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# Peripheral stimulation affects subthreshold Triple Stimulation Technique

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## ARTICLE INFO

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## ABSTRACT

*Background:* Compared to conventional transcranial magnetic stimulation (TMS), the triple stimulation technique (TST) strongly decrease the effects of desynchronization of descending discharges and accompanying phase cancellation that follow TMS and offers a more sensitive method to quantify motor evoked potentials (MEPs).
*New method:* Using the TST, we explored as to whether sub-threshold TMS evokes peripheral motor neuron discharges (MNs). We compared the number of MEPs elicited by TMS and by TST in fifteen healthy participants. We used the subthreshold intensity of 80 % resting motor threshold. To control the TST assessment of the corticospinal tract, we included a peripheral stimulation control condition, which consisted of peripheral stimulation alone, in a subgroup of five volunteers.
*Results:* Compared to TMS, TST at sub-threshold intensities did not detect significantly more responses unequivocally attributable to the cortical stimulation. In contrast, the peripheral supra-maximal stimuli produced confounding effects in the TST condition that were, in part, indistinguishable from cortical responses.
*Comparison with existing methods:* At subthreshold TMS intensities, the TST does not detect more discharges of spinal MNs than conventional TMS and, in addition, it is confounded by effects from peripheral stimulation.
*Conclusion:* The TST can be useful in assessing the integrity of the MN pool and of the corticospinal tract. However, if used at near threshold intensity, the confounding effects of peripheral stimulation need to be considered; for instance, in paired-pulse stimulation paradigms assessing the cortical physiology.

## 1. Introduction

Paired-pulse stimulation paradigms allow the investigations of the cortical physiology of the motor system. Conditioning- and test-pulse intensities are set in reference to the individual motor threshold (MT) in order to account for the inter-subject variability in cortico-motor excitability. In such paradigms, the first subthreshold conditioning stimulus is considered to activate the intra-cortical interneurons, which modulate the response of the MN to the subsequent (second) supra-threshold test stimulus and determine the size of the MEP (Motor Evoked Potential). (Hallett, 2007). However, there is still a matter of debate as to whether this modulatory interaction between the paired pulses occurs exclusively at cortical/ subcortical or whether it can also occur at the spinal level (Reis et al., 2008).

Several factors affect the size and variability of MEPs evoked by TMS. Among them, the desynchronization of the descending action potentials (AP) along their trajectories leads to phase cancellation and results in smaller MEPs, because negative and positive phases of APs cancel each other (Magistris et al., 1999, 1998; Rösler et al., 2002, 2000). Magistris and colleagues (1998) developed the triple stimulation technique (TST) that strongly decrease these effects of desynchronized MN discharges and offers a more sensitive method to quantify motor evoked potentials (MEPs) in comparison with conventional TMS.

The TST is a collision method in which three successive stimuli are delivered: a first one, over the cortical motor hot spot, a second one, over the ulnar nerve at the wrist, and a third one, over the brachial plexus at Erb's point (see Fig. 1). When, in intact corticospinal pathways, all spinal MNs are activated by TMS delivery, the descending discharges collide with the ascending action potentials arising from the simultaneous peripheral wrist stimulation, cancelling each other. Under these circumstances, the depolarization caused by a third stimulation at Erb's point generates a complete M-wave (compound muscle action potential). This is different in the case of a lesion of the corticospinal tract or in the case of a subthreshold stimulation. Under these circumstances, not

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all the ascending action potentials are being cancelled out, which subsequently collide with the action potentials from stimulation at Erb's point. The result is a reduced amplitude of the M-wave.

