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Study of brain plasticity in upper limb amputees with non-invasive brain stimulation techniques

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INTRODUCTION

After the amputation of the upper limb, there is an important reorganization of the primary sensory-motor cortex that can also affect the higher-order and associative sensory-motor areas and inter-hemispheric communication. The reorganization of the primary somatosensory and motor cortex is characterized by a reduction in the expansion of the representative maps of the lost muscles and skin areas and an expansion of the representation of the adjacent body areas.

Despite this reorganization, the "orphan" cortical areas maintain silent connections with the periphery, that can be explored.

In humans, adaptations of the motor cortex after amputation can be studied using non-invasive electrophysiological techniques.

EPIDEMIOLOGY AND ETIOLOGY OF AMPUTATIONS

The loss of a body part can greatly impact a person's life. Despite the advances in conservative methods and antibiotic prophylaxis which have reduced the indications for surgery, the increase in the average lifespan of the population and the considerable incidence of chronic-degenerative diseases of the cardio-vascular system have shifted the main causes of amputation from traumatic to vascular causes (World Health Organization 2004). **Fig.1**

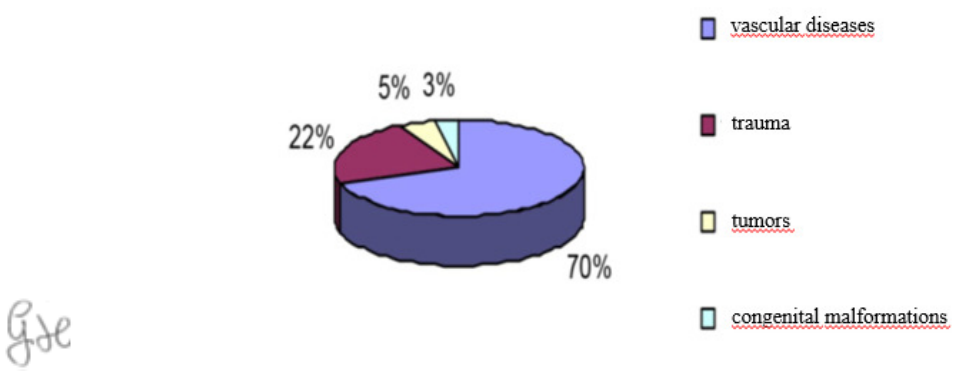


Fig.1 Etiology Amputations. Data from the Ministry of Health 2011.

In the United States, there are approximately 1.7 million people living with limb loss. (Kathryn Ziegler-Graham et al.2008). It is estimated that one out of every 200 people in the U.S. has had an amputation (Patricia F. Adams, et al 1999). The etiology of upper or lower limb amputation is different: in the lower limb, the primary cause is diabetes and peripheral artery disease, occurring in 45 out of 100,000 individuals, most affecting minority individuals (Dillingham et al. 2202; Lefebvre et al. 2011). The amputation of upper limb is much less frequent and affects approximately 41,000 people, or about 3% of the U.S. amputated population. The primary reason for upper limb loss in adults is trauma, followed by cancer (Adams, et al 1999). Other causes of upper limb loss include infections, burns and congenital deformities.

Thanks to modernization processes and industrial work safety laws, rates of traumatic amputations have decreased over the past four decades. Trauma-induced upper limb amputations occur with a frequency of 3.8 individuals per 100,000; finger amputations are the most common (2.8 per 100,000). Trauma hand amputations occur at a rate of 0.02 per 100,000. (Dillingham et al. 2202). Excluding finger amputations,

trans-radial (forearm) and trans-humeral (arm) traumatic amputations are the most common upper limb amputations.

Analysis of the causes of trauma, from the National Trauma database between the years 2000 and 2004, shows that car accidents are the main cause of upper limb amputations compared to lower limb amputations. Machinery, power tools (involving saws or blades), explosions, self-inflicted injuries and assaults are among the most common reasons for traumatic upper limb amputations (Barmparas et al.2010). Men are far more at risk of traumatic amputation than women, demonstrating about 6.6 times the female rate for minor finger and hand amputations (Khurram MF et 2015; Pomares G et al.2018).

Due to conflicts, the number of amputations due to explosive devices has increased, becoming the main reason for the loss of upper limbs in the military (Armstronga et al. 2018). As of July 2011, 14% of major limb losses have suffered in Operation New Dawn, Operation Iraqi Freedom, and Operation Enduring Freedom involved the upper extremity. Between October 1, 2001 and July 30, 2011, there were 225 active soldiers who had their upper limbs amputated. Of these 225, 11 (7%) were bilateral upper limb amputees.

Elbow disarticulations were less common (2.1%) than transradial amputations (47%) (Stuart L et al. 2019). Electric burn is a rare cause of upper limb amputation. Heating causes coagulation necrosis and the passage of electric current through the tissues causes cell membranes to rupture (Sofić et al. 2016).

There are about 3,500 and 5,200 upper limb amputations reported each year in Italy (http://www.salute.gov.it/ricoveriOspedalieri/ric_informazioni/default.jsp) and in the United Kingdom.

This thesis focuses on brain plasticity after upper limb amputations.

Upper limb amputation can be classified as follows:

Minor Upper Extremity Amputations:

- Partial hand amputation or removal of any portion of the hand is a minor upper extremity amputation.

Major Upper Extremity Amputations:

- Wrist disarticulation (separation of the radius from the proximal carpals or separation between the proximal and distal row of carpals).
- Trans-radial amputation (through the radius and ulna), previously known as below-elbow.
- Elbow disarticulation (separation of the humerus from the ulna or amputation through the most distal portion of the humerus).
- Trans-humeral amputation (through the humerus), previously known as above-elbow.
- Shoulder disarticulation (separation of the humerus from the scapula).

- Forequarter (removal of any portion of the thorax, together with any portion of the shoulder girdle and all distal parts). (Cordella et al, 2016). **Fig.2**

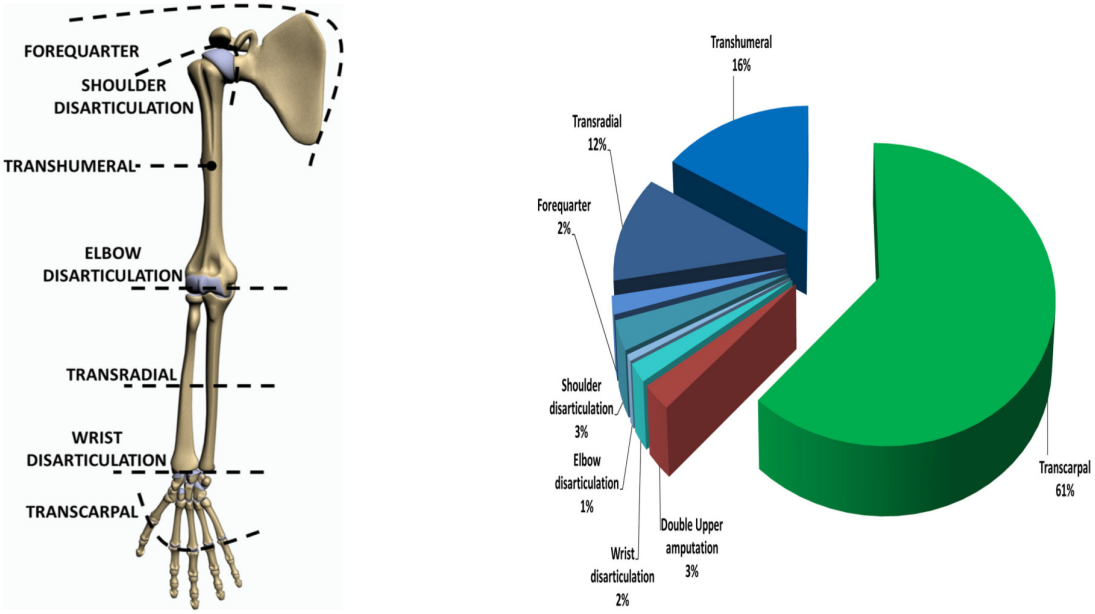


Fig.2

Phantom Limb Syndrome

Phantom limb syndrome (PLS) is very common after amputation (Ramachandran et al. 1993). PLS is characterized by many symptoms that share the perception that the limb is still present; pain is a very frequent symptom of this syndrome, being reported in 50-80% of all amputees (Flor 2002). The pain can manifest itself with different sensations which include burning, gnawing, tearing, pressure and pain. Among the

hypotheses formulated to explain the phenomenon are three main neuroanatomical theories which include peripheral neuromas, hyperexcitability of pain afferents in the dorsal horn, and somatosensory cortical reorganization.

Brief history of prosthetics

The early use of prosthetics goes back to at least the fifth Egyptian Dynasty that reigned between 2750 to 2625 BC. The oldest known splint was unearthed by archaeologists from that period. The earliest known record of a prosthesis being used by man was made by the famous Greek historian, Herodotus. His classic "History," written about 484 B.C., contains the story of the Persian soldier, Hegistratus, who, when imprisoned in stocks by the enemy, escaped by cutting off part of his foot, and replaced it later with a wooden version. One of the earliest known prostheses was fabricated of copper around 300 BC. These early attempts at prosthetic management predate early surgical considerations for lifesaving reasons by many decades. Ambrose Pare (1510–1590), whom many consider the father of modern orthopedic surgery, contributed significantly to the advancement of amputation surgery. It is believed that Pare performed the earliest upper extremity amputation, an elbow disarticulation, late in 1536. In 1863, Dubois L. Parmelee of New York City invented a way to attach prostheses using atmospheric pressure. In 1961 Johannes Schmidl, an austrian technician at the Inail Prosthesis Center of Vigorso di Budrio, invented the first myoelectric prosthesis. In the middle of the 20th century that major

advancements were made in the attachment of lower limbs. New advances to keep an eye involve the growing use of 3-D printing, which has allowed for the fast, precise manufacturing of artificial limbs that traditionally have been custom-built by hand.

Prosthetic rejection rates are high among upper limb amputees (Salminger et al. 2020). The shape of the prosthesis depends on the number of residual muscles and joints available but although a longer abutment offers a better mechanical advantage for prosthetic use, the length of the abutment does not always correspond to an increase in prosthetic function.

Rehabilitation for Persons With Upper Extremity Amputation

Following an upper limb amputation, complex rehabilitation pathways are needed, in equipped facilities and with a multidisciplinary team that includes therapists, prosthetists and doctors with specialized knowledge and experience. Patients must be effectively transferred from the hospital post-surgical unit, sometimes to a hospital rehabilitation unit to an outpatient long-term prosthetic and rehabilitation program. Proper rehabilitation and a comfortable and functional prosthesis will facilitate the functional restoration and quality of life of this condition that often afflicts young, professionally productive people, mainly men. The INAIL Prosthesis Center of Vigorso di Budrio, the main one in Italy and one of the most important in Europe, is an integrated prosthetic-rehabilitation treatment model by virtue of which complete and highly qualified services are provided to the assisted for the recovery andreintegration into relationship life.

