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"Neuroplasticity in People Affected by Major Upper Limb Amputation: a Transcranial Magnetic Stimulation study".

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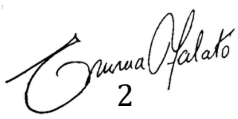

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List of abbreviations:

AMT = Active Motor Threshold;
CF = congenital forearm
COG = Center of Gravity
cTBS: continuous Theta Burst Stimulation
EMG = Electromyography;
fMRI = functional Magnetic Resonance Imaging
INAIL = Italian National Institute for Insurance against Accidents at Work
ISI = Inter-stimulus interval;
MEPs = Motor-Evoked Potentials;
PAS10: Paired Associative Stimulation
PLP = Phantom Limb Pain
PLS = Phantom Limb Sensation
RMT = Resting Motor Threshold;
SD = Standard Deviation
TH = Transhumeral
TMR= Targeted Muscle Reinnervation
TMS = Transcranial Magnetic Stimulation;
TR= Transradial
mULA = major Upper Limb Amputation
ULA = Upper Limb Amputation

BACKGROUND

Major upper limb amputations (mULAs), meaning amputation that affect the upper limb above the wrist accounted for 8% of total upper limb amputations (ULA) in an epidemiological study carried out in the USA (year 2005; Ziegler-Graham et al. 2008). Middle aged men are mostly frequently affected (Maduri and Akhondi, 2022). In Italy, in 2005 (*last official data available*), 5600 new ULA were reported by the Italian National Institute for Insurance against Accidents at Work (INAIL)[1]. The etiology of ULA is most frequently traumatic, the remaining causes including bone or soft tissues tumors, vasculopathies, infections and congenital limb deficiency (Maduri and Akhondi, 2022).

[1] https://www.inail.it/cs/internet/comunicazione/news-ed-eventi/news/p1780018061_sanita_solo_il_5_di_amputazi.html - accessed on 13 March 2022

mULAs have disastrous consequences for amputees' life, in terms of mental, physical, and social well-being (Puranik et al. 2021) and are frequently accompanied by phantom limb pain (PLP), which has an estimated lifetime prevalence of 80% in amputees (Stankevicius et al. 2020).

Despite the advances in medical technology and computer software applied to medicine, people affected by mULA continue to use, as standard of care, prosthesic technologies developed almost half a century ago (Uellendahl 2017).

Table 1 Classes of Upper-Extremity Prostheses *(adapted from Pierre et al. 2018)*

Type	Mechanism	Advantage	Disadvantage
Cosmetic	Socket attached to the residual limb	Cosmetic	No mechanical function
Body-powered	Shoulder motion is captured with a harness and transferred through a cable to operate a distal joint	Inexpensive Highly functional for basic tasks	Only one joint can be operated at a time Heavy and unwieldy, can be physically demanding
Myoelectric*	Electrical signals produced by muscle contraction are captured by surface electrodes and used to operate a motorized arm	Provides a strong grip	Only one function can be performed at a time Heavy Control is not intuitive; may require mode switch to increase degrees of freedom Signal quality is adversely affected by poor socket fit and cross-talk (EMG noise from adjacent muscles that dilutes signal quality)

** A prerequisite for myoelectric prosthetic use is the presence of adequate muscle activity producing electric signals for myoelectric control.*

Even if overall advantageous (Resnik et al. 2020) the use of limb prosthesis is burdened by low satisfaction rates and high rates of rejection (Davidson 2002, Benz et al. 2016) mainly due to the low wearability, poor controllability and the absence of sensory feedback of the current prostheses (Cordella et al. 2016).

Targeted muscle reinnervation (TMR) is a revolutionary surgical approach which consists in the transfer of functioning nerves that have lost their operational target to intact proximal muscles that become biologic amplifiers for electromyographic signals. Then, these novel signals can be used to control advanced myoelectric prostheses, conferring additional degrees of active motion (Kuiken et al. 2004, 2009). A serendipitous outcome of TMR surgery was reduced neuroma pain and phantom limb pain, and it has been hypothesized that these effects might be due to the establishment of a new afferent signal from muscular sensory receptors that closes the efferent-afferent feedback (Dumanian et al. 2019).

The standard TMR technique typically involves the following nerve transfers (Cheesborough et al. 2015):

- in case of elbow disarticulation and transhumeral amputation

NERVE	TRANSFER	SIGNAL PROVIDED
median nerve	end-to-end transfer to the motor nerve of the short head of the biceps	"hand close" signal
distal radial nerve	motor nerve of the lateral head of the triceps	"hand open" signal
ulnar nerve*	motor nerve entering the brachialis muscle	additional hand or wrist control signal
the long head of the biceps, innervated by the musculocutaneous nerve, is left intact to maintain the "elbow flexion" signal		
the long head of the triceps is left intact to provide an "elbow extension" signal		

** if adequate limb length remains and the brachialis muscle is present*

- in case of shoulder disarticulation

NERVE	TRANSFER	SIGNAL PROVIDED
musculocutaneous nerve	clavicular head of the pectoralis major	elbow flexion signal
median nerve	largest motor nerve of the sternal head of the pectoralis muscle	"hand close" signal
proximal branch of radial nerve*	residual triceps muscle (connection)	elbow extension signal without nerve transfer
distal radial nerve**	latissimus dorsi (dividing the thoracodorsal nerve) or serratus anterior (dividing the long thoracic nerve)	"hand open" signal
ulnar nerve*	any remaining muscular targets	additional hand or wrist signal

** optional*

*** Alternatively, if the motor nerve to the pectoralis minor can be reached by the residual radial nerve, this muscle can be disinserted and mobilized laterally to provide a target for the radial nerve distinct from the pectoralis major*

Several case series have described positive functional outcomes with TMR (Pierre et al. 2018, Myers et al. 2020), and studies are ongoing to assess its long-term outcomes and to maximize the potential of this novel therapy (Kang et al. 2018).

The study of the how the nervous system changes after limb amputations can offer fundamental insights for the challenge of improving prosthetic control. Brain plasticity is the ability of the nervous system to change its activity in response to intrinsic or extrinsic stimuli by reorganizing its structure, functions, or connections (Puderbaugh amd Emmady 2022). It is known that the loss of afferent and efferent signals from and to the amputated limb is accompanied by extensive peripheral and central nervous systems reorganization (Di Pino et al. 2009, Gunduz et al. 2020). It is commonly accepted that in subjects affected by ULA, after an initial unmasking of functionally silent synaptic contacts due to the lack of inhibition from the deafferented area, there is the creation and stabilization of new connections in the adjacent CNS regions. These neuroplastic events could have a compensatory nature but also evolve in maladaptive phenomena, such as PLP (Di Pino et al. 2009).

Neurophysiological studies allowed to demonstrate that after limb amputation, the corticospinal excitability (i.e. the strength of the response of neurons of the corticospinal pathway to a given stimulation (Leocani et al. 2000)) of the brain hemisphere contralateral to the amputation side becomes significantly higher compared to the opposite side, as showed by larger motor responses from the muscles near the stump (Cohen et al. 1991). It has also been found that the representation of the targeted muscle above the motor cortex goes through enlargement and shift (Pascual-Leone et al. 1996). However, the view that there is a true reorganization of the motor map of the stump's muscles has been challenged after the demonstration that changes in map size and location could reflect between-side

differences in corticospinal excitability rather than a true reorganization of the map (Gagné et al. 2011).

Functional Magnetic Resonance Imaging (fMRI) studies also showed that extensive temporal and spatial changes occur after upper limb amputation, not only in sensory and motor areas but also in areas responsible for sensorimotor integration and motor planning (Bao et al. 2021). These changes are partially reversed after reinnervation, even if a long-standing deafferentation (Giraux et al. 2001).

Neuroplasticity can exert a key role in the successful outcome of peripheral nerve regeneration and transfers (Mohanty et al. 2015, Socolovsky et al. 2021).