In the TST stimulation a) TMS leads to desynchronized discharges of two MNs. b) Supramaximal wrist stimulation leads to an activation of all three MNs supplying the target muscle (ADM) and action potentials (APs) propagate in a synchronized manner antidromically (away from the ADM muscle) as well as orthodromically (towards the ADM muscle): this leads to the direct wrist response (activation of ADM). Two of the three antidromically propagating APs collide with the two descending APs that were elicited by TMS, thus canceling each other. c) Shortly before the remaining AP from the wrist stimulus arrives at the Erb's point, the third stimulus is delivered at the Erb's point. Again, all MNs supplying the target muscle will be activated, and APs propagate orthodromically and in synchrony towards the muscle. As a consequence, one orthodromically propagating AP from Erb's point will be cancelled out by the antidromic AP elicited by the wrist stimulus, while the remaining two APs will descend to the muscle and evoke the TST response. Since the third peripheral stimulus at the Erb's point activates MNs synchronously, a re-synchronization of the asynchronously elicited action potentials occur: a re-synchronization of those APs that originated in the spinal anterior horn cells in response to TMS. In other words, the same number of motor units are activated with TST or with conventional TMS but synchronously (in the case of TST) and not asynchronously (in the case of TMS).

Rösler et al. (2002) demonstrated that TMS-induced desynchronization caused one third of the reduction in MEP amplitudes during TST. Therefore, the TST is considered to be more sensitive and a better technique, when it comes to estimations of the amount of activated MNs (Rösler et al., 2008). These facts raise questions about the precise nature of MT and about their use in stimulation paradigms; for instance, in paired-pulse stimulation paradigms. Since, to calculate the MT, TMS stimuli of various intensity are delivered to the motor hot-spot in order to find the intensity needed to have 50 % of chances to have a response of more than 50 μV, it is possible than TMS at lower intensities can stimulate the peripheral neurons but that the response elicited will not reach the intensity of 50 μV due to the desynchronization. TST could unmask such responses.

The goal of this study was to explore cortico-motor excitability using the TST as compared to conventional TMS and included a control condition for the TST. In particular, we tested as to whether commonly used subthreshold stimulation intensities can excite spinal MNs. Such excitation would challenge the current hypothesis on the origins of the inhibitory and excitatory paired-pulse phenomenons entirely (i.e., inhibition of excitation form intra-cortical population of interneurons, without significant participation of spinal cord or peripheral phenomenons). (Chen et al., 2008)

## 2. Methods

Fifteen healthy subjects (8 men; mean age 25.13 ± 2.27 SD years) without psychiatric or neurological disorders participated in the present study. They were all right-handed, according to the Edinburgh Handedness Inventory (Oldfield, 1971). All participants gave their written informed consent prior to their inclusion in the study. This study, which conformed to the principles of the Declaration of Helsinki, was approved by the local ethics committee (protocol 311, 2011).

We determined the conventional resting MT with TMS ($rMT_{TMS}$) and then randomly performed a total of twelve trials for each TMS and TST at a sub-threshold intensity of 80 % $rMT_{TMS.}$ In five subjects, we added a control condition to the peripheral stimulations with TST (stimulation of the ulnar nerve at the wrist and the brachial plexus) without TMS.