Brain plasticity

Neurobiological basis of synaptic plasticity

Brain plasticity is the ability of the brain to change its function and structure in response to experience and the external environment. One of the main mechanisms on which this phenomenon is based is represented by synaptic plasticity, consisting in the possibility of modifying the efficiency and the number of synaptic connections between neurons.

Synaptic plasticity is considered the cellular correlate of many cognitive processes, including learning and memory.

The main mechanisms of synaptic plasticity are represented by long-term potentiation (LTP) and long-term depression (LTD). The term LTP refers to a long-term increase in synaptic transmission between two neurons which is obtained, experimentally, by repeated high-frequency stimulation of the presynaptic axon. This physiological process is characterized by a series of molecular modifications of the function and microstructure of the synaptic junction (Kandel et al. 2003).

The phenomenon of LTP was described for the first time in 1973 by Bliss and Lomo (1973) who showed that the application of a short discharge of high frequency stimuli to the afferent pathways of the hippocampus, determines an increase in the amplitude of the excitatory postsynaptic potentials which may last a few hours or even days.

To induce LTP, the postsynaptic membrane must be depolarized in the time interval in which the pre-synaptic terminal releases glutamate. During normal low-frequency synaptic transmission, glutamate is released from the presynaptic terminations and acts on both NMDA and non-NMDA receptors. Non-NMDA receptors are of the AMPA type. The Na + and K + ions, however, only pass through the non-NMDA channels and not into the NMDA ones because, at the resting membrane potential, the NMDA channel receptors are blocked by Mg2 +.

When the postsynaptic membrane is depolarized by the action of non-NMDA channel receptors, the depolarization removes the blockage of Mg2 + ions from NMDA channels. This allows for the flow of Ca2 + through these channels. The resulting increase in Ca2 + at the level of the dendritic spines activates the Ca-dependent kinases and the Fyn tyrosine kinase, whose joint action determines the appearance of LTP. Ca-dependent kinases phosphorylate non-NMDA channel receptors and increase their sensitivity to glutamate, also activating other normally inactive channel receptors. These modifications provide a valid postsynaptic contribution to the maintenance of LTP. Furthermore, when LTP has been induced, it is believed that the postsynaptic cell releases a group of retrograde messengers, one of which could be nitric oxide, which act on the kinases present in the presynaptic termination, followed by a persistent increase in the release of neurotransmitter that allows the maintenance of the LTP (Kandel et al. 2003).

The term LTD refers to an activity-dependent reduction in the effectiveness of the synapses, lasting hours or more, following prolonged synaptic activation, generally by means of low-frequency stimulus trains.

LTD occurs in many areas of the central nervous system with different mechanisms (Massey and Bashir 2007). The neurotransmitter most involved in LTD is glutamate. LTD is believed to result mainly from a decrease in post-synaptic receptor density, although decreased presynaptic neurotransmitter release may play a role in this phenomenon. It has been hypothesized that cerebellar LTD is important for motor learning while hippocampal is important for memory (Malleret et al. 2010; Nicholls et al. 2008).

LTD is one of the many processes that is used to selectively reduce the efficiency of specific synapses and maintain a homeostatic balance within the system. In fact, an excessive level of LTP could inhibit the encoding of new information (Purves, 2012).

Activity-dependent plasticity phenomena (LTP and LTD) were mainly evaluated by in vitro studies. Recently, the introduction of transcranial magnetic stimulation (TMS), a non-invasive electrophysiological stimulation technique, has made it possible to study similar mechanisms in the human brain, using repetitive TMS (rTMS) protocols similar to those used to induce changes in synaptic activity in experimental preparations. (Cooke and Bliss, 2006).

In this manuscript, we refer to the hemisphere contralateral to the amputated limb as the "affected hemisphere" (AH) and to the hemisphere ipsilateral to the amputated limb as the "unaffected hemisphere" (UH). This definition is based on the assumption that the hemisphere contralateral to amputation is directly affected from sensory deafferentation and motor deafferentation.

Brain plasticity phenomena associated with upper limb amputation

Plasticity is often a phenomenon triggered by environmental factors but it can also be the adaptive response to pathologies that directly affect the central nervous system (CNS), such as stroke, or the peripheral nervous system, as in the case of amputations.

In physiological conditions, the constant flow of information directed to the motor and sensory cortex, both from the environment and from specific training, modulates their function (Butefish et al. 2000; Vahdat et al. 2011). After the amputation of a limb and therefore the loss of muscles and tactile and proprioceptive feedback, this exchange of information to and from the periphery is lost, resulting in loss of information by the primary motor cortex (M1) and primary somatosensory cortex (S1).

After the amputation of the upper limb, studies on animal models, such as rats and primates and on humans, using non-invasive neurophysiological methods and

neuroimaging techniques, have shown a reorganization due to neuroplastic phenomena of the motor and somatosensory cortical areas.

Gse

Evidence in animal models

Studies on rats with upper limb or motor nerve amputation with microstimulation of the de-efferented cortical areas, have led to movements in muscles other than those previously controlled by the same area showing their expansion into the de-efferented cortex (Sanes et al. 1988, 1990; Donoghue et al. 1990).

Studies on upper limb amputation in primates showed that stimulation of the de-efferented portion of M1 of AH, produced contraction of preserved proximal muscles and body parts represented in adjacent cortical areas, such as facial muscles (Wu & Kaas 1999; Qi et al. 2000). Replicating the studies on elderly macaques and with long-standing upper limb amputations, these results were replicated by showing that the re-adaptation phenomenon is not exclusive to young age and does not depend on the time elapsed since the amputation (Qi et al. 2000).

As for the sensory areas deprived of afferent information, it has been shown that they become responsive to the tactile stimulation of the stump and adjacent regions, and at the basis of this it is believed that there is a complex reorganization of cortical and subcortical circuits (Pons et al. 1991; Wu & Kaas 1999).

Evidence in humans

In humans, the adaptations of the motor cortex after the amputation can be studied using non-invasive brain stimulation techniques, in which TMS plays a particularly important role. Through TMS cortical maps, usually by stimulating a grid of points spaced 5-10 mm, it is possible to obtain a map of the cortical motor representation of each muscle examined (Wilson et al. 1993). As observed in animals, studies on amputated subjects have been show the expansion of the muscles of the face and of the stump, in the AH : this pattern of reorganization has been observed in amputations both in childhood (Hall et al. 1990) and adulthood (Cohen et al. 1991; Pascual-Leone et al. 1996) and even more than 20 years after the amputation (Röricht et al. 1999), supporting the hypothesis that such phenomena are not exclusive to young age, even if they are morepronounced during the maturation phase of the CNS. Furthermore, the cortical areas corresponding to the stump muscles are hyperexcitable, having wider responses and lower TMS activation thresholds.

The function of the somatosensory cortex can be studied by stimulating the afferent pathways and recording cortical activity using electrophysiological and neuroimaging techniques with different spatial and temporal resolution. Electroencephalography (EEG), magnetoencephalography (MEG) and somatosensory evoked potentials (SEP) that have a high temporal resolution (in the order of 1 ms) and low spatial resolution (in the order of 1 cm or more). Conversely, positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), being sensitive to brain metabolic

activity, have a low temporal resolution (in the order of a few seconds or minutes), while they can provide accurate spatial localization by superimposition with MRI images. The combination of different investigation methods therefore allows you to obtain real-time information on the physiological processes that occur within the CNS with sufficient spatial precision.

With the combination of these techniques, it could be understood that the somatosensory cortex is also subject to plastic changes related to the interruption of afferent transmission. Two of the first studies conducted on adults with upper limb amputation (Elbert et al. 1994; Yang et al. 1994), which made use of the "magnetic source imaging" technique, that is a combination of MEG and MRI data to create functional maps of cortical activation, report that the topographical representation of the facial area within S1 is displaced towards the area that normally receives information from the nerves connected with the hand and fingers. In a PET study of two adults with upper limb amputation above the elbow (Kew et al. 1997), sensory stimulation at the thoracic level was found to activate the S1 area pertaining to the arm and hand. Furthermore, some data indicate that S1 reorganization processes in humans begin very early, in less than 24 h, after amputation (Borsook et al. 1998).

It is very important to emphasize that, despite the reorganization processes described, various studies indicate that the "orphan" cortical areas maintain connections, even if not active, with the periphery, preserving the memory of the motor and sensory functions of the AH. This is evidenced by the fact that stimulation of the dissected

nerve can evoke a cortical component of the SEP in S1 long after the lesion (Mackert et al. 2003). Furthermore, TMS is able to produce phantom limb movements even in subjects who are unable to voluntarily generate them (Mercier et al. 2006), while voluntary phantom hand movements generate a specific pattern of electromyographic activity in the stump muscles (Reilly et al. 2006).

Role of brain plasticity in phantom limb syndrome

Studies aimed at distinguishing between patients with and without PLS suggest that extensive reorganization of cortical function occurs only in the former, while adaptations are much less pronounced in patients without PLS (Flor et al. 1995, 1998; Montoya et al. 1998). A greater expression of plasticity phenomena in M1 was also observed in patients with phantom limb pain (Kew et al. 1994; Pascual-Leone et al. 1996; Karl et al. 2001). In line with these data, subjects with amelia, who generally do not report phantom limb sensations, do not show substantial cortical asymmetry (Reilly & Sirigu 2011). The possibility of interfering in various ways (prosthetic replacement of the amputated limb, restoration of sensory afferents) with aberrant mechanisms of neuroplasticity can also favor the improvement of the symptoms of PLS. As part of a previous experiment conducted at Campus Bio-Medico laboratories on the use of a peripheral neural interface for the control of a robotic hand prosthesis, the motor training associated with the restoration of sensory feedback for the duration of one month determined a significant improvement in PLS (Rossini et al. 2010).

Effects of amputated limb function restoration

After the amputation of the upper limb, we can have maladaptive form of brain plasticity. TMS studies carried out after cadaveric hand transplantation (Röricht et al. 2001; Vargas et al. 2009) showed a physiological reorganization of cortical maps and a reduction of the areas that had previously expanded, supporting the hypothesis that a reintegration of information afferent and efferent can lead to a "functional" recovery thanks to brain plasticity.

The results described seem to confirm the reversible nature of the plastic changes that occur within the sensori-motor cortex in association with the restoration of the efferent and afferent pathways (Chen et al. 2013). In two recent cases of implantation of peripheral intraneural interfaces for the bidirectional control of a robotic hand prosthesis, studied at Campus Bio-Medico, sensory feedback restitution allowed to obtain a complex perception of the shape and consistency of the objects and an improvement grip capacity (Raspopovic et al. 2014); moreover, motor training resulted in a reduction of symptoms of PLS, associated with the normalization of cortical topography, as evidenced by the mapping using TMS (Rossini et al. 2010), by the EEG recording (Di Pino et al. 2012) and by the TMS-EEG co-registration (Ferreri et al. 2014).

The study of the cortical reorganization of the CNS after amputation can therefore provide information on the effectiveness of an active type prosthesis in promoting the restoration of the hand physiological function and in reducing aberrant forms of plasticity (Di Pino et al. 2009). Recent data also suggest that adaptations resulting from upper limb amputation are not limited to the primary motor and sensory areas, but are associated with a large cortical reorganization that includes higher-order and associative sensory-motor areas (Makin et al. 2015) and inter-hemispheric communication (Di Pino et al. 2012). This could constitute the neurobiological basis of complex cognitive processes in which a key role could be played by sensory feedback, such as those at the origin of the symptoms of the phantom limb and the sense of "embodiment" of the hand prosthesis (Di Pino et al. 2020; Di Pino et al. 2014).