Transcranial Magnetic Stimulation (TMS) is a unique neurophysiological technique that provides the opportunity to test and study the function of the human brain allowing a noninvasive, painless brain stimulation through the intact scalp in the awake subject. TMS is based on the Faraday's principle of electromagnetic induction. When a magnetic stimulation is delivered through a coil placed on the scalp, i.e. over the hand representation area of the primary motor cortex, a transient and painless electrical current is induced in the brain. If the intensity of the stimulation is above the threshold for activation of motor pathways, a muscle twitch is evoked in the contralateral targeted muscle, known as Motor-Evoked Potential (MEP), which can be recorded through surface electromyography (EMG) (Di Lazzaro and Falato 2020). The optimal scalp location to evoke MEPs in the targeted muscle is defined as "hot-spot", while the minimum TMS stimulation intensity able to elicit consistent MEPs (with peak-to-peak amplitudes of at least 50 μV in each trial) in at least 5 out 10 consecutive TMS stimuli at rest is defined as resting motor threshold or RMT (Rossini et al. 2015). The mechanisms through which TMS on the primary motor cortex induces MEPs are partially understood, due to the complexity of cortical and subcortical circuits. Animal recordings revealed that a single electrical stimulus delivered to the motor cortex could produce a high-frequency (>600 Hz) repetitive discharge of corticospinal axons originating both from direct (D wave) and indirect (I waves) trans-synaptic activation of pyramidal cells (Adrian and

Moruzzi, 1939, Patton et al. 1954). It has been demonstrated through epidural high cervical electrodes recordings that also in humans the TMS-induced corticospinal descending activity is made by multiple descending high-frequency waves, and that the composition of the corticospinal volleys in terms of D- and I-waves is influenced by the stimulation parameters (stimulation intensity, coil type, and coil orientation) and by changes in cortical excitability (Di Lazzaro et al. 2012). Specific TMS protocols can be used to study corticospinal excitability, brain plasticity, and to map motor functions (Di Lazzaro et a. 2018, Di Lazzaro and Rothwell, 2014). Moreover, protocols of repetitive TMS can induce changes in brain excitability that outlast the time of stimulation. Among them, continuous Theta Burst Stimulation (cTBS) and Paired Associative Stimulation-10 (PAS_{10}) can temporary reduce corticospinal excitability, through mechanisms wich remail largely unknown (Huang et aal. 2009). cTBS which consists in bursts of high-frequency stimulation (50 Hz) repeated at the theta frequency (5 Hz) for 40s (Huang et aal. 2005), selectively suppressed the I1-wave, suggesting that cTBS has its major effect on a single source of inputs to corticospinal cells, which is responsible for the I1-wave production (Di Lazzaro et al. 2014). PAS 10, which requires the combination of an electrical peripheral nerve stimulation and cortical magnetic stimulation, leads to a decrease in the excitability of cortical mechanisms that generate later I waves in response to single TMS pulses.

The stability and reliability of TMS measures across time support the use of TMS in studying cortical plasticity in amputees (Hetu et al., 2011).

EXPERIMENTAL STUDY:

"Neuroplasticity in People Affected by Major Upper Limb Amputation:

a Transcranial Magnetic Stimulation study"

RATIONALE OF THE STUDY

The aims of this study are:

- Studying hemispheric differences in corticospinal excitability in a population of subject with chronic (≥1year) mULA using active control prosthesis.

  To this end, we will use the following measures of basal corticospinal excitability:

  - Resting Motor Threshold (RMT)

  - MEPs peak-to-peak amplitude

- Evaluating in the same population the effects of "acute" neuromodulatory interventions (cTBS and PAS_{10}) in terms of inhibition of corticospinal excitability.

  We will assess the effects of the neuromodulation comparing the amplitude of the MEPs recorded before and after neuromodulation.

- Moreover, we wanted to study, for the first time in literature, the neuroplastic changes occurring at the primary motor cortex before and after TMR surgery. To this end, we recorded bilateral TMS motor maps in 1 patient affected by transhumeral mULA who underwent TMR surgery.

STUDY DESIGN

Nonrandomized, interventional study

METHODS

Participants' identification and recruitment:

Participants have been recruited in collaboration with INAIL prosthesis center of Vigorso di Budrio (BO, Italy) and its branch facility in Rome (Cto Hospital 'A. Alesini', Rome, Italy)

Inclusion and exclusion criteria:

People aged 18 years and older, with chronic (≥ 1 year after amputation) stabilized mULA already using an active control arm prosthesis (body powered or myoelectric) and who signed the informed consent ("AMP-PLAST15" protocol) were enrolled in the study.

Exclusion criteria: we excluded people affected by cognitive impairment, contraindications to TMS (i.e. , history of epilepsy, brain implanted electrodes or stimulators, cardiac pacemaker, aneurysm clips or coils, arterial stents, metallic implants, spinal or bladder stimulators, previous skull opening or trauma, presence of metallic foreign bodies) psychiatric comorbidities, other comorbidities that could affect the study results.

Study setting:

Recordings have been made at the Neurophysiology and Neuroengineering of Human-Technology Interaction Lab (NeXT-Lab), Campus Bio-Medico University of Rome, between November 2017 and Nov 2021.

During TMS stimulations, participants were at rest, comfortably seated, with eyes opened gazing at a fixed point. Recordings took place during morning sessions.

Transcranial Magnetic Stimulation:

TMS was performed using a MAGSTIM 200^2 stimulator (Magstim Co., Whitland, Dyfed, UK) and a standard figure-of-eight coil with external loop diameters of 9 cm, delivering a monophasic magnetic pulse. The coil was held tangential to the scalp, with the handle

pointing backwards and laterally at a 45° angle from the sagittal midline (posterolateral–anteromedial orientation). The induced monophasic current in the brain flowed in a posterior-to-anterior direction. Intensities were expressed as a percentage of the maximum output of the stimulator. The **"target muscle"** was the biceps for transradial amputations; the biceps or trapezius for transhumeral amputations, according to the amputation level. The optimal site over motor cortex for eliciting consistent MEPs in the target muscle ("hotspot") was identified bilaterally, according to the current standards (Rossini et al., 2015). The TMS hotspots locations was marked bilaterally over the swimming cap to ensure constant coil positioning throughout the experiment. Motor evoked potentials (MEPs) were recorded through surface EMG (cf EMG recordings).

The **TMS protocols** used are:

- resting motor threshold (**RMT**) assessment: RMT was defined as the lowest stimulator's output able to elicit reproducible MEPs (at least 50 μV in peak-to-peak amplitude) in at least 50% of 10 consecutive stimuli (Rottini et al. I.F.C.N. Committee 2015)

- active motor thresholds (**AMT**) assessment: AMT was defined as the lowest stimulator's output able to elicit reproducible MEPs (at least 0.2 mV in peak-to-peak amplitude) in at least 50% of 6 consecutive stimuli while the subject is maintaining a voluntary contraction of about 20% of maximum (Rossini et al., I.F.C.N. Committee 2015)

- **single-pulse TMS**: 12 stimulations were delivered with an intensity of 120% RMT at each hotspot.

- **cTBS**: a standard cTBS protocol (Huang et al. 2005), with bursts of high-frequency stimulation (50 Hz) repeated at the theta frequency (5 Hz), continuously for 40s, was delivered with an intensity of 80% AMT in the hotspot of the target muscle. cTBS was delivered through the DuoMAG XT rTMS system (Rogue Resolutions) stimulator, with a biphasic current. cTBS was delivered unilateral (only in the hemisphere contralateral to the amputation side).

12 single-pulse stimulations were erogated in the hotspot of the target muscle, bilaterally, for

each of the following 3 blocks:

- before neuromodulation with cTBS
- 5min after the end of the neuromodulation
- 10min after the end of the neuromodulation

- **PAS10**: single electrical stimuli were delivered to the residual ulnar nerve of the amputated arm (just above the elbow for transradial amputation and at the axilla for transhumeral amputation) at 300% of the perceptual threshold, followed by TMS at an intensity sufficient to produce an unconditioned response amplitude of approximately 1 mV in the resting target muscle. Ninety pairs of stimuli were delivered at 0.05 Hz at an interstimulus interval (ISI) of 10 ms. The ISI were related to the individual latency of the N20 component of the somatosensory evoked potential of the residual ulnar nerve.

12 single-pulse stimulations were delivered in the hotspot of the target muscle, bilaterally, for each of the following 3 blocks:

- before neuromodulation with PAS
- 5min after the end of the neuromodulation
- 10min after the end of the neuromodulation

Note: cTBS and PAS10 protocols were performed in two separate sessions, at least 1 weak apart. The subject remained at rest, with eyes closed, during the 10 minutes after neuromodulation with cTBS or PAS10.

- **TMS motor mapping**: a grid was drawn on a swimming cap placed on the patient's head. Starting from Cz (half of the distance between nasion and inion, measured over the center line of the scalp, according to the standard 10-20 EEG electrodes positioning system[2]), we marked 11 points forward, 4 points backward and 9 points laterally (bilaterally) from Cz. The distance between the points in the grid was 1cm (*cf figure below*). A total of 304 points were marked, of which 16 on the midline and for 144 in each emisphere (9 columns of 16

[2]https://www.trans-cranial.com/docs/10_20_pos_man_v1_0_pdf.pdf

rows in each hemisphere, plus 16 points on the midline). A mean of 3 stimuli (single pulses) were applied per position with an intensity of about 130% RMT (RMT assessed at the hotspot of the biceps on the hemisphere contralateral to the amputation side; the same intensity was used for the two hemispheres). The coil was held tangential to the scalp, with the handle pointing backwards. Stimulation was delivered in order, starting from the most posterior point in the midline, in a forward direction, following each column (the hemisphere contralateral to the amputation side was stimulated first).

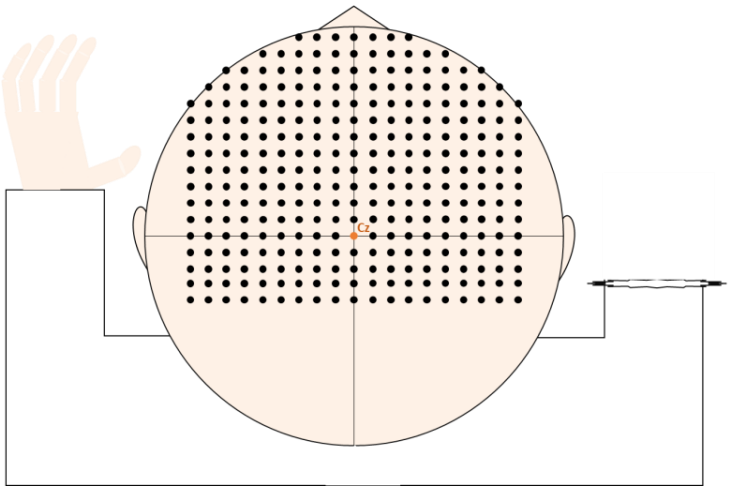


Figure 1 TMS motor mapping experimental setting. MEPs from biceps, triceps, deltoid and trapezius muscles were recorded bilaterally.