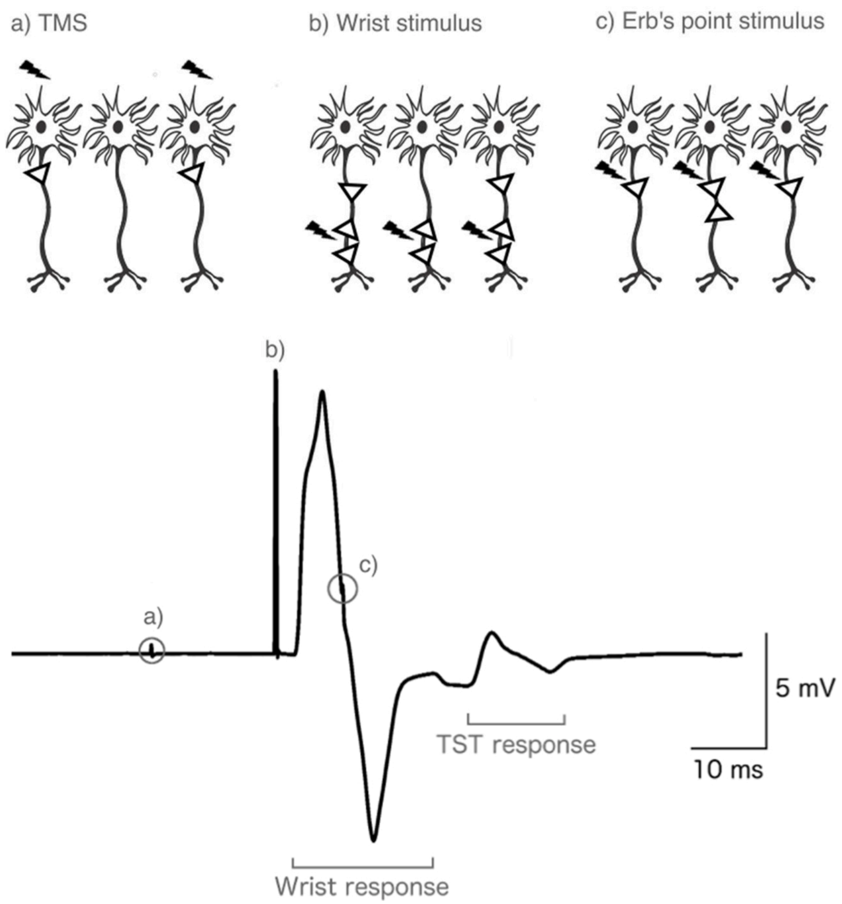

**Fig. 1.** Example of a pathological Triple Stimulation Technique stimulation in an MS patient. Recordings were obtained from an MS patient recorded at our clinic. We recorded the abductor digiti minimi (ADM) using the same methodology described in the study except for the TMS stimulation, which was delivered with a round coil at 100 % of the stimulator output. In the superior part of the picture, an illustration of a neuron is displayed. The black lightning bolt represents the magnetic or electrical stimulation, while white arrows represent moving action motor potentials and their direction. In the inferior part of the picture, the recording of the ADM is displayed with the artifact due to the TMS stimulus a), followed by the artifact due to the wrist stimulation b) and the resulting M-wave. Then the artifact of the Erb's point stimulation c) and the resulting M-wave.

### 2.1. *EMG recordings*

The EMG recordings were obtained from the right abductor digiti minimi (ADM) muscle, with conventional surface electrodes in a belly-tendon montage. The signals were amplified, band-pass filtered (1 Hz – 5 kHz), sampled at a rate of 25 kHz and stored for off-line analysis. We used a custom-built amplifier with an overall gain of 1000 (Sci-Consulting, Switzerland). Throughout the experiment, to ensure the absence of artifacts, the baseline was monitored through visual feedback on the Nicolet Viking apparatus (Nicolet Biomedical, Madison, WI, USA). Nevertheless, potential 50 Hz artifacts were identified and removed based on a Fourier transformation analysis of the digitized signal. In contrast to a conventional Notch filter, we applied a filter that allows the complete removal of the 50 Hz artifacts without altering the other signal components. The custom-made acquisition and pre-/post-processing software were written in LabVIEW by Sci-Consulting, Switzerland, and by Nguyet Dang, NIH, Bethesda, MD).

### 2.2. *Peripheral nerve stimulation*

The ulnar nerve was stimulated both at the wrist and proximally at the Erb's point. At the wrist, a bipolar stimulation electrode was taped over the ulnar nerve, proximal to the pisiform bone with the cathode (diameter 0.8 cm) in 8 cm distance from the active electrode over the ADM muscle belly, and the anode proximal from the cathode in a fixed 2 cm distance. At the Erb's point, a monopolar handheld small cathode was slightly pressed on the skin over the brachial plexus (diameter 0.8 cm) and a large anode electrode (surface 80 $cm^2$) was taped over the scapula. The ulnar nerve and the brachial plexus were stimulated at supramaximal intensities eliciting maximal M waves (Magistris et al., 1998; Roth and Magistris, 1987). To determine supramaximal intensities, the stimulation intensity was gradually increased, up to a value at which the M wave amplitude did not grow any further. The stimulation intensity of the peripheral nerves was then set at 120 % of the minimal intensity needed to elicit the maximal M-wave, individually calculated for each nerve and each single subject. All fingers of the participants' right hand but the thumb were taped together and their hands held in place by linen sheets.