Non- invasive Brain Stimulation Techinques

Background

The first studies of cortical stimulation in animals began at the end of the 19th century, thanks to the studies of Fritsch (1870) and Ferrier (1876). Later Bartholow (1874) but especially Penfield and Jasper (1954) identified the distribution of the various cortical motor areas in humans through electrical stimulation during neurosurgery.

At the same time Gualtierotti and Peterson (1954) by applying trains of electrical stimuli on the intact scalp obtained contralateral motor responses to the stimulus but with enormous dispersion of the electric current and high pain for the patient.

Later Merton and Morton (1980) applied a single high voltage discharge that allowed effective penetration of the current into the brain tissue with a small leakage. Cohen and Hallet (1988) using this method of transcranial electrical stimulation (TES) reproduced Penfield's motor homunculus. Although it is painful for the subject, it is still today a valid brain stimulation technique used for neurophysiological studies.

It was Barker and collaborators who in 1985 developed and used the electromagnetic stimulator. In fact, it was understood that, like an electrical stimulus, even a transient magnetic field of short duration is able to activate the cerebral cortex. Thus a new method became available which, in addition to retaining the advantages of transcranial electrical stimulation, allows to stimulate the brain areas in an even simpler and less invasive way. For these reasons, the TMS has undergone a great development in the following years, being still widely used both for diagnostic purposes in pathologies of the cortico-spinal system, and for neurophysiological investigations.

Even more recently, techniques of repetitive transcranial magnetic stimulation (rTMS) or transcranial stimulation with direct current (tDCS) have been widely used, capable of determining lasting effects on brain functions by inducing neuroplasticity phenomena.

Transcranial electrical stimulation

Transcranial electrical stimulation (TES) is made by applying two subcutaneous needle electrodes to the scalp. Stimulation is defined anodic or cathodic in relation to the electrode, respectively the anode or the cathode, positioned in correspondence of the area to be stimulated. For example, the anodic electrical stimulation of the motor area involves positioning the cathode at the vertex and the anode 7 cm laterally with respect to the cathode. Stimulation is generally conducted using stimuli lasting 100-200 μs and intensity up to 3-4 mA. TES preferentially activates the axon of pyramidal cells (Di Lazzaro et al., 2004a). This technique can be considered minimally invasive and, with the highest stimulation intensities, can be associated with a slight painful sensation, linked to the resistance of the structures of the scalp to the transmission of electric current.

Transcranial magnetic stimulation

Transcranial magnetic stimulation (TMS) is based on the principle of mutual induction between electric and magnetic fields. It is made by placing on the scalp, on the motor area, an electromagnetic coil that delivers an alternating current generated by a capacitor. This produces a time-varying magnetic field lasting 100-200 μs. The intensity of the magnetic field generated is about 2 tesla, a value corresponding approximately to 40,000 times the Earth's magnetic field or, approximately, to that used in magnetic resonance. The magnetic field in turn induces a flow of current in the nervous tissue sufficient to produce neuronal depolarization. **Fig.3-4**

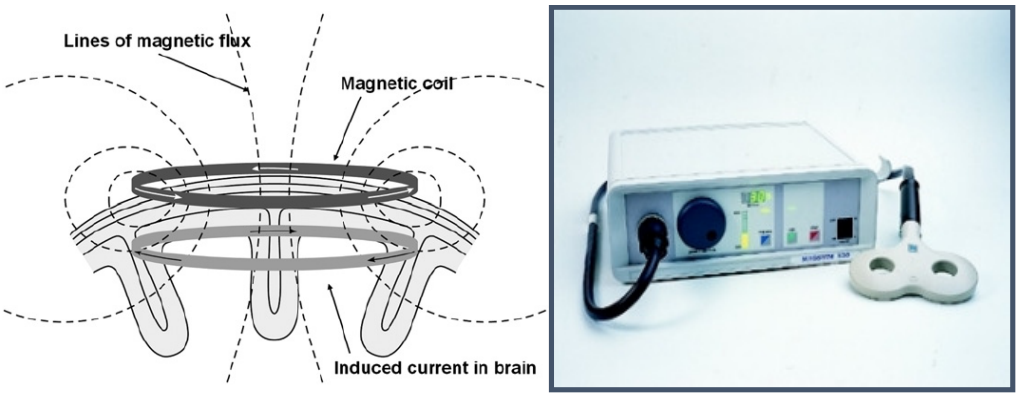


Fig.3 : Mutual electro-magnetic induction in TMS (Hallett 2007)

Fig.4 : Magnetic stimulator

Unlike the electrical stimulus, the magnetic field is not influenced by the high impedance of the structures of the scalp and therefore does not cause nociceptive sensations. However, it is important to underline that the effects of TMS are not a direct consequence of the applied magnetic field, but they derive from the electric field induced in the nervous tissue causing neuronal depolarization.

Unlike TES, which preferentially activates the axon of pyramidal cells, TMS predominantly activates the pyramidal cells by transynapic connections.

The orientation of the coil on the scalp, determining the direction of the induced current, also affects the pattern of neuronal activation.

Main parameters for evaluating the effects of TMS

Motor evoked potential (MEP).

The activation of the pyramidal cells of the motor cortex determines a muscular response, the motor evoked potential, which can be recorded using surface electrodes. This response consists in a variation of the potential difference, which reflects the simultaneous depolarization of all the activated muscle cells. The muscular response evoked by the magnetic stimulus depends on cortical excitability, subcortical structures, and the spinal cord. The main parameters evaluated are latency, depending on to the conduction speed along the cortico-spinal pathways of large diameter and along the peripheral motor pathways, and the amplitude, related to the number of activated motor units.

Motor threshold.

Resting motor threshold (RMT) was defined as the minimum stimulus intensity producing a miminal MEP, about 50 μV in 50% of 10 trials, at rest. The active motor threshold (AMT) is defined as the minimum stimulus intensity producing a miminal MEP (about 200 mV in 50% of 10 trials) during isometric contraction of the tested muscle at 20% of maximum voluntary strength. Because muscle contraction increases the probability of spinal motor neuron firing, AMT is lower than RMT.

Cortico-spinal volleys.

Direct recording of cortico-spinal activity is possible in patients with an epidural electrode, at the level of the cervical or dorsal cord, for the treatment of chronic pain. The activity that is recorded at the level of the cortical spinal tract is a more complex

discharge than the MEP recorded from the muscle, and it consists of a train of descending waves 1.5 ms apart, therefore at high frequency. In relation to coil orientation of stimulation intensity, it is possible to evoke an early wave called **D wave,** expression of the direct activation of the corticospinal axon, and a series of waves called **I waves,** expression of the transynaptic activation of pyramidal neurons (Di Lazzaro et al., 2004a). This type of recording, although limited to a very small number of subjects, has the advantage of allowing the direct recording of the output of the motor cortex, not influenced by changes in spinal excitability. **Fig. 5**

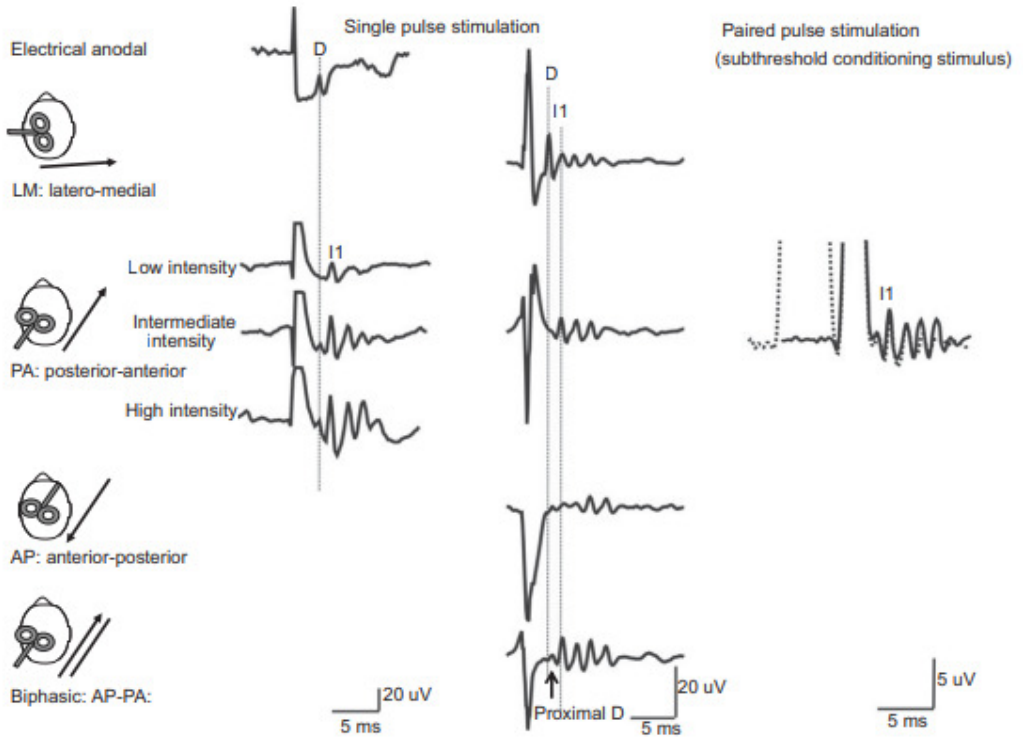


Fig. 5 : Descending volleys evoked by electrical and magnetic stimulation and by paired pulse magnetic stimulation (from Di Lazzaro V. and Rothwell JC 2014 - with permission)

Main clinical and experimental uses of TMS

Diagnosis of pathologies affecting the pyramidal pathways.