Surface electromyographic (EMG) recording:

Superficial EMG for MEPs was recorded through Ag/AgCl electrodes (diameter 10 mm) filled with conductive jelly paste (Ten20 conductive paste gel) through a 8 channel amplifier (D360, Digitimer, Welwyn Garden City, UK) and a a CED 1401 analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK). The sampling rate was 10kHz, EMG band pass 3–3000 Hz.

EMG recordings were made from:

- the "target muscle" (the biceps for transradial amputations; the biceps or trapezius for

transhumeral amputations, according to the amputation level) for cTBS and PAS_{10} studies

- biceps, triceps, deltoid and trapezius bilaterally for the TMS motor mapping. The same electrodes positions for EMG recording were used at baseline and 21m after TMR surgery

The active electrodes were placed over the belly of each muscle, the reference electrode over the nearest tendon. Standard skin preparation with abrasive gel (Nuprep, Weaver and Co.) was made before the electrodes' placement.

Data processing and Statistical Methods:

MEP traces were visually inspected on the Signal software v.5.08x86. MEPs with visible artifacts were excluded from the extraction (<5%). Then, the peak-to-peak MEPs' amplitude was measured through the same Signal software and copied on Microsoft Excel (2018) software. For each subject, MEPs that exceeded three standard deviations were excluded from the analysis (<0.5%).

For the TMS motor maps, the MEPs that were recorded for each stimulation point, were averaged, and placed on an Excel table; then, MEPs averages were normalized (divided by 0.05) to keep real MEPs excluding background noise. Then, the following maps parameters were calculated (Gunduz et al. 2020):

- map area: number of scalp positions which, on being stimulated with equal intensities, elicited MEPs (EMG responses of at least 0.05 mV in peak-to-peak amplitude)

- map volume: sum of all MEPs average normalized amplitudes

- center of gravity (COG): x and y COG coordinates of the maps were calculated by using the MEPs average normalized amplitude and their position on the map, creating an amplitude weighted mean of the map

Area, volume and COGs modifications were described.

The graphical representation of the motor maps was obtained through the contour plot of matrix on Matlab version R2018a (MathsWorks); the same scale was used for all the maps' colorbars, to allow qualitative comparison.

Non-averaged MEPs data recorded from each of the 8 muscles (biceps, triceps, trapezius and deltoid, bilaterally) were used for ANOVA and t-tests.

ANOVA were performed on the JASP software v. 0.16.1 (by JASP Team, 2022). Normality of data has been examined using Shapiro-Wilk test. ANOVA and post hoc tests have been performed on natural log-transformed values. In the presence of significant interactions, corrected pairwise comparisons have been performed by nonpaired t-tests. Bonferroni corrections have been applied for multiple comparisons. Correlation analysis has been performed with Kendall's tau and Spearman's rank correlation coefficients. The significance level has been set at $P < 0.05$.

RESULTS:

1) Basal corticospinal excitability:

Demographic characteristics of the sample: data from 1 subject were excluded from the analysis because of major artifacts of basal recordings. The remaining recordings were from 12 subjects (11M, 1F), age (mean$\pm$ SD)=49.6 $\pm$ 17.19 years old (range 21-80), level of amputation: 6 transhumeral (TH), 5 transradial (TR), 1 congenital forearm amputation (CF), years after amputation (mean$\pm$ SD)= 12,6$\pm$12,1 (range 1-49). 3/12 were affected by phantom

limb pain (frequency from sporadic to occasional); 10/12 reported non-painful phantom limb sensation.

Table 2 *Investigated patients. TR = transradial; TH = tranhumeral; CF= congenital forearm; N=no; Y= yes; PLP = phantom limb pain; PLS = phantom limb sensation*

			Amputation				
Subject #	**Gender**	**Age (y)**	**Years from amput**	**Side**	**Level**	**PLP**	**PLS**
1	M	65	49	R	TR	N	Y
2	M	30	6	L	TR	Y	Y
3	M	38	15	R	TH	N	Y
4	M	47	4	R	TH	N	Y
5	M	80	1	R	TH	N	Y
6	F	49	2	L	TR	N	N
7	M	21	21	L	CF	N	Y
8	M	48	12	L	TH	N	Y
9	M	56	43	R	TR	N	Y
10	M	60	26	L	TH	N	Y
11	M	60	5	L	TR	Y	N
12	M	39	9	R	TH	Y	Y

1a) MEPs amplitude

Basal corticospinal excitability assessed through MEPs peak-to-peak amplitude (recorded at target muscles, bilaterally) was significantly higher for the brain hemisphere contralateral to the amputation side, independently from the level of amputation

Table 3

BASAL CORTICOSPINAL EXCITABILITY - ANOVA Summary

Effect	df	ChiSq	p
HEMISPHERE	**1**	**9.309**	**0.002**
LEVEL OF AMPUTATION	2	0.203	0.904
HEMISPHERE ⁎ LEVEL OF AMPUTATION	2	4.625	0.099

Note. Generalized linear mixed model with gamma family and log link function.
Note. Model terms tested with likelihood ratio tests method.
Note. Type III Sum of Squares

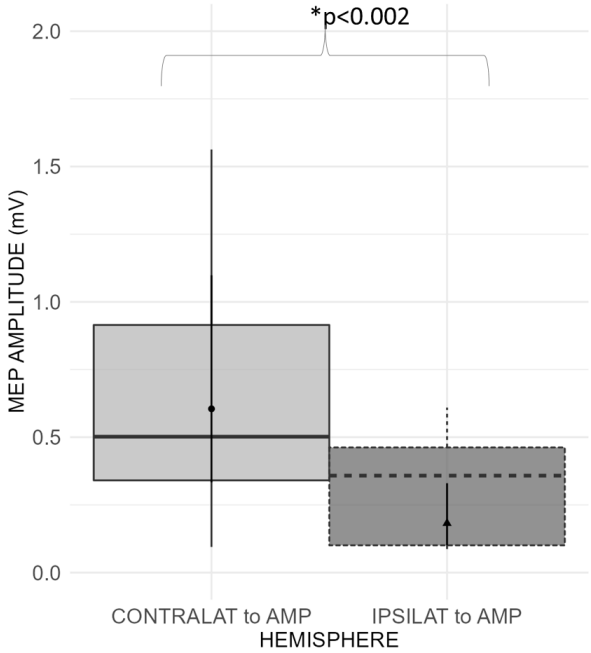


Figure 2: basal corticospinal excitability assessed through MEPs amplitude.
Average data from all the 12 subjects are shown.

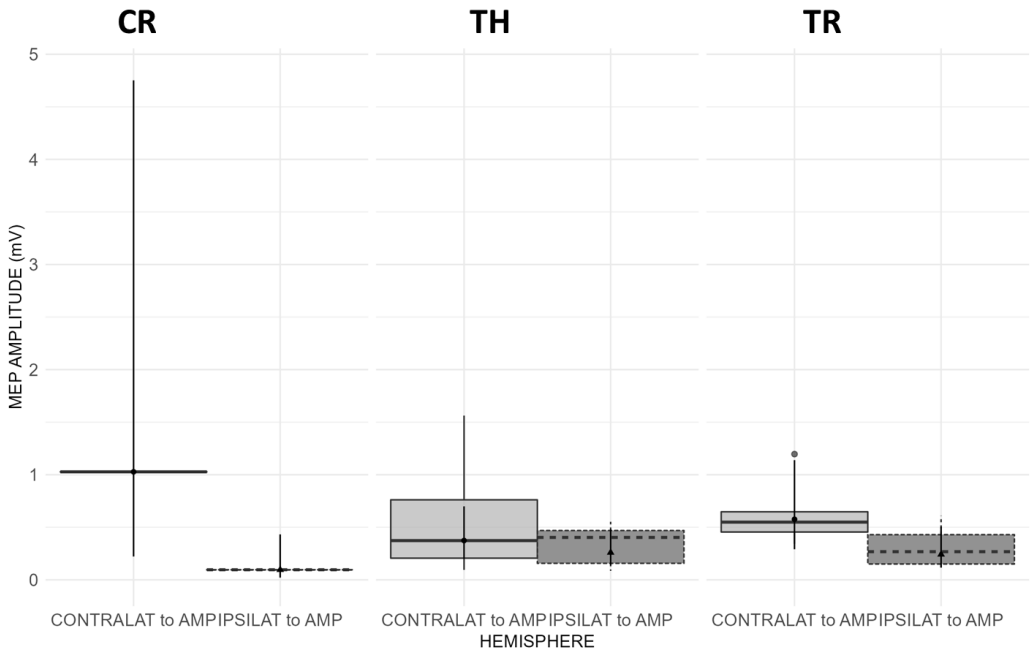


Figure 3 the statistically significant difference in MEPs amplitude between the two hemispheres was was mainly driven by the transradial group

Contrasting the basal corticospinal excitability in TR and TH subgroups reveals that the overall effect is mainly driven by TR patients (TR group: p=0.06, TH group: P=0.28, p values not adjusted)

1b) Motor thresholds (hotspot: target muscle):

- for the hemisphere contralateral to the amputation side, RMT (mean ±SD) was 63, 1± 9,2; mean AMT was 53,0±11,1

- for the hemisphere ipsilateral to the amputation side, RMT (mean ±SD) was 66, 1± 10,1; mean AMT was 51,4±10,3

No statistically significant difference was found between the two hemispheres.