### 2.3. *Transcranial magnetic stimulation*

The TMS was applied over the hand area of the left motor cortex through a circular, 90 mm hand-held coil, using a Magstim 200 monophasic stimulator (Magstim, Whitland, UK). Current flow in the coil was oriented counter-clockwise seen from above with the handle pointing posterior, 0° to the midline. The site where the largest MEPs could be evoked ("motor hot spot") was determined as follows: MEPs of 0.5–1 mV were first elicited at an initial estimate 5 cm lateral and 1 cm frontal to Cz. Additional three TMS pulses were then delivered at each of four sites around the initial estimate, 1 cm frontal, posterior, medial, and lateral. To ensure the correct positioning of the coil throughout the experiment, the motor hot spot was marked with a pen over a cap the patient had to wear during the experiment. Threshold estimations were performed according to the software-based maximum likelihood threshold-hunting procedure described by Awiszus (2003) (Motor Threshold Assessment Tool, MTAT, version 2.0: http://www.clinicalresearcher.org/software). This 'adaptive method' estimates the probability to evoke a MEP at a given stimulation intensity and is presumed to be more accurate, with the same number of trials, than the classical 'relative frequency' procedure (Groppa et al., 2012; Rossini et al., 2015). To determine the rMT, a MEP $\geq 50$ μV peak-to-peak amplitude was fed back to the software as a valid response.

### 2.4. *Triple stimulation technique*

Triple stimulation was triggered by a commercially available software package for the Nicolet Viking apparatus (Nicolet Biomedical, Madison, WI, USA). Delays between the stimuli were calculated as follows: Delay I = ['minimal MEP latency' *minus* 'M-wave$_{wrist}$ latency']; Delay II = ['M-wave$_{Erb}$ latency' *minus* 'M-wave$_{wrist}$ latency'] (Magistris et al., 1998). Background EMG activity was assessed by visual inspection prior to each trial.

The control condition consisted of a triple stimulation with a pseudo-sham TMS stimulation: The stimulation coil was hand-held over the scalp, exactly as it was done during the test stimulation, but this time with the stimulator output set to 0%. Pseudo-sham TMS was followed by supramaximal ulnar stimulation at the wrist that it was in turn followed by a supramaximal stimulation of the brachial plexus at Erb's point. Between stimulations, the same delays as in the conventional TST-condition were used.

### 2.5. *Direct and indirect responses*

The single pulse supra-threshold TMS generates a direct response: a motor evoked potential (MEP). This MEP results from single, and under certain circumstances, from repetitive motor neurons discharges (Magistris et al., 1998; Z'Graggen et al., 2005). The supra-maximal stimulation of the ulnar nerve at the wrist or at Erb's point evokes a direct response: a compound motor action potential (CMAP), and also an indirect late spinal response, an F-wave, which arises from the antidromic depolarization and the reactivation of a few proximal axons.

### 2.6. *Analysis*

For analyses, we differentiated the responses depending on their latencies: a) early responses (E), presumed to be either direct responses from cortical stimulation (i.e. TMS) or the stimulation of the brachial plexus (Erb's point), and b) delayed responses (D), presumed to correspond to late indirect spinal responses from the supramaximal stimulation of the Erb's point. Furthermore, we defined as intermediate responses (I) all those responses that could be classified as either direct or indirect if they followed a proximal stimulation at the Erb's point and occurred within a short delay. The timeframe for responses was defined from the shortest F-wave latency and the longest duration of the CMAP$_{Erb}$ (brachial plexus stimulation). We calculated an approximate timeframe of 4 ms as follows:

[CMAP$_{Erb}$ duration *minus* the minimal latency of F-waves] (See Fig. 2).

The shortest latency expected for the F-waves deriving from the plexus stimulation was estimated as follow F wave latency$_{ERB}$:l [F-wave latency$_{wrist}$ minus the difference between the delays of the M-wave derived from the stimulation at the brachial plexus and at the ulnar nerve].

The delay of 4 ms separating E- from I- responses was chosen in a conservative way, by comparing this delay to the one of the 2 subjects for whom we actually measured the F-wave delays. On the one hand, this is in agreement to Day et al. (1987), who consider 4 ms to be the variability of the latency of MEPs evoked by electric cortical stimulation: using a collision technique, they observed late responses 4 ms after the initial MEP-response. On the other hand, this is also in agreement to Magistris et al. (1998) who observed repetitive motor neuron discharges after a TMS-stimulus with the earliest onset at 5.4 ms post stimulation, in a study of single motor units.