MEP recording allows to calculate the central motor conduction time (CMCT), by stimulating the motor cortex and the paravertebral regions. Therefore, MEP allow to detect conduction delays along the central motor pathways (Di Lazzaro et al. 1999a; Chen et al. 2008). **Fig. 6**

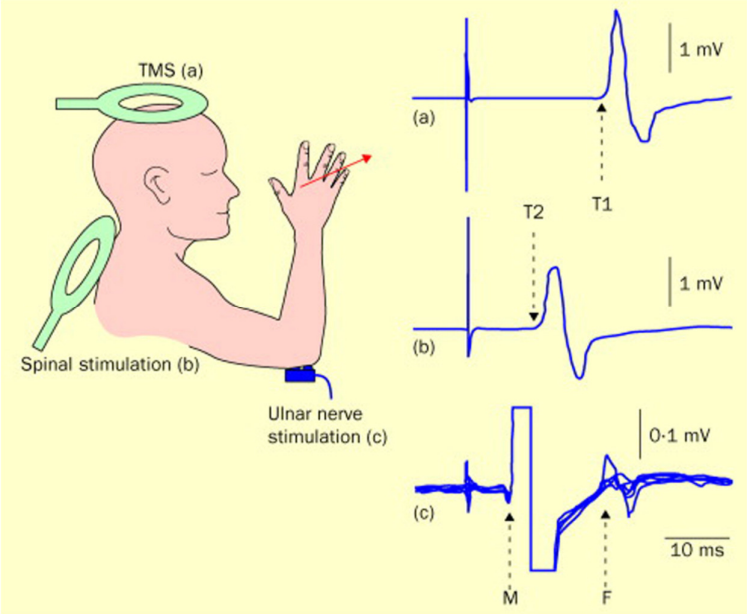


Fig. 6 (from Kobayashi and Pascual-Leone, 2003 – with permission). Schematic representation of the calculation of central motor conduction time (CMCT) (from Kobayashi and Pascual-Leone, 2003 – with permission). Motor evoked potential induced by TMS. (b) MEP after cervical spinal root stimulation. (c) F-waves after ulnar nerve electric stimulation. CMCT is estimated by onset latency of T1 minus onset latency of T2. By use of F-wave latency CMCT can be estimated more precisely as T1 – (F + M-1/2) . T1 = onset latency of MEP elicited by TMS; T2 = onset latency of MEP elicited by the coil placed on the back of cervical spine.M = onset latency of M-wave by electrical ulnar nerve stimulation. F = onset latency of F-wave by electrical ulnar nerve stimulation.

Function of specific intracortical circuits.

In the experimental studies, the amplitude of the MEPs and cortico-spinal volleys provides a estimate of the excitability of the motor cortex. Furthermore, paired stimulation protocols allow the study of specific intracortical circuits and have made it possible to clarify many aspects relating to the physiology of the excitatory and inhibitory phenomena of the motor cortex (Di Lazzaro et al. 2003a).

Modulation of cortical functions.

Instead of single stimuli or pairs of stimuli, it is possible to deliver a series of magnetic stimuli, with a specific temporal succession, on a single point of the scalp, in the form of repetitive transcranial magnetic stimulation (rTMS). RTMS is essentially based on the same principles as traditional TMS. From a technical point of view, rTMS has been made possible by the introduction of appropriate stimulators capable of delivering sequences of stimuli in rapid succession (current repetitive stimulators can reach stimulation frequencies of 100 Hz). The potential of this stimulation method consists in the possibility not only of activating certain neuronal populations, but of modulating their activity for a prolonged time, similar to what obtained with protocols used for the induction of plasticity in vitro. This has led in recent years to evaluate its possible therapeutic applications in the neurological and psychiatric disorders.

Study of cognitive processes.

It has been shown that by TMS, in particular with high stimulation frequencies and intensities it is possible to induce a temporary disruption of the function of certain areas of the cerebral cortex. Protocols aimed at determining a disruption of specific cortical functions have been used in numerous neuropsychological studies and have made it possible to better clarify the role of the areas involved in particular cognitive tasks (Jahanshahi and Rothwell 2000; Siebner et al. 2009).

Study of cortical excitability modifications

General principles of paired-pulse TMS protocols

For the study of intra-cortical inhibition and facilitation in healthy subjects and in patients, it is possible to use different paradigms of paired conditioning-test stimulation (CS-TS) in which the modulatory effect of the conditioning stimulus on the motor response of the test stimulus depends on the intensity stimulation of both, on the interstimulus interval (ISI) and on the level of muscle contraction. **Fig. 7.**

We can study different brain circuits as summarized in **Fig. 8**. The target muscles are usually at rest, as their contraction could alter the result, for example contraction results in significant reduction of short interval intracortical inhibition (SICI) (Ridding et al., 1995b) and EMG monitoring is always recommended. To calculate the intensity of the conditioning stimulus, we can try different levels of intensity, or use percentages

calculated on the thresholds at rest or in activation. The TS intensity is set typically at amplitudes of 0.5–1 mV for hand muscles or 110–120% of resting MT. Using a test stimulus that produces MEP at the middle value of the Input–Output curve is optimal and allows equal amounts of inhibition and facilitation (Kukke et al., 2014).

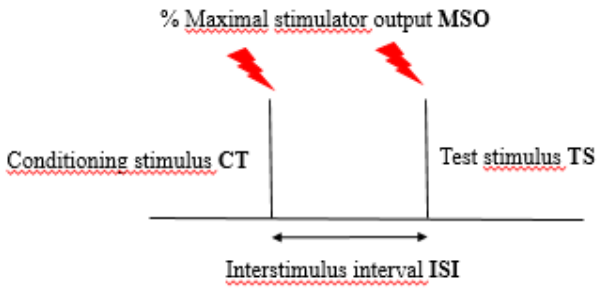


Fig.7

Summary paired-TMS methods.

Method	Cortical circuit								
	SICI	LICI	SICF	ICF	SIHI	LIHI	SAI	LAI	CBI
Conditioning/first stimulus	Sub-threshold TMS	Supra-threshold TMS	Supra-threshold TMS	Sub-threshold TMS	Supra-threshold TMS–contra M1	Supra-threshold TMS–contra M1	Median nerve ES	Median nerve ES	Sub-threshold TMS-contra cerebellum
Test stimulus/second stimulus to M1	Supra-threshold	Supra-threshold	Sub-threshold or threshold	Supra-threshold	Supra-threshold	Supra-threshold	Supra-threshold	Supra-threshold	Supra-threshold
Interstimulus interval (ms)	1–6	50–200	1.0–1.5, 2.3–3.0, 4.1–5.0	8–30	8–12	40–50	~20–25	~200	5–8
Proposed neurotransmitter/ receptor	$GABA_A$ DA	$GABA_B$	GLU $GABA_A$	GLU NE	Not known	$GABA_B$	ACh $GABA_A$	Not known	Not known

ACh = acetylcholine; CBI = cerebellar inhibition; contra = contralateral; DA = dopamine; ES = electrical stimulation; GABA = γ-aminobutyric acid; GLU = glutamate; ICF = intracortical facilitation; LAI = long latency afferent inhibition; LICI = long interval intracortical inhibition; LIHI = long latency interhemispheric inhibition; M1 = primary motor cortex; NE = norepinephrine; SAI = short latency afferent inhibition; SICF = short interval intracortical facilitation; SICI = short interval intracortical inhibition; SIHI = short latency interhemispheric inhibition.

Fig.8 (Rossini et. Al. 2015)

Short interval intracortical inhibition (SICI)

A Conditioning Stimulus (CS) administered on the motor cortex at an intensity lower than the resting threshold (RMT), not evoking any MEP, can suppress the motor

response of the target muscle given by a suprathreshold Test Stimulus (TS), if delivered before and with an ISI between 1 and 5 ms (Short interval intracortical inhibition - SICI) while with an ISI of 6-20 ms, there will be a facilitation (IntraCortical Facilitation - ICF) (Kujirai et al. 1993). The facilitation is observed only with the muscle completely relaxed otherwise we will have a slight inhibition at ISI from 6 to 15 ms (Ridding et al. 1995)

The cortical origin of SICI and ICF has been confirmed by the recording of TMS evoked corticospinal activity at the cervical epidural level (Di Lazzaro et al . 1998b, Kaneki et al. 1996, Nakamura et al. 1997).

Several studies have confirmed that TMS is able to selectively activate inhibitory circuits within the motor cortex. If the coil of the conditioning stimulus is moved anteriorly or posteriorly but keeping the test stimulus on the motor hot spot, it has been show that the percentage of inhibition decreases (Kujirai et al. 1993).

There are two phases of SICI, peaking at ISIs of 1 ms and 2.5 ms. SICI at 1 ms may partly be related to neuronal refractoriness but may also involve synaptic inhibition (Fisher et al., 2002; Hanajima et al., 2003; Roshan et al., 2003). SICI at 2.5 ms likely represents post-synaptic inhibition mediated by gamma-aminobutyric acid type A (GABAA) receptors because drugs that enhance GABAAergic neurotransmission increase SICI (Ziemann et al., 1996a; Di Lazzaro et al., 2007). **Fig. 9**

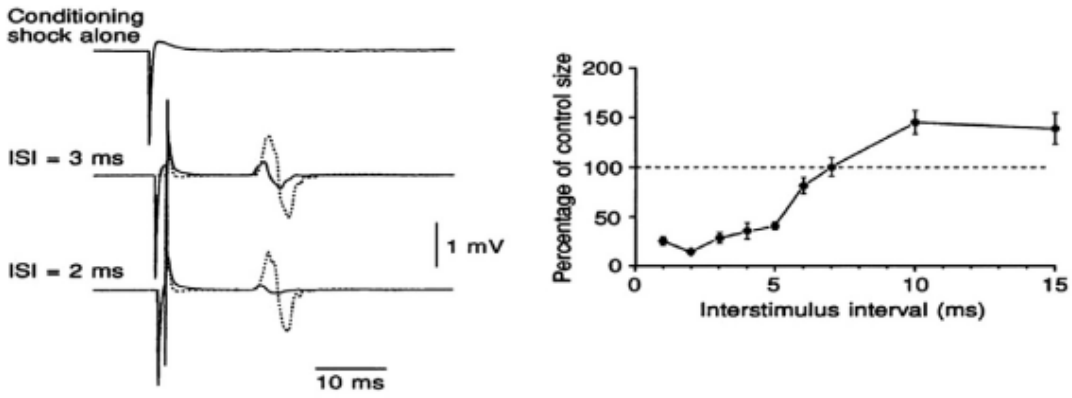


Fig. 9 (from Kujirai et al., 1993).

Intracortical facilitation (ICF)

ICF is performed with a similar protocol at SICI but at longer ISIs of 6-30 ms (Kujirai et al., 1993). Facilitation is reduced by glutamate NMDA antagonists (Liepert et al. 1997, Ziemann et al. 1998a, 2004) and it has been suggested that this form of facilitation could derive from the recruitment of additional cortical circuits, different from those more easily activated by single pulse stimulation (Di Lazzaro e Rothwell, 2014). However, the physiological basis of the ICF is still little understood (Di Lazzaro et al., 2006).

Intracortical inhibition appears to be involved in the reorganization of the motor cortex following amputation. Studies on the stump muscles of subjects with unilateral upper limb amputation showed a reduced inhibition (Chen et al. 1998) correlated with their cortical expansion (Fuhr et al. 1992).

Schwenkreis et al., 2000 studied the excitability of the motor cortex and the

functional reorganization in patients with upper limb amputation: they observed a highly significant reduction of intracortical inhibition at shorter intervals on the AH in the forearm group but not in the arm group, and an increase in ICF in arm amputees on the UH. This study confirms that there are changes in the activity of intracortical circuits following the amputation of the upper limb which may depend on the level of amputation, and they are probably the functional basis of cortical reorganization.

The same authors later investigated the role of NMDA-mediated mechanisms in cortical excitability changes after limb amputation and their possible relationship to PLP (Schwenkreis et al., 2003). By administering the NMDA antagonist memantine and evaluating SICI and ICF, they concluded that NMDA-mediated mechanisms influence the changes in SICI and ICF but these changes in cortical excitability and PLP pain are independent of each other.