1c) Correlations:

The difference (expressed as %) in corticospinal excitability between the two hemispheres (measured at target muscles) did not correlate with age (Kendall's tau-B -0,023, p=0,932), time after amputation (Pearson's r=0,022, p=0,952), level of amputation (Kendall's tau-B -0,098, p=0,713), side of amputation (Kendall's tau-B -0,025, p=0,925), presence of phantom limb pain (Kendall's tau-B -0,062, p=0,830), presence of phantom limb sensation (Kendall's tau-B -0,031, p=0,916).

2) Acute induction of neuroplasticity (cTBS and PAS10):

2a) cTBS

Demographic characteristics of the sample: the same 12 subjects described above

cTBS protocol results: cTBS (delivered on the brain hemisphere contralateral to the amputated side) induced a significant inhibition of MEPs amplitude (recorded at the target muscles) only for MEPs elicited from the brain hemisphere contralateral to the amputated side. Significant inhibition was still present 10 min after cTBS.

Table 4

cTBS ANOVA Summary

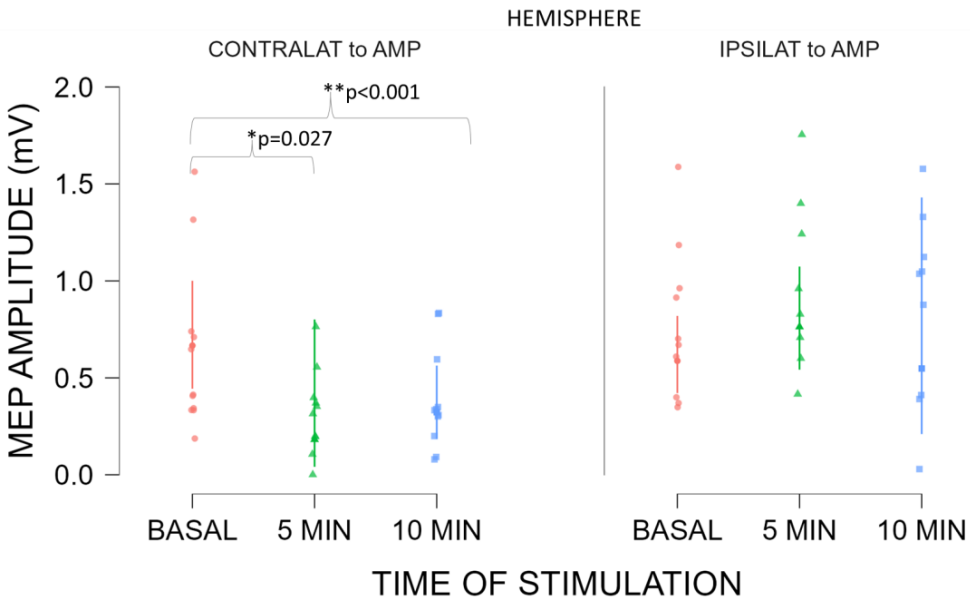
Effect	df	ChiSq	p
TIME OF STIMULATION	2	3.092	0.213
LEVEL OF AMPUTATION	2	2.946	0.229
HEMISPHERE	**1**	**4.284**	**0.038**
TIME OF STIMULATION ✻ LEVEL OF AMPUTATION	4	3.436	0.488
TIME OF STIMULATION ✻ HEMISPHERE	**2**	**6.224**	**0.045**
LEVEL OF AMPUTATION ✻ HEMISPHERE	2	1.500	0.472
TIME OF STIMULATION ✻ LEVEL OF AMPUTATION ✻ HEMISPHERE	4	7.548	0.110

Note. 2 observations were removed due to missing values.
Note. Generalized linear mixed model with gamma family and log link function.
Note. Model terms tested with likelihood ratio tests method.
Note. Type III Sum of Squares

The ANOVA showed a significant difference in MEPs amplitude between the two hemispheres, and a significant interaction between the hemisphere and the time of stimulation.

Post-hoc comparisons showed that there was a significant difference in MEPs amplitude in the hemispheres contralateral to the stimulation site 5 minutes ($p=0.027$) and 10 minutes ($p<0.001$) after cTBS, compared to the basal MEPs amplitude (Bonferroni corrections for multiple comparisons were applied)

Figure 4 cTBS induce a significant reduction in MEPs amplitude from the stimulated M1



2b) PAS 10

Participants: 6 of the 12 subjects, all males, age (mean± SD)=50.1 ± 15.9 years old (range 21-65), level of amputation: 3TH, 2TR, 1CF, years after amputation (mean± SD)=19,7±16,8 (range 5-49) participated to the PAS session. 1 was affected by phantom limb pain (sporadic); 5/6 reported non-painful phantom limb sensation.

No significant modifications in MEPs amplitude and no significant interactions were observed

Table 5

PAS10, ANOVA Summary

Effect	df	ChiSq	p
HEMISPHERE	1	0.721	0.396
TIME OF STIMULATION	2	0.067	0.967
HEMISPHERE ✻ TIME OF STIMULATION	2	0.434	0.805

Note. Generalized linear mixed model with gaussian family and log link function.
Note. Model terms tested with likelihood ratio tests method.
Note. Type III Sum of Squares

3) TMS motor mapping before and after TMR surgery:

Participant: a 35-year-old man, who suffered traumatic transhumeral amputation of the right arm at the age of 23, was enrolled as part of the study "PCR 1/2:New methods in the treatment of limb amputations aimed at the application of bionic prostheses", ongoing at Campus Bio-Medico University of Rome in collaboration with INAIL.

At the time of the interviews, he was wearing a cosmetic prosthesis and a traditional myoelectric prosthesis, unsatisfactorily.

On the 11th of September 2019, he underwent unprecedented surgery, performed at the Orthopedic Unit of Campus Bio-Medico University Hospital, combining osteointegration, Targeted Muscle Reinnervation (TMR) and Targeted Sensory Reinnervation (TSR) surgery in a single time.

In more details, all the following were performed:

- revision of the amputation abutment with implantation of a percutaneous osseointegrated intramedullary prosthetic system in the right humerus (OPRA™ Implant System, Integrum, Sweden)
- TMR surgery

  Nerve transfers:

  • Median nerve to the motor branch for the brachial muscle

  • Ulnar nerve at the entry point of the motor branch of the short head of the biceps

  • Median nerve bundle for the thumb to the medial cutaneous nerve of the forearm

  • Medial fascicle of the deep branch of the radial nerve to the fascicle for the lateral head of the triceps muscle

  • Lateral fasciculus of the deep branch of the radial nerve to the fasciculus for the brachio-radial muscle

- TSR surgery: to recreate an area of exclusive sensitivity for the thumb on the amputation stump, the sensory fascicle for the thumb of the median nerve was transposed to the medial cutaneous nerve of the forearm.

The man participated to a specific rehabilitation protocol without the use of active prosthesis) between November 2019 and April 2020 and to a 1-week rehabilitation cycle in July 2020. Then, due to the ongoing Covid-19 pandemic, he participated (unconstantly, due to technical limitations in electrodes placing) to a home training with remote monitoring.

3a) Corticospinal excitability

The study of brain excitability (average of MEPs amplitude recorded bilaterally for TMS motor maps) revealed that there was a statistically significant difference between the two hemispheres, independently from the time point. The MEPs from all muscle recorded (biceps, triceps, deltoid and trapezius) were included in the analysis.

Table 6

Subject's corticospinal excitability _ ANOVA summary

Effect	df	ChiSq	p
TIME POINT (before surgery, 21m after surgery)	1	0.620	0.431
HEMISPHERE	**1**	**17.457**	**< .001**
TIME POINT ∗ HEMISPHERE	1	2.080	0.149

Note. Generalized linear mixed model with gamma family and log link function.
Note. Model terms tested with likelihood ratio tests method.
Note. Type III Sum of Squares

More specifically, corticospinal excitability of the hemisphere contralateral to the amputation side was significantly higher compared to the opposite hemisphere before TMR surgery. The difference between the two hemispheres was even increased 21m after TMR surgery.