### 2.7. *Statistical analysis*

Mixed-effects linear regression models were applied to assess the stimulation effects (i.e. the E-, the D- and the I-responses), the type of stimulation technique (conventional TMS-, TST-, control condition) and their potential interactions. Significance-level was set at $p \leq 0.05$. Significance levels for multiple comparison testing were corrected with the Benjamini-Hochberg procedure. (Benjamini and Hochberg, 1995) and

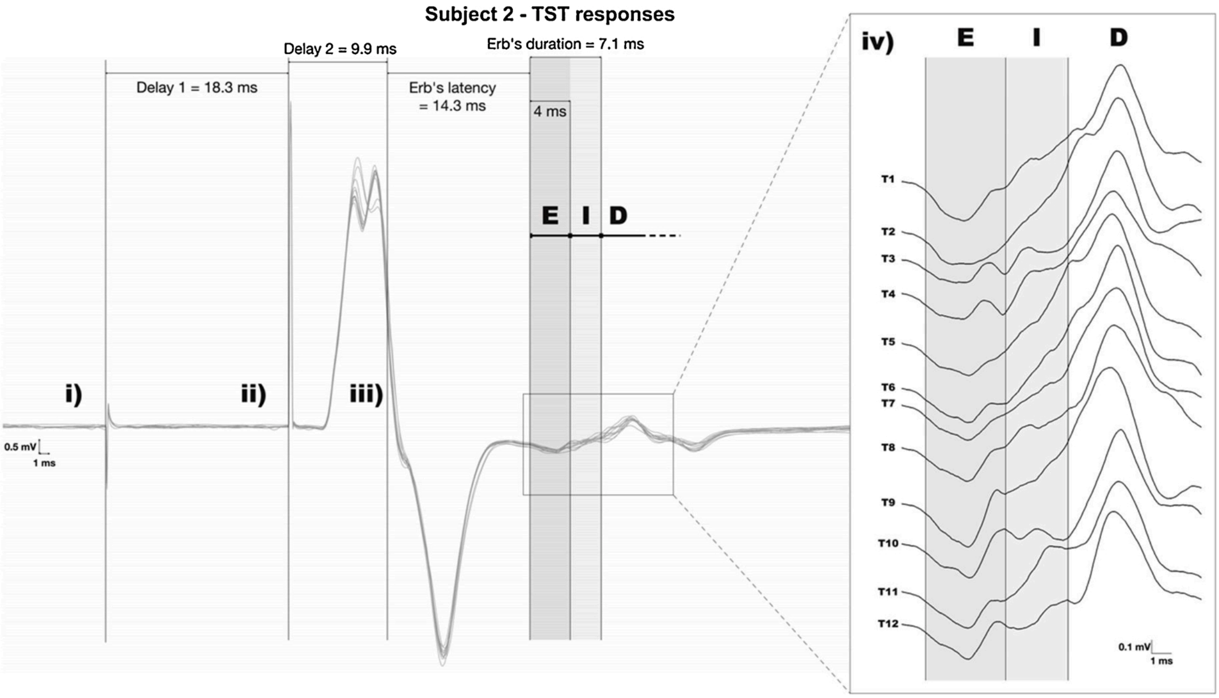

**Fig. 2.** Representative TST recordings of subject 2. Stimulation at 80 % of the $rMT_{TMS}$ during resting condition. i) TMS magnetic stimulation ii) Ulnar nerve stimulation, iii) Brachial plexus stimulation. iv) Magnification of the timeframes showing at which latencies responses were expected. The overall grey area represents the timeframe for responses deriving from the brachial plexus stimulations. The dark grey area represents the timeframe within which only direct responses from the brachial plexus stimulation were expected (Early responses). The light grey area depicts the time frame were direct responses and other late responses (i.e. probably F-waves) can superimpose (I- responses). Responses outside the grey area derive from other sources and are not a direct response to electric stimulation (Delayed responses). Please note that for simplicity, TST-trials were numbered from T1 to T12 here; but they were interspersed and presented randomly, in combination with TMS-trials during experimental sessions. We applied the same stimulations parameters in each trial.

yields a P threshold of 0.025. P values are presented without correction. Data were analyzed using Stata software version 16.0.