Interhemispheric Inhibition (IHI)

In interhemispheric inhibition, the CS is applied to the motor cortex of one hemisphere and the TS to the motor cortex of the contralateral hemisphere. Both stimuli are delivered at the same suprathreshold intensity, therefore both are able to evoke MEPs from both hemispheres. IHI is a cortical phenomenon (Di Lazzaro et al., 1999b) that is likely produced by interhemispheric excitatory pathways through the corpus callosum and synapse onto local inhibitory circuits in the target M1 (Ferbert et al., 1992; Wahl

et al., 2007; Ni et al., 2009), although there might be some subcortical contribution (Gerloff et al., 1998).

It has been observed that at ISIs between 6 and 50 ms there is an inhibition of the MEP evoked by the TS (Ferbert et al. 1992, Chen et al. 2003). The maximum inhibition was found at an ISI of 10 ms (Short Latency IHI, SIHI) and 50 ms (Long Latency IHI, LIHI) (Chen et al, 2009). The IHI and in particular the LIHI represent the projection to the contralateral motor cortex of various cortical areas, including the dorsolateral prefrontal cortex, the dorsal premotor cortex and somatosensory cortex (Ni et al., 2009). Pharmacological studies suggest that LIHI is mediated by post-synaptic GABAB receptors (Irlbacher et al., 2007). Interhemispheric facilitation from the premotor cortex to M1 has also been demonstrated (Bäumer et al., 2006). **Fig. 10**

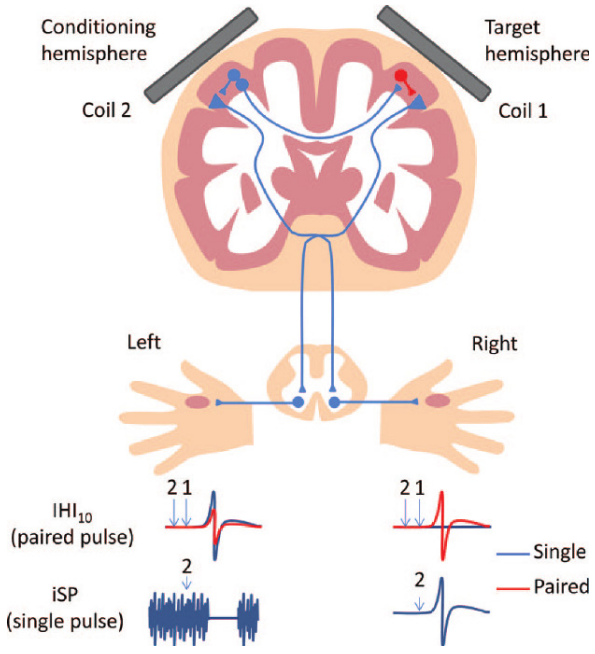


Fig. 10

Short latency afferent inhibition

Cutaneous or mixed afferent input from the hand can inhibit MEPs evoked in resting muscles by TMS of M1 at short latencies. Reduction of cortical excitability has been confirmed also by epidural spinal cord recordings (Tokimura et al., 2000). Short - atency afferent inhibition (SAI) is obtained with a peripheral conditioning electrical stimulus and a magnetic TS. The median nerve at the wrist and the first dorsal interosseous muscle (FDI) are usually used. SAI is obtained if median nerve stimulation at the wrist precedes stimulation of the contralateral motor cortex at ISIs around the latency of the N20 component of the somatosensory evoked potential. Maximum inhibition occurs in normal subjects at N20+2 to 4 ms (Tokimura H et al. 2000). Pharmacological studies showed that SAI involves cholinergic (Di Lazzaro et al., 2000) and GABAAergic (Di Lazzaro et al., 2005) circuits. **Fig. 11**

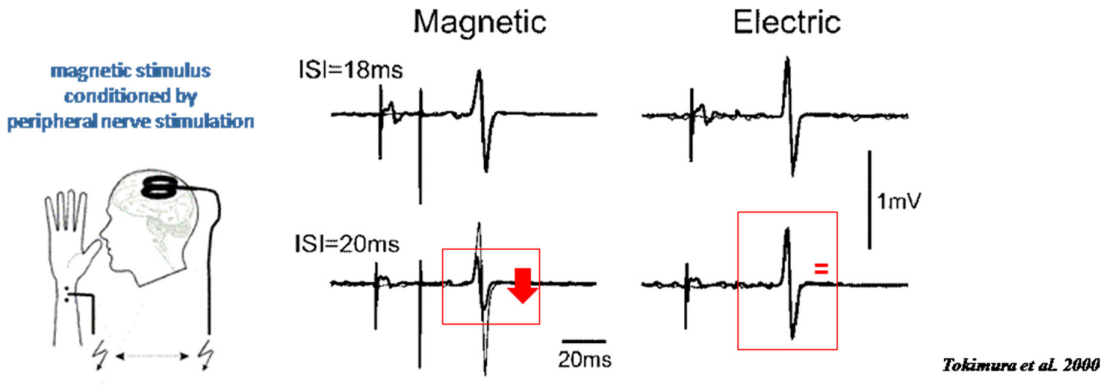


Fig. 11

EXPERIMENTAL STUDY

Experimental design

The aim of this project is to evaluate by TMS the excitability of the primary motor cortex and of the excitatory and inhibitory intracortical circuits in chronic upper limb amputation, in both hemispheres. The project was carried out in collaboration with the INAIL Prosthesis Center.

Understanding the changes in intracortical excitability in amputees provides information on the reorganization of the brain in the post-amputation phase, thus obtaining useful information for rehabilitation and for brain-computer interfaces (BCI).

Materials and Methods

Patients

18 patients with upper limb amputation were recruited (1 female; mean age: 47 years ± 15.5 SD; Age at amputation: 34 years ± 17.2 SD). Of these 13 were trans-radial (2 *Amelia*) and 5 trans-humeral. Excluding patients with *Amelia*, manual preference before amputation was right-side (n = 17) and left-side (N = 1) and most patients have a long-standing amputation (16 years ± 15.7 SD) with an average of years of use of the prosthesis of 17 years ± 15.5 SD in14 patients **(Table 1)**

ID	AMP	SIDE	AGE	SEX	HOT SPOT MUSCLE	AGE AT AMPUTATION	YEARS ELAPSED FROM AMPUTATION TO STUDY	YEARS OF USE OF THE PROSTHESIS	MANUAL PREFERENCE BEFORE AMUTATION
1	TR	DX	61	M	BB	16	45	44	DX
2	TR	SN	52	M	BB	47	5	2	DX
3	TR	DX	47	M	BB	20	27	23	DX
4	TH	SN	33	M	TPZ	20	13	8	DX
5	TR	SN	29	M	BB	24	5	2	DX
6	TH	SN	59	M	TPZ	34	25	22	DX
7	TR	DX	46	M	BB	43	3	0	DX
8	TOH	DX	38	M	TPZ	30	8	7	DX
9	TR	DX	25	M	BB	25	0	0	DX
10	TR	SN	59	M	BB	55	4	0.5	DX
11	TH	SN	48	M	TPZ	36	12	2	DX
12	TH	DX	60	M	TPZ	31	29	26	DX
13	TR	DX	57	M	BB	20	37	26	DX
14	TR/ *AMELIA*	SN	20	M	BB	0	20	0	-
15	TR/ *AMELIA*	DX	29	M	BB	0	29	29	-
16	TR	SN	49	F	BB	47	2	2	DX
17	TR	DX	56	M	BB	13	43	43	DX
18	TR	DX	80	M	BB	79	0.5	0	DX

Table 1 : clinical and demographic characteristics of patients.
TH: transhumeral amputation; TR: transradial amputation; TPZ: trapezius muscle; BB: biceps brachialis muscle.

The study was approved by the Ethics Committee of Campus Bio-Medico University and was conducted in accordance with the Declaration of Helsinki.

Inclusion criteria:

1. Stable amputation of the upper limb

2. Use of an active hand prosthesis, of the kinematic or myoelectric type

3. Age between 18 and 65 years

Exclusion criteria:

1. Presence of contraindications to exposure to magnetic fields (e.g., cardiac pacemakers, other stimulators or internal non-paramagnetic metallic objects)

2. Inability to express consent to participate in the study

3. Pathologies of any nature that compromise the motor function of the non-amputated limb

4. Pathologies and/or therapies that alter the functions of the central nervous system

5. Cognitive impairment (i.e., MMSE score <24)

6. Epileptic syndromes

Electrophysiological evaluation

We evaluated the excitability modifications of the motor cortex in the hemisphere contralateral to amputation "affected hemisphere" (AH) and in the ipsilateral "unaffected hemisphere" (UH).

Magnetic stimulation was performed with a high-power Magstim 200^2 (MagstimCo., Whitland, Dyfed). A figure-of-eight coil with external loop diameters of 9 cm was held over the motor cortex at the optimum scalp position to elicit MEPs in the distal muscles available in the stump and in the same muscles of the contralateral limb. The induced current flowed in a postero-anterior direction. Intensities were expressed as

a percentage of the maximum output of the stimulator. MEPs were recorded via two 9-mmdiameter Ag-AgCl surface electrodes. The electromyogram (EMG) was amplified and filtered (bandwidth 3 Hz-3 kHz) by D360 amplifiers (Digitimer, Welwyn Garden City, UK). Data were collected on a computer with a sampling rate of 10 kHz per channel and stored for later analysis using a CED 1401 analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK).

Subjects were given audiovisual feedback of the EMG signal at high gain (50 V / D) to assist in maintaining complete muscular relaxation.

In the case of paired-pulse stimulation, the mean amplitudes of the conditioned MEPs at the various ISIs were expressed as a percentage of the mean amplitude of the unconditioned MEP test.

Traces contaminated by EMG activity were discarded.

The following parameters of cortical excitability were evaluated in both cerebral hemispheres:

- Resting motor threshold (RMT)
- Active motor threshold (AMT)

RMT and AMT are given as percentages of maximum stimulator output (%MSO).

