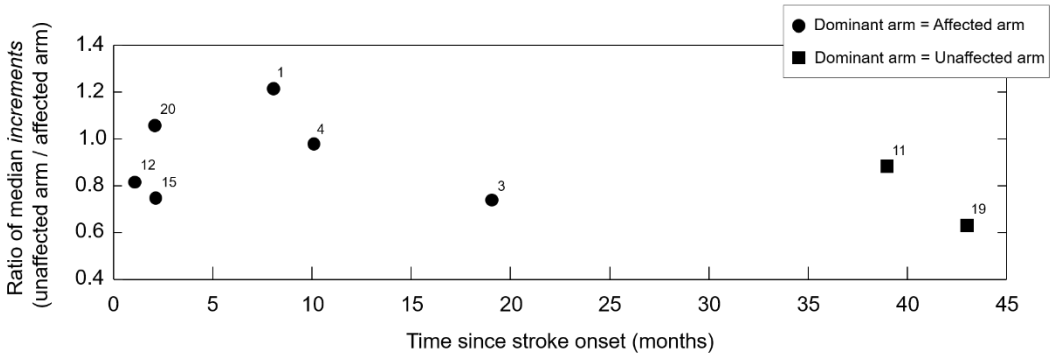

Figure 14: Correlation of the ratio between the median *increments* of the unaffected and affected arms with the time elapsed since the stroke onset (months). Circles and squares indicate whether the dominant arm of the patient was the affected or the unaffected one, respectively. Numbers close to circles and squares refers to the patients' identification (see Table 3).

4.4 Discussion

In this study we investigated whether, during incremental stimulation of the biceps brachii in stroke patients, changes in the amplitude of M wave between consecutive stimulation levels are greater in the affected than in the unaffected side. M waves were recorded through surface EMGs positioned in the distal portion of the muscle, while electrical stimulation was applied in the proximal portion. Current intensity was increased gradually up to the maximal intensity tolerated by each subject. Our findings showed a clear trend towards greater changes in the amplitude of M waves between consecutive stimulation intensities in the affected than in the unaffected arm of most of the stroke patients evaluated.

4.4.1 Preliminary considerations

First of all, to ensure that the biceps brachii muscles evaluated in this study were somewhat affected following stroke, we decided to disregard patients whose Fugl-Meyer (biceps brachii) score was above 90% of the maximum score. Methodological considerations with respect to the experimental protocol applied here are the same discussed for the study of chapter 3 (see subsection 3.4.1). Similarly to this previous study, here we also applied the linear interpolation to match the number of stimulation levels and, therefore, of *increments* within the

current range between the arms of each patient. The interpolation was performed to avoid a possible bias in the results found (Figure 13A) due to distinct number of *increments* between arms (see chapter 3, subsection 3.2.4 for more details) and to ensure like-with-like comparison between affected and unaffected muscles of each patient. Although differences in the *current range* between arms seemed to have been negligible for most of the patients evaluated (for five out of eight patients *current range* differed between arms from 0 to 4 mA), as confirmed by the statistical analysis (cf. Figure 13B), for three patients the difference between arms was about 16 to 18 mA (i.e., eight to nine stimulation levels). By performing the linear interpolation, however, we probably assumed that motor units were recruited between consecutive stimulation levels, which could not be true. Thus, it is possible that median *increments* had been underestimated in patients' arm which stimulus-response curve was interpolated.

4.4.2 Variations in M-waves amplitude differed between biceps brachii of affected and unaffected arms

Structural adaptations in the motor units' size of muscles affected after stroke were indirectly investigated by comparing the size of *increments* in the amplitude of M waves elicited in biceps brachii of affected and unaffected sides of stroke patients. Variations in the M-wave amplitude is usually assumed to reflect a change in the number of motor units activated (McComas et al. 1971) and, therefore, in the number of muscle fibers activated. Assuming that collateral reinnervation takes place in muscles affected after stroke (Martínez et al. 1982; Lukács et al. 2009), we expected that increases in M-waves amplitude, evaluated by means of median *increments*, would be greater in affected rather than unaffected muscles for similar, relative increases in current intensity. In this case, it is reasonable to expect a greater slope of the stimulus-response curve of affected muscles with respect to the curve of unaffected muscles. Indeed, differences in the curve slope and, therefore, in the sizes of *increments* between affected and unaffected muscles were somewhat observed in the stimulus-response curves of most of the patients (cf. Figure 12). For patients 4, 12 and 15, for example, greater *increments* in affected than unaffected muscles seemed to be more evident in the low stimulation levels, while for patients 11, 19 and 20 differences appear in the high stimulation levels. Although statistical analysis did not show significant difference ($p < 0.05$), our findings showed a clear trend towards greater median *increments* in muscles of affected with respect to unaffected arms for most of the stroke patients evaluated (cf. Figure 13A). In other words, increases in M-waves amplitude seemed to have