## 3. Results

Delay I was on average 18.52 ± 2.09 ms and Delay II 8.98 ± 0.88 ms. Table 1 shows the individual number of Early-, Intermediate- or Delayed responses for each participant and each condition. There was no significant difference in Early responses when comparing the three different techniques (TMS vs TST $p = 0.157$; TMS vs Control $p = 0.505$ and TST vs Control $p = 0.105$). Intermediate- and Delayed responses were significantly different when comparing TMS to the TST and the Control conditions (for Intermediate responses: $p < 0.00$; for D- responses: $p = 0.001$). Differences were not significant when comparing Intermediate responses with Delayed responses between TST and the Control conditions ($p = 0.792$ and $p = 0.665$ respectively) (See Fig. 3).

**Table 1**
Results. From left to right: Left panel TMS-condition; middle panel TST-condition, right panel Control condition. E: number of responses observed after the plexus delay; responses were too fast to be contaminated by late responses. I: responses with a timeframe in which a direct response to the plexus stimulation or response contamination from a late response would be also possible. D: responses triggered after the end of the timeframe estimated for a response triggered in response to plexus stimulation.

| Subject | TMS | | | TST | | | Control | | |
|---|---|---|---|---|---|---|---|---|---|
| | E | I | D | E | I | D | E | I | D |
| 1 | 0 | 0 | 0 | 0 | 7 | 10 | | | |
| 2 | 2 | 0 | 0 | 11 | 7 | 12 | | | |
| 3 | 0 | 0 | 0 | 0 | 11 | 11 | | | |
| 4 | 0 | 0 | 0 | 0 | 9 | 9 | | | |
| 5 | 0 | 0 | 0 | 0 | 11 | 3 | | | |
| 6 | 5 | 0 | 3 | 0 | 5 | 12 | | | |
| 7 | 6 | 3 | 2 | 10 | 12 | 0 | | | |
| 8 | 0 | 0 | 0 | 11 | 2 | 11 | | | |
| 9 | 0 | 0 | 0 | 11 | 11 | 0 | | | |
| 10 | 1 | 0 | 0 | 0 | 4 | 9 | | | |
| 11 | 4 | 2 | 1 | 0 | 10 | 12 | 0 | 8 | 10 |
| 12 | 0 | 0 | 0 | 0 | 10 | 2 | 0 | 12 | 11 |
| 13 | 6 | 2 | 2 | 9 | 11 | 1 | 3 | 12 | 1 |
| 14 | 3 | 0 | 1 | 0 | 7 | 0 | 0 | 1 | 12 |
| 15 | 1 | 0 | 0 | 0 | 7 | 2 | 0 | 11 | 1 |
| **Mean** | **1.87** | **0.47** | **0.6** | **3.47** | **8.27** | **6.27** | **0.6** | **8.8** | **7** |
| **Median** | **1** | **0** | **0** | **0** | **9** | **9** | **0** | **11** | **10** |
| SD | 2.33 | 0.99 | 0.99 | 5.1 | 2.99 | 5.11 | 1.34 | 4.66 | 5.52 |