- Short latency intracortical inhibition (SICI)

SICI was studied using the technique of Kujirai et al. (1993). Two magnetic stimuli were given through the same stimulating coil over the motor cortex at an ISI of 2-3 ms, and the effect of the first CS on the second TS was investigated. Ten single-pulse stimuli and ten each paired stimuli at 2-3 ms ISI in a pseudorandomized order were delivered. The CS was set at an intensity of 5% MSO below AMT. The intensity of the TS was adjusted to elicit an unconditioned test MEP in the relaxed target muscle of ~ 1 mV in peak-to-peak amplitude. These data were averaged across all ISIs to obtain a grand mean single value of SICI. **Fig. 11**

- Intracortical facilitation (ICF):

We also evaluated ICF by analyzing the facilitatory interaction that occurs between pairs of magnetic stimuli given over the motor cortex at 15-ms ISI. Ten single-pulse stimuli and ten paired stimuli at 15-ms ISI in a pseudorandomized order were delivered. **Fig. 12**

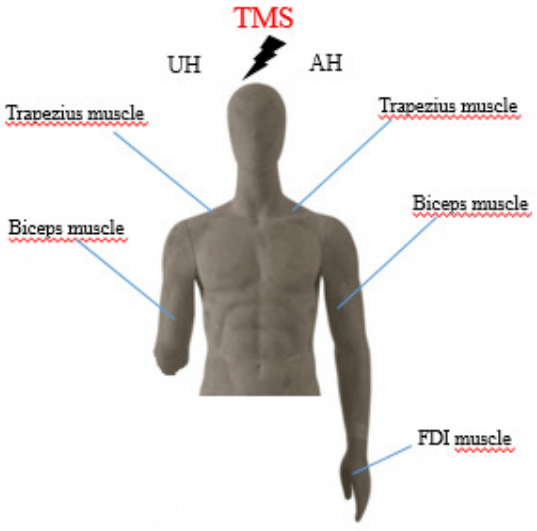


Fig. 12 : representation of registered muscles

- Short latency afferent inhibition (SAI)

SAI was studied using the technique described by Tokimura et al. (2000). Conditioning electrical pulses (constant current square-wave pulses; duration 200 μs) were applied through a bipolar electrode to the ulnar nerve at the elbow (cathode proximal). The intensity of the TMS TS over the motor cortex was adjusted to evoke an unconditioned MEP in the relaxed target muscle an adequate Mep response.The CT to the ulnar nerve preceded the TS by ISIs that were related to the individual latency of the N20 component of the ulnar nerve somatosensory evoked potential. To record somatosensory evoked potentials, the active electrode was attached 3 cm posterior to C4 or C3 (according to the 10–20 international EEG system) and the reference was positioned 3 cm posterior to C3 or C4 , respectively. Five hundred responses were averaged to identify the latency of the N20 peak. ISIs corresponding to the N20 latency plus 2, 3, and 4 ms were investigated (Tokimura et al. 2000) with five repeats per ISI in a pseudorandomized order. These data were averaged across all ISIs to obtain a grand mean value of SAI. **Fig. 13**

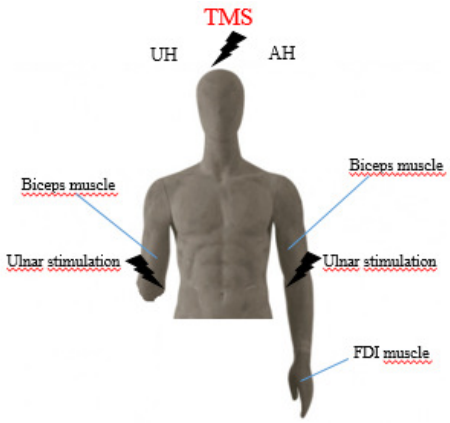


Fig. 13: representation of registered muscles and peripheral stimulation site.

- Interhemispheric inhibition (IHI):

Magnetic stimulation was performed with two high-power Magstim 200^2 each connected to a figure-of-eight coil. The coils were held over the right and left motor cortex (at the optimum scalp position to elicit motor responses in the target muscle) with the induced current flowing in a posteroanterior direction. IHI was studied using the technique of Ferbert et al. (1992). The effect of the first CS given over the AH motor cortex on the second TS given over the UH motor cortex was investigated and viceversa. Both CS and TS intensity were adjusted to evoke a muscle response in relaxed target muscle with an amplitude of ~ 0.5 mV peak-to-peak. ISIs of 8,10 and 15 ms were investigated with ten repeats per ISI in a pseudorandomized order . These data were averaged across all ISIs to obtain a grand mean value of IHI. **Fig. 14**

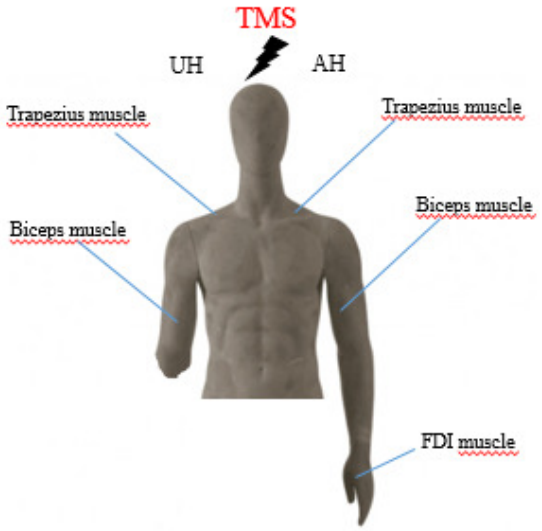


Fig. 14: representation of registered muscles and bilateral cortical stimulation site.

For all the above described paired-pulse TMS protocols, the amount of inhibition/facilitation at the various ISIs was expressed as a percentage of the mean amplitude of the unconditioned test MEP, based on the following formulas:

```
INHIBITION = (AMP_MEP_TEST - AMP_MEP_COND) * 100 / AMP_MEP_COND

FACILITATION = (AMP_MEP_COND - AMP_MEP_TEST) * 100 / AMP_MEP_COND
```

Statistical analysis

The MEP values were analyzed with a generalized linear mixed-effects model with logarithmic link. The participants were modelled as the random effects factor, and level of amputation (transradial and transhumeral level), stimulation side (AH and UH side), stimulation condition (test and conditioned stimulus) were modelled as the

fixed effects factors. Therefore, the data were processed in an ANOVA-like analysis, resulting in a 2 × 2 × 2 model design. This has several advantages over the classic, repeated measures ANOVA approach: it allows to effectively fit large and unbalanced data sets (e.g., missing data) and requires less restrictive assumptions to run the analysis properly (Baayen 2008).

Post-hoc pairwise comparisons were performed on significant interaction and significance values were Bonferroni-corrected. Additionally, post-hoc pairwise comparisons between stimulation conditions were performed for different level of stimulation and stimulation side (4 comparisons); significance values were Bonferroni-corrected.

GJe

RESULTS

Corticospinal excitability (motor thresholds)

The excitability of the cortical motor area of the target muscle (the most distal muscle available in each subject) was assessed by measuring the motor threshold at rest (RMT) and the motor threshold during muscle activation (AMT). There were no significant differences in the value of RMT and AMT between subjects with trans-radial amputation (n = 13) and with trans-humeral amputation (n = 5) (RMT: 59.1 ± 3.3 % vs 68.0 ±3.4 %, p= 0.14; AMT: 47.3 ± 2.7 % vs 54.4 ± 1.7%, p= 0.14) **Fig. 15.**

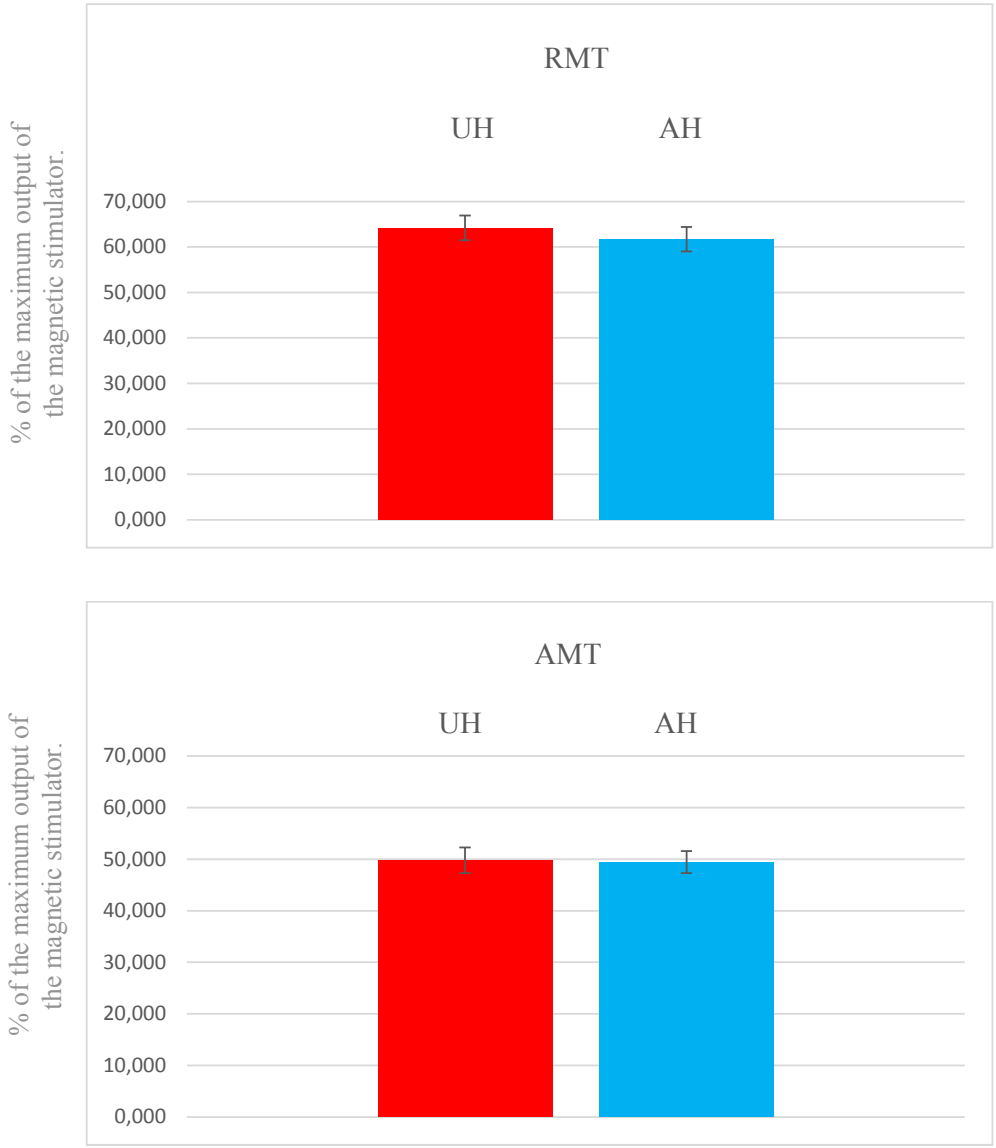
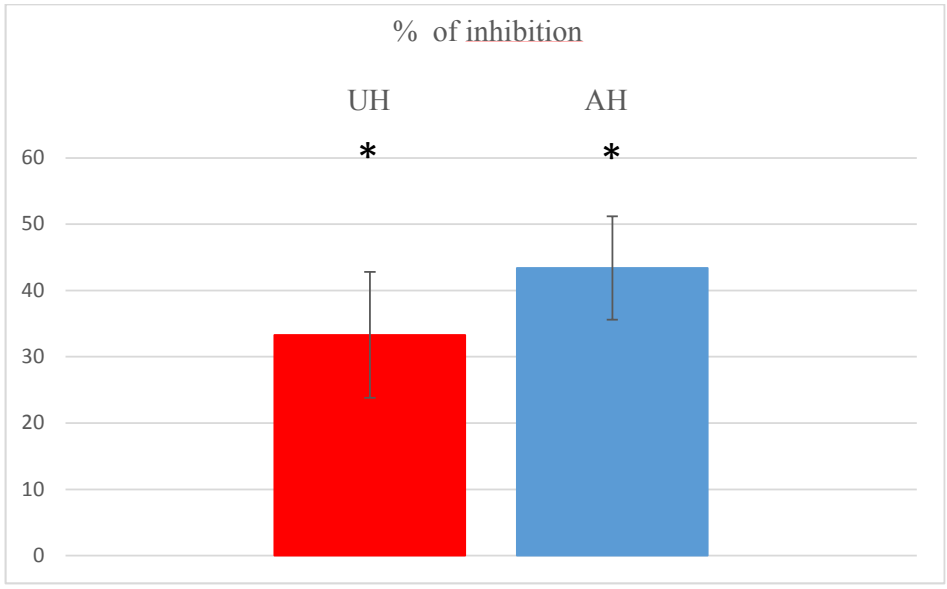