been progressively larger in affected than unaffected biceps brachii for similar, relative increases in the current intensity. Indeed, *P* value (0.08) was close to the one used to define the statistical significance (*P*=0.05). By increasing the sample of patients, maybe a statistical difference would be observed.

Before interpreting the differences observed between affected and unaffected muscles, a consideration should be made: are these side-differences an outcome of stroke or are they also related to effects of upper-limb dominance on the muscles responses? In our previous study, reported in chapter 3, we performed the same experimental protocol of this study with 16 healthy, young subjects and found significant smaller median *increments* in biceps brachii of dominant than non-dominant arms (cf. chapter 3, subsection 3.3). Hence, to understand whether the side-differences found in the stroke patients may have any relation with dominance, patients were organized in three different groups: (Group 1) the four patients whose dominant arm was the affected one and median *increments* were greater in the affected than unaffected arm; (Group 2) the two patients whose dominant arm was the unaffected one and median *increments* were greater in the affected than unaffected arm; (Group 3) the two patients whose dominant arm was the affected one and median *increments* were smaller in the affected than unaffected arm.

For the four patients in (Group 1), results found for median *increments* agreed with our hypothesis of greater variations in the amplitude of M waves elicited in biceps brachii of affected than unaffected arms. Such results were likely an outcome of stroke, since with respect to dominance, they were contrary to the results found for the healthy subjects (cf. chapter 3, subsection 3.3), i.e., the dominant arm of these patients showed higher median *increments* with respect to the non-dominant arm. Median *increments* were about 0.1-1.3 % greater in affected than unaffected biceps brachii muscles. This finding seems to be consistent with the notion that collateral reinnervation takes place after stroke, increasing the number of muscle fibers per unit (innervation ratio) and, therefore, the magnitude of the muscle responses. It corroborates with previous studies (Martínez et al. 1982; Lukács et al. 2009) which showed higher fiber density in muscles affected after stroke with respect to healthy, unaffected muscles.

For the two patients in (Group 2), it is not clear whether the greater *increments* observed in the affected muscles of these patients may reflect changes in the motor units' size due to stroke or whether they may be a result of side-differences related to dominance. The muscles affected in these patients were those of their non-dominant arms. Thus, greater median *increments* were observed in muscles of non-

dominant that dominant arms, as verified for the healthy subjects evaluated in chapter 3. Due to the reduced number of patients in this group, we could not perform a statistical analysis in order to verify whether the side-differences found for these patients are compared with those found for the healthy subjects. However, comparing the difference between the median *increments* of arms of each patient (cf. Figure 13A) with the average difference found between arms of the healthy subjects (cf. Figure 9A of chapter 3), distinct patterns were observed. For one patient, side-difference (0.2 %) was smaller than the average difference found between arms of healthy subjects (0.7 %), while for the other patient it was higher (1.2 %). A greater sample of subjects would be necessary to verify whether the effect of stroke overcomes the effect of the dominance on side-differences of median *increments*.

For the two patients in (Group 3), results were contrary to our hypothesis and were consistent with the findings for the healthy subjects, about dominance. Median *increments* were 0.2 % and 0.4 % smaller in the affected than unaffected muscles of these patients, with the affected sides corresponding to the dominant arms. These results suggest that a muscle reinnervation process may not necessarily occur for all muscles affected by stroke. Indeed, the inter-subjects variability observed in our findings, with respect to side-differences of neuromuscular responses of stroke patients, was also reported by previous studies, which did not consider the patients' dominance though. Briefly, Kallenberg and Hermens (2009) and Li et al. (2013) analyzed the absolute amplitude values of surface EMGs recorded in muscles of affected and unaffected arms of stroke patients, during isometric contractions performed at different force levels. For part of the patients they evaluated, muscles responses were greater in affected than unaffected sides and, although statistical differences have not been significant, the authors suggested the collateral reinnervation to explain such difference. However, for the other part of the patients, muscle responses were smaller in affected than unaffected sides. Kallenberg and Hermens (2009) suggested that the greater responses observed in unaffected muscles may have been due to an increase in fiber diameter on unaffected muscles as a consequence of overuse. While Li et al. (2013) suggested the muscle fiber atrophy as a possible reason for the smaller responses observed in affected muscles. In our study, however, differences between the muscle fibers' diameters in affected and unaffected biceps brachii do not explain the results we found, since M-waves amplitude were normalized with respect to the minimal and maximal muscle responses, compensating the effect of anatomical differences between arms on the surface EMGs (Farina et al. 2002).