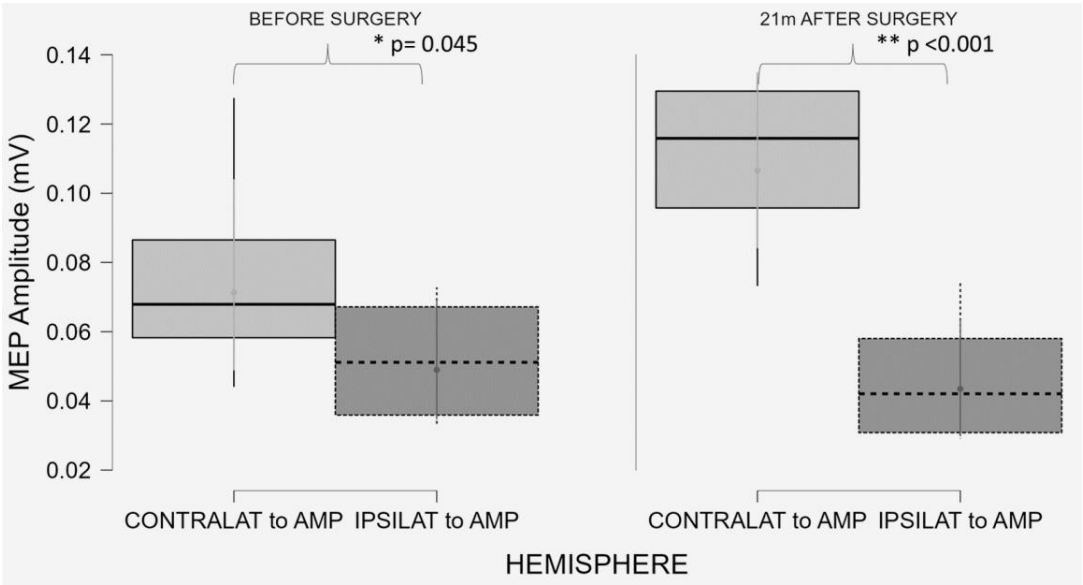
Table 7 post-hoc analyses showed a statistically significant difference between the two hemispheres at baseline and after TMR surgery

Contrasts	**Estimate**	**SE**	**df**	**z**	**p†**
Contralat vs Ipsilat BEFORE SURGERY	0.022	0.010	∞	2.279	**0.045***
Contralat vs Ipsilat 21m AFTER SURGERY	0.063	0.012	∞	5.238	**< .001****

Note. Results are on the response scale.

† P-values are adjusted using Bonferroni adjustment.

Figure 5: statistically significant difference between the two hemispheres before and after TMR surgery. The MEPs recorded for the motor map from all muscles (biceps, triceps, deltoid and trapezius) were included in the analysis.



Then, T-TEST (two tails, unpaired data) was performed on homologous muscles pairs. Results are shown in the table below.

Table 8 t-test comparison between muscles pairs. H=hemisphere

	T-TEST comparisons	p-value
BEFORE SURGERY (Contralat vs Ipsilat)	H Contralat vs H Ipsilat (muscle: biceps)	0,003*
	H Contralat vs H Ipsilat (muscle: triceps)	0,76
	H Contralat vs H Ipsilat (muscle: deltoid)	0,029*
	H Contralat vs H Ipsilat (muscle: trapezius)	<0,001*
21m AFTER SURGERY (Contralat vs Ipsilat)	H Contralat vs H Ipsilat (muscle: biceps)	<0,001*
	H Contralat vs H Ipsilat (muscle: triceps)	<0,001*
	H Contralat vs H Ipsilat (muscle: deltoid)	<0,001*
	H Contralat vs H Ipsilat (muscle: trapezius)	<0,001*
BEFORE SURGERY vs 21m AFTER SURGERY	H Contralat BEFORE vs H Contralat 21m AFTER SURGERY(muscle: biceps)	0,11
	H Contralat BEFORE vs H Contralat 21m AFTER SURGERY (muscle: triceps)	<0,001*
	H Contralat vs H Ip Contralat 21m AFTER SURGERY silat (muscle: deltoid)	<0,001*
	H Contralat vs H Contralat 21m AFTER SURGERY (muscle: trapezius)	<0,001*
	H Ipsilat BEFORE vs H Ipsilat 21m AFTER SURGERY(muscle: biceps)	<0,001*
	H Ipsilat BEFORE vs H Ipsilat 21m AFTER SURGERY (muscle: triceps)	<0,001*
	H Ipsilat vs H Ip Ipsilat 21m AFTER SURGERY silat (muscle: deltoid)	0,23
	H Ipsilat vs H Ipsilat 21m AFTER SURGERY (muscle: trapezius)	<0,001

3b) TMS motor maps

The TMS motor map recorded 21months after TMR surgery compared to the TMS motor map recorded before TMR surgery showed:

- enlargement of the area of biceps, triceps, deltoid and trapezius contralateral to the stimulation side
- volume increase of triceps, deltoid and trapezius contralateral to the stimulation side
- area and volume reduction of biceps, triceps and deltoid ipsilateral to the amputation side
- area and volume increase of the trapezius ipsilateral to the amputation side
- a significant difference between map's volumes (cumulative volumes of biceps, triceps, deltoid and trapezius) between the two hemispheres 21 months after TMR surgery (p= 0.016, volume higher in the hemisphere contralateral to the amputation side)
- x COG lateral shift for biceps and triceps areas bilaterally
- y COG backward shift of the trapezius areas bilaterally

Stimulation intensities was 80% of the maximal stimulator output for both the motor maps recorded before and after TMR surgery.

Table 9 T-Test Comparisons: Maps' Area and Volumes.

		Mean	SD
AreaContraBEFORE	*(mean map area of the 4 muscles BEFORE surgery, Hemisphere contralat to Amp)*	30,7	28,8
AreaIpsiBEFORE	*(mean map area of the 4 muscles BEFORE surgery, Hemisphere Ipsilat to Amp)*	45,5	41,2
AreaContraAFTER	*(mean map area of the 4 muscles AFTER surgery, Hemisphere contralat to Amp)*	70,5	48,4
AreaIpsiAFTER	*(mean map area of the 4 muscles AFTER surgery, Hemisphere Ipsilat to Amp)*	44,1	43,2
VolumeContraBEFORE	*(mean map volume of the 4 muscles BEFORE surgery, Hemisphere contralat to Amp)*	152,4	52,6
VolumeIpsiBEFORE	*(mean map volume of the 4 muscles BEFORE surgery, Hemisphere Ipsilat to Amp)*	117,2	38,0
Volume ContraAFTER	*(mean map volume of the 4 muscles AFTER surgery, Hemisphere contralat to Amp)*	241,6	84,9
VolumeIpsiAFTER	*(mean map volumee of the 4 muscles AFTER surgery, Hemisphere Ipsilat to Amp)*	114,9	49,6

	tTEST
AreaContraBEFORE vs AreaIpsiBEFORE	p = 0,516
AreaContraAFTER vs AreaIpsiAFTER	p = 0,438
AreaContraBEFORE vs AreaContraAFTER	p = 0,085
AreaIpsiBEFORE vs AreaIpsiAFTER	p = 0,962
VolumeContraBEFORE vs VolumeIpsiBEFORE	p = 0,232
VolumeContraAFTER vs VolumeIpsiAFTER	**p = 0,016***
VolumeContraBEFORE vs VolumeContraAFTER	p = 0,088
VolumeIpsiBEFORE vs VolumeIpsiAFTER	p = 0,947

Table 10: description of motor maps parameters changes after TMR surgery

MUSCLE	SIDE	MEASURE	21m AFTER TMR surgery
BICEPS	CONTRLAT to AMP	**Area**	**↑ 100%**
		Volume	**↓ 0.4%**
		x COG	lateral shift
		y COG	forward shift
	IPSILAT to AMP	**Area**	**↓ 26.7%**
		Volume	**↓ 19.1%**
		x COG	lateral shift
		y COG	=
TRICEPS	CONTRALAT to AMP	**Area**	**↑ 277.4%**
		Volume	**↑121,4%**
		x COG	lateral shift
		y COG	=
	IPSILAT to AMP	**Area**	**↓83.1%**
		Volume	**↓45,7%**
		x COG	lateral shift
		y COG	=
DELTOID	CONTRALAT to AMP	**Area**	**↑116,7%**
		Volume	**↑71.7%**
		x COG	=
		y COG	=
	IPSILAT to AMP	**Area**	**↓50.0%**
		Volume	**↓2.1%**
		x COG	lateral shift
		y COG	=
TRAPEZIUS	CONTRALAT to AMP	**Area**	**↑46,3%**
		Volume	**↑92.6%**
		x COG	=
		y COG	backward shift
	IPSILAT to AMP	**Area**	**↑623.1%**
		Volume	**↑94,7%**
		x COG	lateral shift
		y COG	backward shift

Figure 6: graphical representation of motor maps before TMR surgery.
MEPs average amplitude (in mV) for each stimulation point are plotted for each hemisphere. The same scale is used for the colorbar for all muscles.