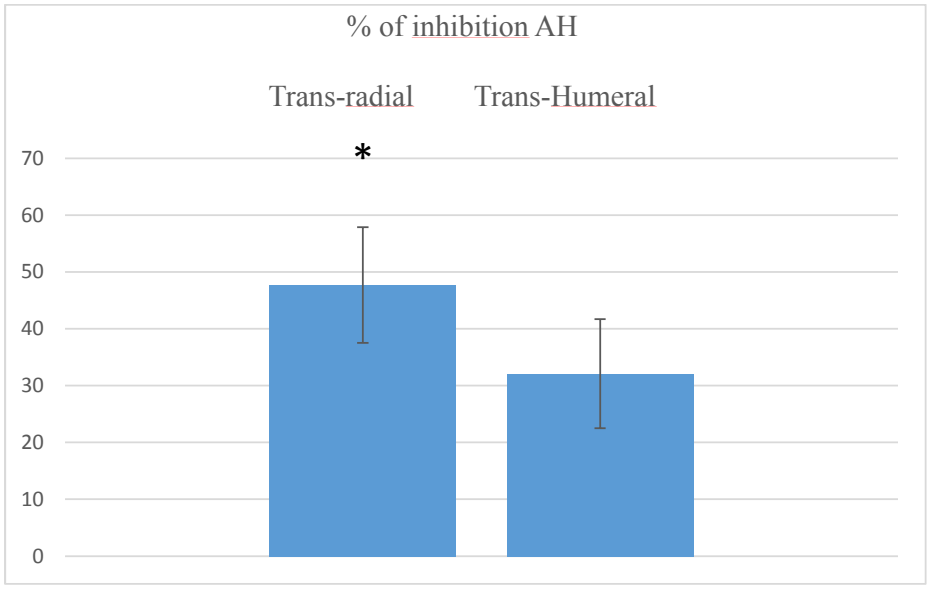
Fig. 15: Average values (± SD) of the motor threshold at rest (RMT) and in activation (AMT) measured in UH and AH. Values are expressed as percentage of the maximum output of the magnetic stimulator.

Short-interval intracortical inhibition (SICI)

We studied 18 patients (13 TR - 5 TH). The generalized linear mixed model analysis highlighted a significant effect of stimulation condition ($\chi 2$ (1) = 10.19, $p<0.001$) and a significant interaction between stimulation side and level of amputation factors ($\chi 2$ (1)= 4.05, $p<0.044$). In particular, test MEP amplitude was significantly higher than

conditioned MEP amplitude ($p<0.001$); test MEP amplitude was significantly higher ($p<0.001$) than in conditioned MEP amplitude in AH of trans-radial amputee group ($p=0.005$); the other comparisons between test and conditioned responses were not significant **Fig. 16.**





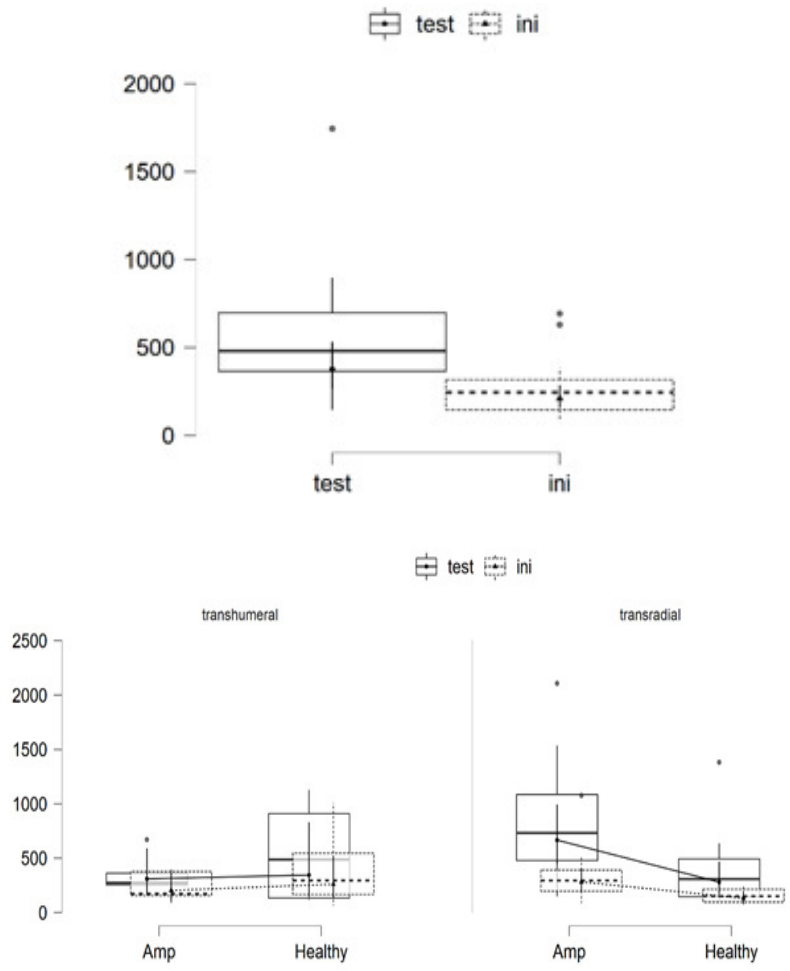
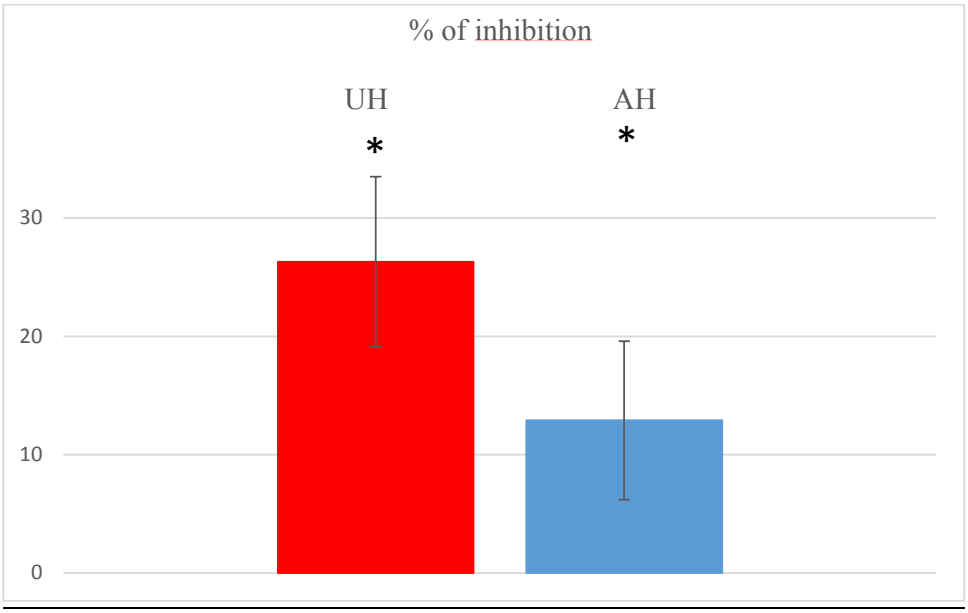
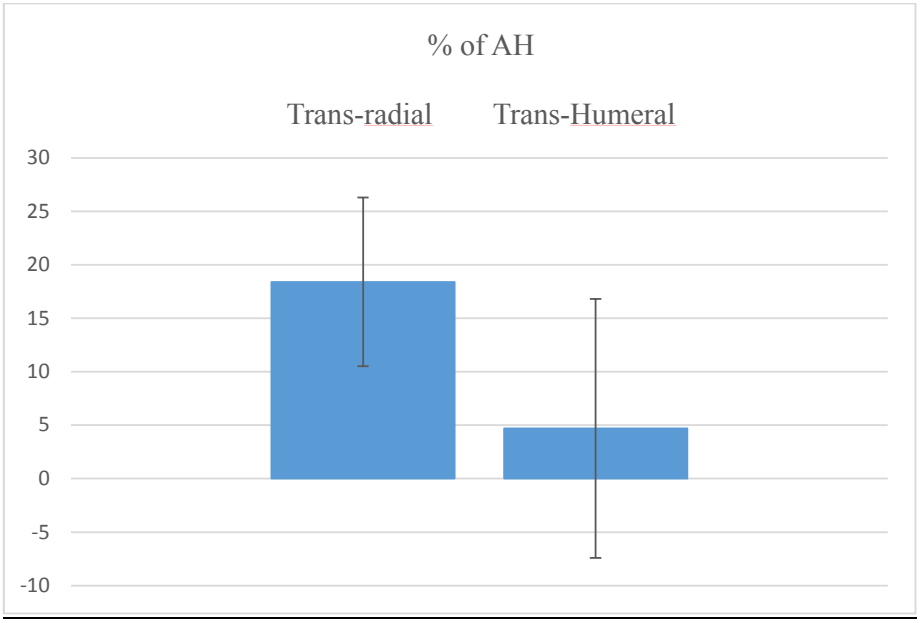


Fig. 16: Percentage of inhibition measured in subjects between UH side and AH and percentage of inhibition measured between trans-radial and trans-humeral amputation.

Inter-hemispheric inhibition (IHI)

We studied 10 patients (6 TR/4 TH). The generalized linear mixed model analysis highlighted a significant effect of stimulation condition ($\chi 2$ (1)= 10.19, $p<0.001$). In particular, test MEP amplitude was significantly higher ($p<0.001$) than conditioned MEP amplitude. Considering that significant interaction was not found, no post-hoc analysis was performed **Fig. 17.**





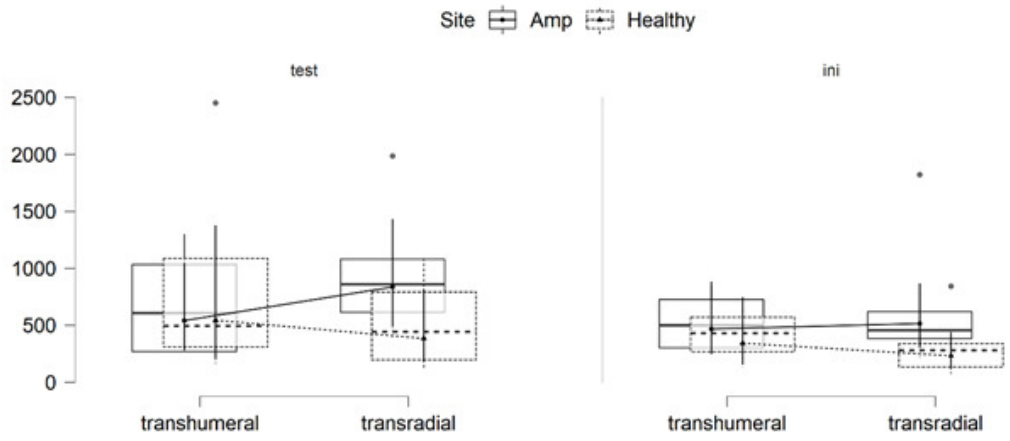


Fig. 17: Percentage of inhibition measured in subjects between UH and AH and percentage of inhibition measured between trans-radial and trans-humeral amputation.