Besides dominance, we also verified the relationship between the time elapsed since the stroke onset of patients and side-differences in muscle responses, evaluated by means of the ratio between median *increments* on unaffected and affected muscles (ratio of median *increments*). No correlation was found between the ratio of median *increments* and the time elapsed since the stroke onset of the patients evaluated (Figure 14), probably due to the reduced sample of subjects and the variability among them. However, observing the individual patient results (cf. Figure 14), for two patients whose results indicated a muscle reinnervation have occurred after stroke (patients 12 and 15), the time elapsed since the stroke onset ranged between one and two months, suggesting muscle reinnervation may be already observed after few months following the stroke onset. Such assumption corroborates with Martínez et al. (1982) and Lukács et al. (2009) findings, which suggested that motor unit reorganization following stroke already occurs in the first months after the stroke onset. Briefly, the authors of both studies evaluated the fiber density of hand muscles affected in stroke patients, through single-fiber EMG. Martínez et al. (1982) found increased fiber density in patients with only two months since the stroke onset. Lukács et al. (2009) observed that for patients with less than ten months from the stroke onset, there was a gradual increase in the muscle fiber density as patients had more time elapsed since the stroke onset.

4.4.3 Physiological implications

Notwithstanding the reduced sample of patients evaluated in this study, we verified a trend towards greater variations in the amplitude of M waves elicited in affected than unaffected biceps brachii. Although our findings are not conclusive, they seem to provide evidence of changes in the organization of the neuromuscular system following stroke, specifically of increases in the innervation ratio of muscles affected after stroke likely due to muscle fiber reinnervation. Such possible increase in the motor units' size in muscles affected after stroke, however, would imply that the surviving motoneurons may produce higher force values than before stroke. Focusing attention on patients whose variations in M-waves amplitude were greater in affected than unaffected biceps brachii, the maximum side-difference in median *increments* was 1.3 % (cf. Figure 13A). Such a value can be considered as indicative of the greater amount of additional motor units elicited in the affected muscle with respect to the unaffected muscle for similar, relative increases in the stimulation intensity. In this hypothesis, if we translate this side-difference in terms of incremental force, biceps brachii of affected arms would produce greater relative force increments with respect to unaffected arms. Hence, the collateral

reinnervation process following stroke may hinder the ability of stroke survivors to produce increments in force as finely as healthy muscles.

4.4.4 Study limitation

Given that previous findings suggest that after stroke the number of motor units may be on average 20-60% smaller in affected than unaffected muscles (McComas et al. 1973; Hara et al. 2004; Li et al. 2011, 2014a; Kouzi et al. 2014), one could expect a much clear difference in the M-waves responses between affected and unaffected biceps brachii evaluated in this study. The subtle difference we observed between the median *increments* of affected and unaffected muscles (Figure 13A) may put in doubt whether the stimulus-response curve analysis we performed is effective to assess structural adaptations of muscles affected after stroke due to a collateral reinnervation process. Before drawing such conclusion, however, an important consideration must be highlighted: we do not know the degree of collateral reinnervation in the biceps brachii muscles affected in the patients we evaluated. If the number of motor units in a muscle is reduced in 50% after stroke, for example, this does not mean that all the muscle fibers denervated in this muscle would be reinnervated by the survivors motoneurons. Perhaps only part or none of the muscle fibers denervated are reinnervated. In our opinion, such uncertainty with respect to the occurrence of a reinnervation process in the muscles evaluated is the main limitation of the present study and, probably, of previous studies which also investigated alterations in the neuromuscular system following stroke (Kallenberg and Hermens 2009; Li et al. 2013). Indeed, as commented in the discussion, such studies as well as ours found controversial results among the patients assessed, which may be associated with different degrees of a reinnervation process in muscles affected after stroke. Therefore, more investigation should be necessary in order to verify the effectiveness of stimulus-response curve analysis performed in the present study.

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