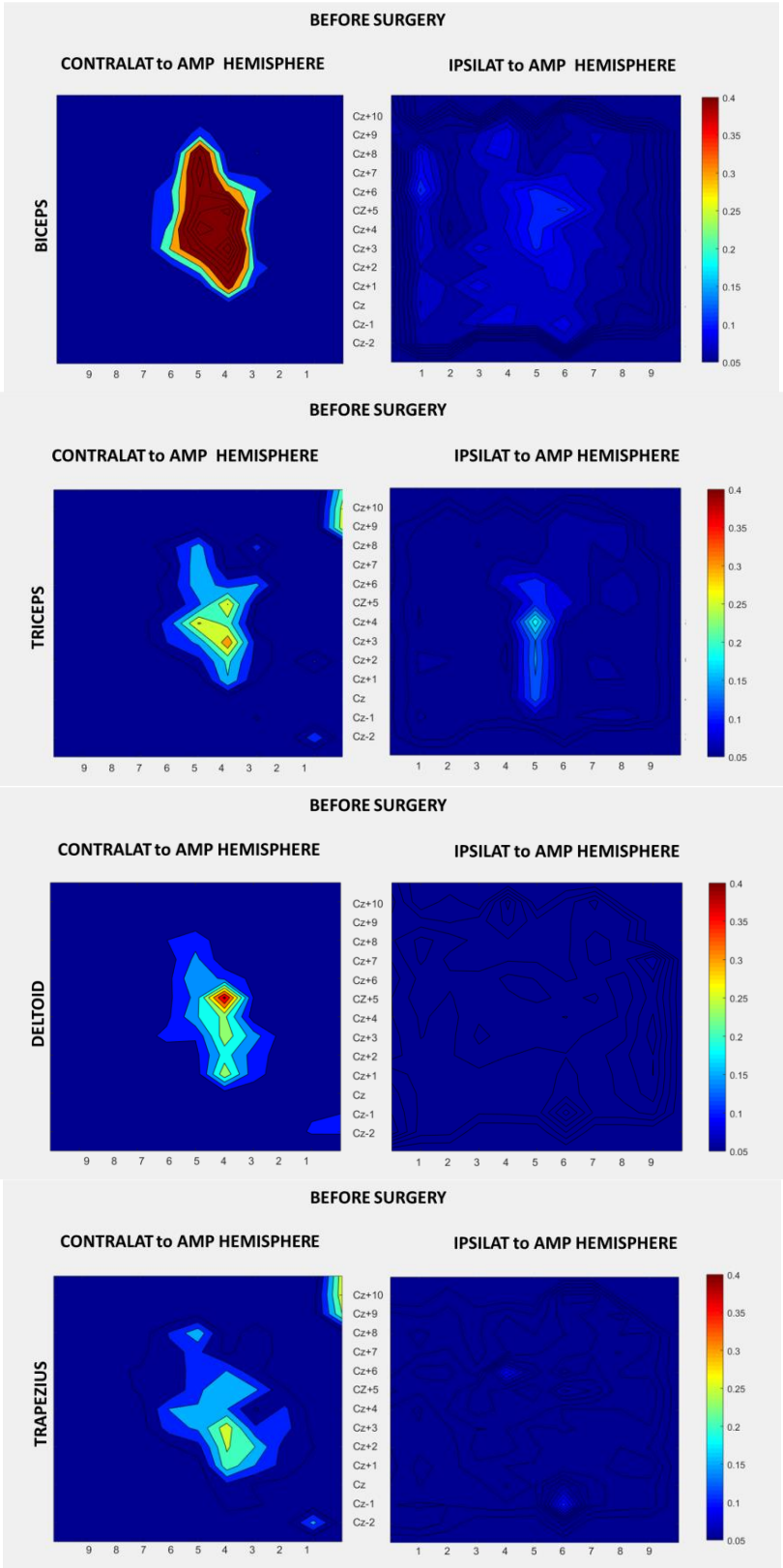


Figure 7: graphical representation of motor maps before TMR surgery.
MEPs average amplitude (in mV) for each stimulation point are plotted for each hemisphere. The same colorbar scale used in the previous map is here used for all muscles.

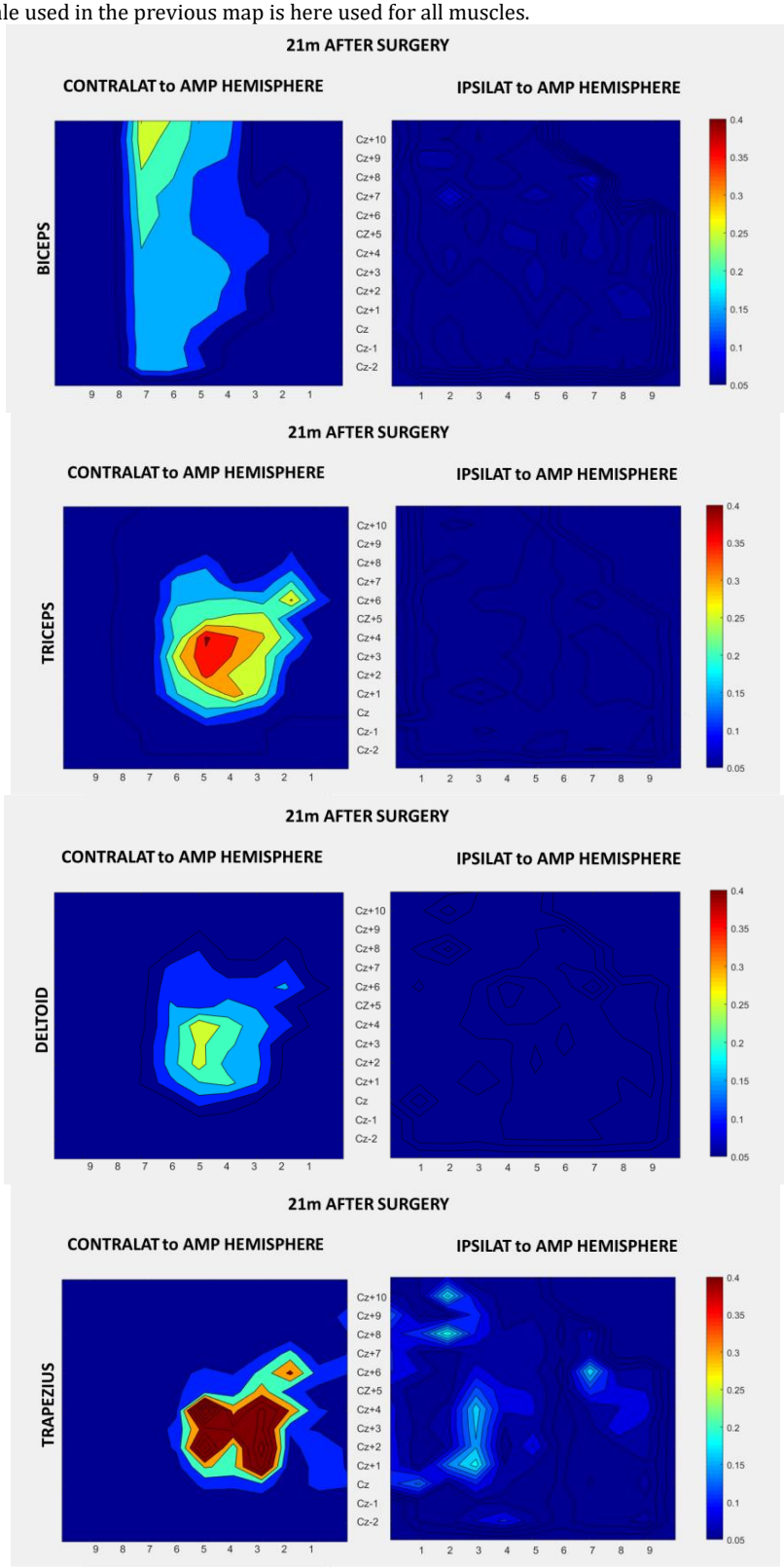


Table 11: quantitative data. TMS motor maps.

MUSCLE	HEMISPHERE	MEASURE	BEFORE TMR SURGERY	21 months AFTER TMR SURGERY	% difference
BICEPS	**CONTRALAT to AMP**	**MEP average amp (mV)**	**0,128**	**0,103**	**-19,6**
		MEP SD	0,245	0,075	
		Area	**33**	**66**	
		Area normalized	0,367	0,564	
		Volume	**242,381**	**241,387**	
		Volume normalized	1,000	0,839	
		x COG	4,387	5,122	
		Y COG	4,444	5,058	
	IPSILAT to AMP	**MEP average amp (mV)**	**0,073**	**0,053**	**-27,4**
		MEP SD	0,018	0,011	
		Area	**90**	**66**	
		Area normalized	1,000	0,564	
		Volume	**153,528**	**124,165**	
		Volume normalized	0,633	0,432	
		x COG	4,524	5,529	
		Y COG	4,104	4,011	
TRICEPS	**CONTRALAT to AMP**	**MEP average amp (mV)**	**0,063**	**0,128**	**+103,2**
		MEP SD	0,097	0,105	
		Area	**31**	**117**	
		Area normalized	0,344	1,000	
		Volume	**126,935**	**281,046**	
		Volume normalized	0,524	0,977	
		x COG	3,599	4,116	
		Y COG	4,851	4,363	
	IPSILAT to AMP	**MEP average amp (mV)**	**0,065**	**0,029**	**-55,4**
		MEP SD	0,033	0,017	
		Area	**71**	**12**	
		Area normalized	0,789	0,103	
		Volume	**145,502**	**78,927**	
		Volume normalized	0,600	0,274	
		x COG	4,300	6,269	
		Y COG	4,566	4,125	
DELTOID	**CONTRALAT to AMP**	**MEP average amp (mV)**	**0,044**	**0,073**	**+65,9**
		MEP SD	0,069	0,074	
		Area	**18**	**39**	
		Area normalized	0,200	0,333	
		Volume	**90,964**	**156,235**	
		Volume normalized	0,375	0,543	
		x COG	3,798	4,206	
		Y COG	3,898	4,170	