Intracortical facilitation (ICF)

We studied 3 TR-amputated patients. The generalized linear mixed model analysis did not highlight any significant difference. Considering that significant interaction was not found, no post-hoc analysis was performed **Fig. 17.**

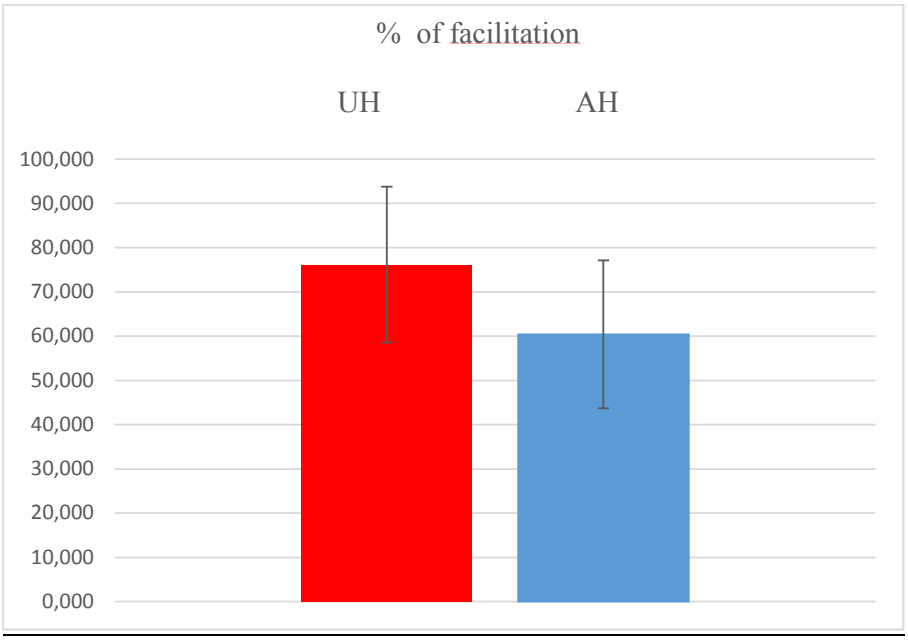


Fig. 18: Percentage of facilitation measured in subjects between UH and AH.

Short-latency afferent inhibition (SAI)

We studied 11 TR-amputated patients. Amputees showed a very low or absent SAI, both in the UH (-0,018 ± 0,111 ES) and in the AH (0,125 ± 0,072 ES).

The generalized linear mixed model analysis did not highlight any significant difference. Considering that significant interaction was not found, no post-hoc analysis was performed **Fig. 19.**

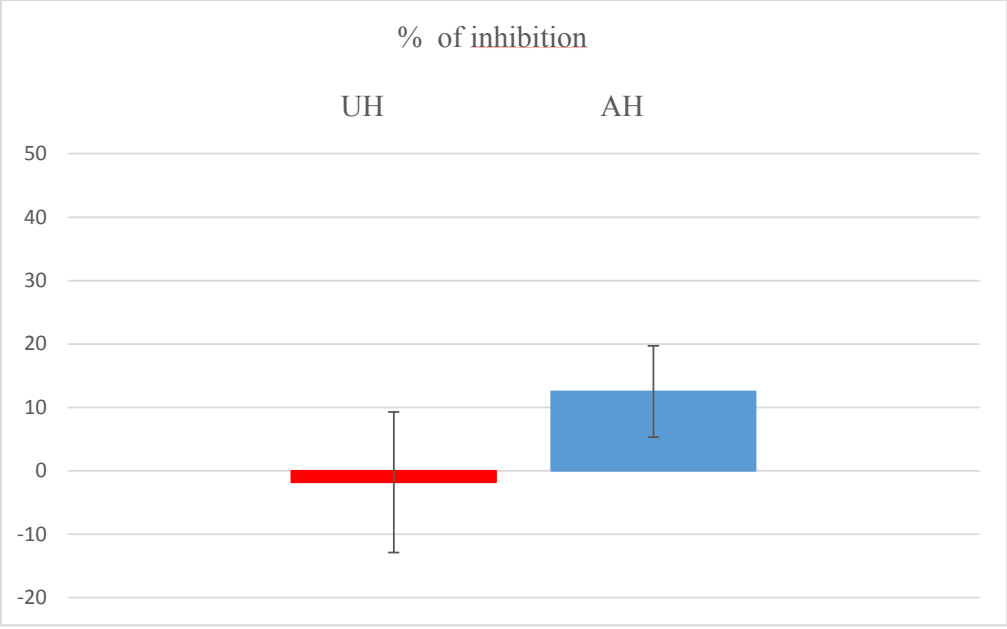


Fig. 19: Percentage of inhibition measured in subjects between UH and AH.

DISCUSSION

After the amputation of the upper limb, sensorimotor re-adaptation phenomena occur in the cortex corresponding to the amputated limb and in the adjacent areas that expand and become more excitable (Cohen et al. 1991; Pascual-Leone et al. 1996; Röricht et al. 1999).

To study these phenomena of neuroplasticity we used a non-invasive brain stimulation technique, Transcranial Magnetic Stimulation (TMS), that has the

property of activating corticospinal neurons as well as cortical interneurons. We used single and paired-pulse stimulation protocols to evaluate changes in corticospinal excitability and intracortical circuits.

As regards the phenomena of gabaergic inhibition (as tested by the SICI protocol), our results show a significant inhibition of motor response in both hemispheres; the amount of inhibition was significantly greater on the AH in the trans-radial patients' group. A recent review that analyzed studies on intracortical inhibition in subjects with amputation showed that most authors reported a decreased SICI in the "affected" hemisphere, while others found no difference between the two hemispheres (Santos et al. 2020). These data are in contrast with our results, but the heterogeneity of the studies needs to be evaluated. Indeed, while decreased SICI in the amputation hemisphere could support plastic changes underlying vicariation of the deafferented cortex by surrounding areas (i.e., disinhibition), compensatory adaptations due to chronic amputation, leading to an opposite phenomenon of increased inhibition, could also be considered. It should also be considered that it is not known if and to which extent SICI contributes to functional adaptations or if it just represents an epiphenomenon.

Glutamatergic excitatory phenomena (as tested by the ICF protocol) were investigated only in a very small sample (n=3): facilitation was present in both hemispheres but we cannot draw specific conclusions.

As regards the phenomena of afferent cortical inhibition mediated by cholinergic circuits (as tested by the SAI protocol), we were not able to obtain a physiological inhibition of the MEP, neither in the AH nor in the UH. One possible explanation could be that SAI has been studied usually by stimulating peripheral nerves in the wrist and by recording MEPs in distal muscles; therefore, we cannot exclude that the inhibitory effect is less pronounced when tested at proximal levels. This should be further investigated by increasing the sample size and by comparing with appropriate

control subjects. As for the AH, it could also hypothesized a greater excitability of M1 and adjacent areas which therefore becomes more difficult to suppress. Alterations of the peripheral afferents should also be considered, but it does not seem to explain altered SAI, since subjects did not exhibit substantial sensory alterations on the AH, and SAI was also impaired in the intact side.

Interhemispheric inhibition is fundamental in the control of bimanual movement, which involves bilateral cortical and subcortical areas through the corpus callosum. Our results showed a conserved interhemispheric inhibition mechanism in both directions, and with no differences between trans-radial and trans-humeral amputees.

There aren't data in the literature on interhemispheric inhibition in subjects with amputation, so we hypothesize that the cortico-cortical projections through the corpus callosum remain preserved.

Interestingly, a generally higher test MEP amplitude in the AH, across different paired-pulse TMS protocols, is coherent with the already described phenomenon of increased M1 excitability following amputation.

Overall, our data suggest increased gabaergic inhibition in the motor cortical areas contralateral to amputation: this finding, combined with a possible hyperexcitability state following amputation, might represent a compensatory phenomenon associated with central rearrangements in the chronic amputation condition.

One limitation of the study is the lack of a control group of intact subjects. Indeed, we acknowledge that changes in cortical excitability might occur also in the hemisphere ipsitaleteral to the amputation, due to interhemispheric interactions and/or compensatory phenomena. A similar condition of excitability imbalance between AH and UH is described in chronic stroke. One study on patients with chronic stroke (Murase et al. 2004), in which the IHI was tested bilaterally, showed increased inhibition of UH on the AH, secondary to disinhibition of the UH and

consistent with a model of interhemispheric competition in the motor and sensory systems.

It should also be considered that the time from amputation and the amount of training with prosthesis can play a role in the plastic changes of the sensorimotor cortex (Di Pino et al. 2009).Our patients' group is mostly composed by long-term amputees who have been using prostheses for many years. A study by Röricht S. in 1999 (Röricht et al. 1999) showed that plastic changes can occur after 20 years from amputation, even if with different results in different muscles of the stump that could be due to varying ipsilateral corticospinal projections.

The two subjects with hand agenesia might represent a specific condition in terms of plastic adaptations, due to hemispheric deafferentation occurring during the developmental period.

The side of amputation is another factor that might influence subsequent adaptations in the CNS. Our subjects were all right-handed and the side of the amputation was the right in N=9 and left in N=7, so the sample can be considered quite homogeneous. One study by Xie et al. (2013) suggests that manual preference before amputation should not lead to different plasticity phenomena in those who use the prosthesis on the dominant or non-dominant side. In this study, fMRI of chronic forced non-dominant hand use by unilateral amputees showed that, with chronic and exclusive forced use, the speed and quality of nondominant hand precision endpoint control in drawing can achieve levels nearly comparable with the dominant hand. A study by Williams L. in 2016 (Williams L et. Al. 2016) carried out by electroencephalogram on patients with amputation of the right upper limb, to study whether the laterality of motor activity is influenced by neural reorganization after amputation, showed there is a remodeling of activations from traditional contralateral motor areas to the posterior parietal areas for motor planning and execution when using the amputated limb.

In conclusion, neurophysiological assessment, other than characterizing a static condition, could represent a useful tool for monitoring each patient's plastic changes of the motor cortical areas following prosthetic rehabilitation procedures. The study of the cortical reorganization of the CNS following amputation can therefore provide information on the effectiveness of an active prosthesis in promoting the restoration of the physiological function and in reducing aberrant forms of plasticity (Di Pino et al. 2009). The surgical, neurophysiological and technological developments could then contribute to improve the amputee person's quality of life, compared to the past. The search for neurophysiological markers of cortical reorganization after amputation can help in choosing the timing and characteristics of prosthetic rehabilitation in an increasingly personalized and patient-friendly medicine.

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