	IPSILAT to AMP	**MEP average amp (mV)**	**0,033**	**0,031**	**+6,1**
		MEP SD	0,010	0,010	
		Area	**8**	**4**	
		Area normalized	0,089	0,034	
		Volume	**76,794**	**75,197**	
		Volume normalized	0,317	0,261	
		x COG	4,588	5,680	
		Y COG	4,065	4,012	
TRAPEZIUS	**CONTRALAT to AMP**	**MEP average amp (mV)**	**0,073**	**0,133**	**+82,2**
		MEP SD	0,090	0,206	
		Area	**41**	**60**	
		Area normalized	0,456	0,513	
		Volume	**149,430**	**287,751**	
		Volume normalized	0,617	1,000	
		x COG	3,535	3,582	
		Y COG	4,547	3,821	
	IPSILAT to AMP	**MEP average amp (mV)**	**0,037**	**0,074**	**+100**
		MEP SD	0,025	0,048	
		Area	**13**	**94**	
		Area normalized	0,144	0,803	
		Volume	**93,124**	**181,314**	
		Volume normalized	0,384	0,630	
		x COG	3,869	6,123	
		Y COG	5,080	4,192	

4) Safety reporting:

TMS stimulation, neuromodulation protocols (cTBS, PAS10) and TMS motor mapping were well tolerated. No adverse effects were reported by patients.

DISCUSSION AND CONCLUSIONS:

We studied through TMS the motor cortex output in subjects with chronic mULA who were using active prosthetic devices.

We were interested in assessing the basal corticospinal excitability of the two brain hemispheres and the response to two acute inhibitory neuromodulation protocols (cTBS and PAS10) applied on M1.

The **demographic characteristics** of our sample - composed of 12 subjects affected by chronic mULA, of which 11 males and mean age 49.6±17.19 years - reflect literature epidemiological data, according to which mULAs mostly affect middle-aged men (Maduri and Akhondi, 2022). The low prevalence of PLP in our sample (3/12) could be related to the use of active control prostheses (Lotze et al. 2009), required by our inclusion criteria, and to the time after amputation (Bosmans et al. 2010). Even if the only female of our sample was affected by PLP, a recent literature metanalysis did not find a correlation between gender and PLP (Limakatso et al. 2020).

The results of the basal **corticospinal excitability** assessment showed that the MEPs recorded from the target muscle (biceps or trapezius, according to the amputation level) elicited from the hemisphere contralateral to the amputation side were significantly higher in amplitude compared to the opposite side, independently from the amputation level. These results are in line with previous studies on smaller groups of patients (Cohen et al. 2011, Hall et al. 1990, Pascual-Leone et al. 1996). They are also in line with one study (Roricht et al. 1999) on 15 patients of mean age 60.9±9.7 years (higher than the mean age of our sample, $p<0.01$) tested at least 22 years after mULA (mean 38.7±17.5 years), that is a longer interval compared to our study ($p<0.01$), where the mean interval between amputation and TMS study was of 12,6±12,1 years. Therefore, our findings confirm and extend to a sample representative of a younger population previous observations of higher corticospinal excitability in the hemisphere contralateral to the amputation side.

Even if there was no statistically significant interaction at the ANOVA between the factors hemisphere and level of amputation ($p=0.09$), the direct contrast between the basal

corticospinal excitability in TR and TH subgroups suggested that the overall effect (higher corticospinal excitability in the hemisphere contralateral to the amputation side) is mainly driven by TR patients (*TR group: p=0.06, TH group: P=0.28, p values not adjusted*).

A **different reorganization pattern in patients with more proximal arm amputations,** consisting in balanced brain excitability or reversed pattern of corticospinal excitability (i.e. higher in the hemisphere ipsilateral to amputation), has been observed in amputees and linked to stronger ipsilateral corticospinal inputs into the motoneuron pools of more proximal muscles (Roricht et al. 1999, Freund et al. 1985). Moreover, it is known from healthy subjects that proximal muscles of the upper limb have bilateral corticospinal innervation, which may contribute to synergies during upper limb reaching tasks (Bradnam et al. 2010). Indeed, the outputs from the ipsilateral M1 project bilaterally to alpha motoneurons innervating homologous proximal muscles bilaterally (Kuypers 1964; Lemon 2008). Further, ipsilateral MEPs have a longer latencies than contralateral MEPs in proximal muscles (Alexander et al. 2007; Bawa et al. 2004; MacKinnon et al. 2004), indicating that ipsilateral MEPs are mediated by indirect pathways such as the ipsilateral reticulospinal tract or cervical propriospinal system (Chen et al. 2003; Ziemann et al. 1999). Also, projections from ipsilateral M1 converge onto interneurons in the spinal cord in common with those from the contralateral corticospinal tract (Kuypers 1964; Lemon 2008; Bradnam et al. 2010).

In contrast with MEPs amplitudes, the **difference in RMTs between the two hemispheres was not statistically significant.**

MEPs' amplitude reflects the excitability of the motor cortex, the number of recruited motoneurons, the integrity of the corticospinal tract, spinal and peripheral nerve excitability. Instead, RMTs provide information about a central core of neurons in the muscle representation at the level of the motor cortex, and are likely to reflect neuronal membrane excitability, as well as non-N-methyl-D-aspartate receptor-mediated glutamatergic neurotransmission (Nardone et al. 2015). Significantly different MEPs' amplitudes and no

significant changes in RMT suggest that there could be a subcortical, spinal, contribution to the changes in corticospinal excitability. Input-output curves, high-density EMG and H-reflex studies could be used to disentangle the relative contribution of motor cortex and spinal circuits to the corticospinal excitability.

In our sample, the difference (expressed as %) in corticospinal excitability between the two hemispheres (assessed through MEPs amplitude of the target muscles) did not **correlate** with age (Kendall's tau-B -0,023, p=0,932), time after amputation (Pearson's r=0,022, p=0,952), level of amputation (Kendall's tau-B -0,098, p=0,713), side of amputation (Kendall's tau-B -0,025, p=0,925), presence of phantom limb pain (Kendall's tau-B -0,062, p=0,830), presence of phantom limb sensation (Kendall's tau-B -0,031, p=0,916). In literature, a correlation between PLP and sensorimotor cortical reorganization following arm amputation has been described, first by Flor et al. (1995) by magnetoencephalography, although a number of studies have reported no relationship between reorganization, pain and deafferentation (Jutzeler et al. 2015). These heterogeneous results have been related to methodological differences in the assessment of cortical reorganization (Andoh et al. 2020). One TMS study on 6 subjects found that MEPs in the hemisphere contralateral to the amputation side were higher in amputees with PLP compared to amputees without PLP (Karl et al. 2001), but several TMS map studies did not found a significant correlation between cortical mapping and intensity of PLP (Gunduz et al. 2020).

As second aim, we tested the **acute response of the hyperexcitable motor cortex to acute neuromodulation interventions** applied to the primary motor cortex contralateral to the amputation side (cTBS and PAS10, performed at least 1 week apart).

MEPs were recorded at the target muscles at three-time points (before neuromodulation, 5 minutes after neuromodulation, 10 minutes after neuromodulation).

All 12 subjects participated in **the cTBS protocol**. cTBS, delivered according to the standard protocol (Huang et al. 2005), induced a significant decrease in the corticospinal excitability

of the target muscle up to 10 minutes after stimulation, as shown by a reduced amplitude of the MEPs elicited from the hemisphere contralateral to the amputation side (*at the ANOVA p=0.038 for the factor hemisphere and p=0.045 for the interaction time of stimulation*hemisphere; post-hoc comparisons: significant difference in MEPs amplitude elicited from the hemispheres contralateral to the stimulation site 5 minutes (p=0.027) and 10 minutes (p<0.001) after cTBS, Bonferroni corrections for multiple comparisons were applied*).

cTBS is a noninvasive neuromodulation protocol that has been shown to decrease up to 1 hour the excitability of the targeted area (hand muscles) in healthy subjects, with a mechanism of action intrinsic to the motor cortex (Huang et al., 2005).

cTBS effects have also been described on the non-stimulated M1 in healthy subjects, with non-congruent results. Ishikawa et al. (2007) found reduced MEPs in both target and contralateral M1, while Stefan et al. (2008) reported that cTBS reduced MEPs in the target M1 but increased them in the non-stimulated M1. Suppa et al. (2008) studied more deeply the effects of cTBS on contralateral non-stimulated M1, using a stimulation intensity of 80% AMT (as in Ishikawa) and two orientations of the TMS coil: PA (posterior-to-anterior) and AP (anterior-to-posterior). PA stimulation (the same used by Ishikawa and Stefan and in the present study) had no significant effects on the non-stimulated M1 (in contrast with Ishikawa and Stefan, but in line with our results). Instead, AP cTBS stimulation increased MEPs and reduced SICI in the non-stimulated M1, possibly due to lasting changes in the ongoing activity of interhemispheric connections (reduction in the amount of ongoing inhibition or increase in facilitation) and, regarding SICI reduction, to an increase in the net facilitation (or reduced inhibition) from the stimulated M1 (Suppa et al. 2008).

No previous studies on the effects of cTBS in amputees have been published, to the best of our knowledge.

Our results (reduced corticospinal excitability only in the stimulated M1) support the view that the response to cTBS of both, the stimulated and non-stimulated M1 in amputees has a

similar behaviour than that observed in healthy subjects (Huang et al. 2005, Suppa et al. 2008), suggesting that intracortical excitatory and inhibitory networks related to cTBS are, almost in part, preserved in amputees. The clinical impact of these results must be clarified by further data. At this time, our results suggest that the imbalance in corticospinal excitability between the two hemispheres in chronic mULA can be transiently reversed through cTBS, a neuromodulatory intervention which acts on cortico-cortical monosynaptic ptojections to the corticospinal neurons (Di Lazzaro et al. 2010) . If this imbalance is maladaptive, then cTBS stimulation might have a positive impact on PLP and functional restoration.

Very little data exist about the effects of cTBS on proximal muscles in healthy subjects: one study (Bolton et al. 2012) found that cTBS over the motor region of the distal hand muscles led to a focal attenuation on the targeted hand responses, with negligible impact on the proximal arm muscles. Another study, although using a different experimental setting (motor task), found that cTBS of M1 can alter the excitability of neurons controlling ipsilateral proximal musculature and degrade ipsilateral upper limb motor control (Bradnam et al. 2010).

Studying cTBS effects on proximal muscles, with different coil orientations and with longer time point of assessments, will help to clarify and strengthen our results.

Regarding the absence of significant MEPs amplitude modifications after the **PAS10** protocol, no definite conclusions can be drawn at this time, due to the limited number of subjects included and to the lack of normative data from proximal muscles.

A limitation of the our study of acute M1 neuromodulation through cTBS and PAS10 protocols is the non-controlled study design.

Lastly, we assessed the **motor maps** of one subject before and 21 months after TMR surgery. The subject, a 35-year-old man who suffered traumatic transhumeral amputation of the right arm at the age of 23, had, at baseline, higher corticospinal excitability in the

hemisphere contralateral to the amputation side (*as evidenced by the ANOVA in which it was included the MEPs' amplitude of all the 4 muscles studied bilaterally: biceps, triceps, deltoid, trapezius, p<0.001, and as evidenced by t-test comparisons between homologous muscle couples, showing a significant difference at baseline between MEPs' amplitude of biceps, trapezius, deltoid and their homologous contralateral, p<0,001*).

Our subject had a negative history for PLP and reported non-painful PLS. Therefore, the imbalance in corticospinal excitability was not accompanied by PLP.

21 months after surgery, the imbalance in corticospinal excitability was still present (*as shown by the ANOVA in which it was included the MEPs' amplitude of all the 4 muscles, with significant factor hemisphere p<0.001 and no interaction between hemisphere and time point, and by t-tests comparisons between muscles pairs, showing highly significant differences in MEPs amplitude between all homologous muscles pairs at 21 months, p<0,001*).

To compare corticospinal excitability between the two-time points, we run unpaired, two-tailed t-tests between the MEPs' amplitudes of homologous muscle pairs before and after TMR surgery, which showed a significant increase in corticospinal excitability for all muscles (p<0,001) except for the biceps contralateral to the amputation site (p=0,11) and for the deltoid ipsilateral to the amputation site (p=0,23).

To further characterize the changes in corticospinal excitability, we computed for each muscle the following parameters: map area (= number of scalp positions which, on being stimulated with equal intensities, elicited MEPs), map volume (= sum of all MEPs average normalized amplitudes) and COG, calculated by using the MEPs average normalized amplitude and their position on the map, creating an amplitude-weighted mean of the map (Gunduz et al. 2020).

The differential behaviour of the biceps contralateral to the amputation site (whose average MEP's amplitude was not statistically different at 21m compared to baseline p=0,11) can be better understood considering that the short head of the biceps was reinnervated by ulnar

nerve. The motor map of biceps contralateral to the amputation side underwent an increase of 100% compared to baseline, while the map volume remained relatively stable, thus indicating that the MEPs were of similar amplitude, but the muscular response could be elicited by a doubled area (x2 compared to the baseline). A similar pattern was shown by the deltoid ipsilateral to the amputation side.

At 21m after TMR surgery, the area of biceps, triceps, deltoid and trapezius contralateral to the amputation side were enlarged, however with no significant change detectable through the unpaired t-test comparisons, which included all the 4 muscles of each hemisphere (p=0,08), probably due to the low number of observations. Map volumes of triceps, deltoid and trapezius contralateral to the stimulation side also increased at 21m after stimulation, while there was an area and volume reduction of biceps, triceps and deltoid ipsilateral to the amputation side. Unpaired t-test comparison between maps' volumes showed a significant difference in the volume of the map between the two hemispheres at 21m after TMR surgery (*p = 0,016*). Biceps' and triceps' x COG had lateral shift bilaterally, while trapezius' y COG had a backward shift, bilaterally. No quantitative COG analysis was possible due to the number of observations. Graphical representation of the maps suggests that the motor map of biceps, triceps and trapezius on the hemisphere contralateral to the amputation side were mostly changed after TMR surgery.

This is the first TMS study after TMR surgery. Overall, our observations on TMS motor maps cannot be generalized, including only one subject and only two-time points. Given these premises, the major evidence is that after TMR surgery the imbalance in the corticospinal excitability between the two hemispheres was not reduced, as might have been expected. Indeed, Chen et al. (2013) in high-density electroencephalography (EEG) study on 2 patients who underwent TMR surgery, evidenced that after TMR there was an effective restoration of peripheral function, which was linked to the return to a more physiological cortical expression for the missing limb. A fMRI study in one subject showed that graft of both hands reversed the functional reorganization of the limb cortical map 4 years after

amputation (Giraux et al. 2001). Change in motor thresholds could partly influence our results (Gagné et al. 2011). The stimulation intensities were the same for the two recording sessions (80% of the maximal stimulator output).

The motor maps presented here have been recorded 21m after surgery, in the absence of PLP. Therefore, PLP seems not related to the persistence of the imbalance in corticospinal excitability.

A role in the increased corticospinal excitability of the hemisphere contralateral to the amputation side could have been played by the intensive rehabilitation protocol started by the patient after surgery. The rehabilitation protocol included a first phase finalized at improving the integration of the bone-implant that lasted about 7 months and involved a task of lifting increasing weights (from 50g to 1Kg) attached to the osseointegrated implant and a task of applying pressure on a scale. This first rehabilitation phase was concluded about 6 months before our TMS map recording session. After the first rehabilitation phase, the patient started a daily home-based training focused on the affected limb, with virtual reality and EMG superficial electrodes. The home training included motor exercises with the amputated limb (100 repetitions/day): elbow flexion, supination of the wrist, hand closing, elbow extension, pulse pronation, hand opening. The patient was following, with low consistency, the home-based training in the weeks before our TMS motor maps study, with some issues related to a co-contraction between hand closing and elbow flexion, and a generalized activation of the EMG electrodes after the request of hand opening. It is poorly understood how the nervous system responds to chronic muscular training. Little literature data support that chronic exercise could increase corticospinal excitability, probably through a spinal mechanism (Philpott et al. 2015). However, another study did not find significant changes in motor maps after chronic training (Maeo et al. 2021).

TMR itself caused temporary deafferentation, followed by reafferentation from transferred nerves, in non-physiological positions. Brachialis, brachioradialis, biceps and triceps muscles were reinnervated by median, radial, ulnar and radial nerve, respectively. This

change in motor control requires a central adaptation to altered peripheral connections (Malessy et al. 2003).

Therefore, it is plausible that the neuroplastic changes we observed are primarily the epiphenomenon of the recent widespread reinnervation, and reflect plastic phenomena both in the sensory input and motor output activity that take place to allow the control of the reinnervated muscles (Shen 2022).

Our results do not allow us to determine the level of reorganization (i.e., cortical or subcortical). However, they are compatible with an increased density of corticomotoneuronal (CM) connections targeting reinnervated muscles and muscles proximal to the stump. The analysis of maps recorded at multiple time intervals after TMR surgery ad of MEPs latency data (recorded, here not presented) and further follow-up recordings will allow to better clarify the outcome of TMR surgery on the corticospinal output in our patient. fMRI data could add meaningful information. Similar studies in other subjects are necessary for the generalizability of the results. The potential effects of central nervous system stimulation in improving the outcome of peripheral nerve transfers could be further explored.

In conclusion, replicating with prostheses the capacity of the human arm is still a long way to go. Advancing our understanding of how the nervous system changes after mULA can give major contributions to technical advancements and to the optimization of rehabilitation protocols.

Conflicts of Interest

None

Ethical And Regulatory Considerations

Declaration of Helsinki: This study has been conducted in accordance with the principles of the Declaration of Helsinki.

Approvals: The protocol, informed consent form and participant information sheet received written approval by the Ethics Committee of Campus Bio-Medico University of Rome ("AMP-PLAST15" protocol)

Participant's Confidentiality: Participants' anonymity has been maintained. The study complied with the General Data Protection Regulation (GDPR) (EU) 2016/679

Discontinuation/Withdrawal of Participants from Study: Each participant had the right to withdraw from the study at any time.

Expenses and Benefits: Participants travel expenses have been reimbursed.

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