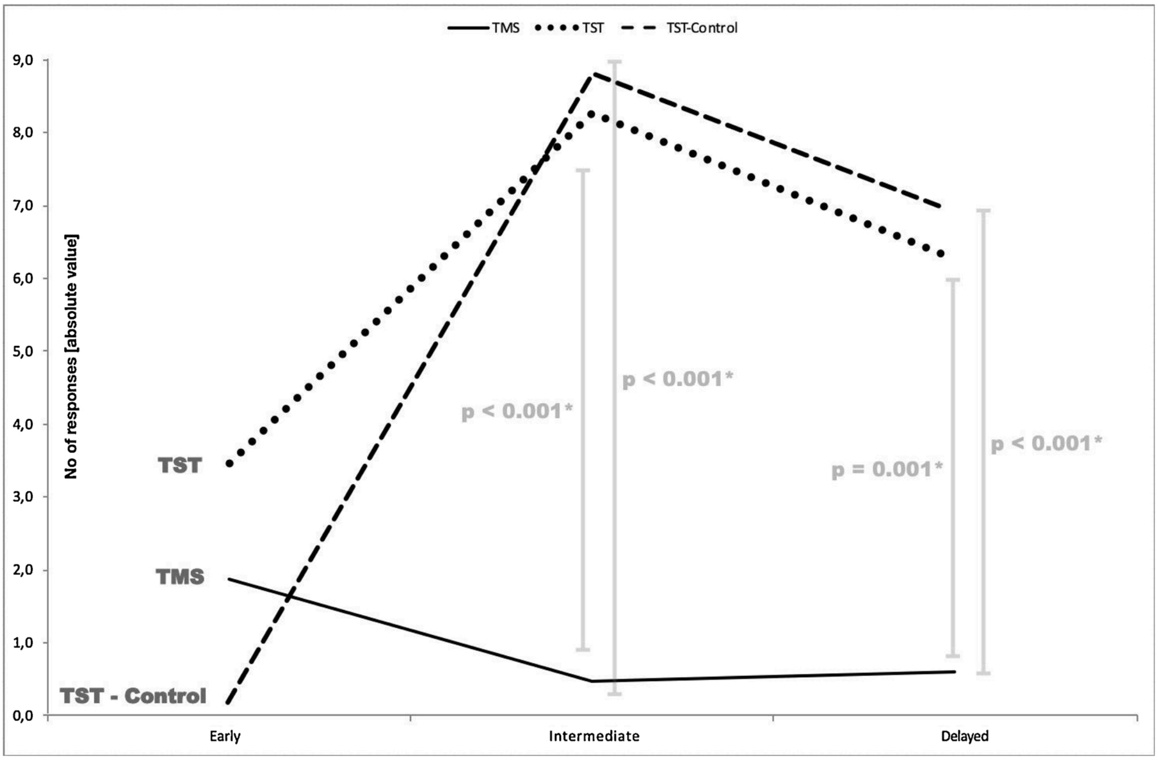

**Fig. 3.** E-, D- and I-responses with TMS, TST and Control condition.
The dotted line represents the TST, the plain line represents the TMS and the dashed line represents the Control condition. Note that p-lines are just connecting lines and not error bars.

Mixed-effects linear regression models showed that there was a significant effect when setting TMS-Early-Responses as a reference in comparison to all other responses: TST-Intermediate responses (Coef 6.2 [95 % conf. int. 2.82–9.60]; $p < 0.001$), TST-Delayed responses (Coef 4.07 [95 % conf. int. 0.68–7.45]; $p = 0.018$), Control-Intermediate (Coef 9.6 [95 % conf. int. 4.82–14.38]; $p < 0.001$) and Control-Delayed (Coef 7.67 [95 % conf. int. 2.88–12.45]; $p = 0.002$). When TST-Intermediate responses was set as a reference, there was a significant effect in comparison to TMS-Early-Responses (Coef 6.2 [95 % conf. int. 2.82–9.58]; $p < 0.001$) but not when compared to TMS-Delayed responses (Coef 2.13 [95 % conf. int.−1.25 – 5.52]; $p = 0.216$), Control-Early-Responses (Coef -3.4 [95 % conf. int.−8.18 – 1.38]; $p = 0.164$) or to Control-Delayed responses (Coef 0.2 [95 % conf. int.−4.58 – 4.98]; $p = 0.935$).

In only 1.87 ± 2.33 out of 12 trials, direct MEPs responses were elicited by TMS at an intensity of 80 % RMT, whereas, with the TST set at the same stimulation intensity, Early responses were elicited in 3.47 ± 5.1 trials. However, there was an important variability between subjects, with 10 out of 15 subjects showing no Early responses and other 5 subjects showing a high number of Early responses (a minimum of 9 out of 12 trials). (See Table 1).

In the Control condition, almost no Early responses were elicited (0.6 ± 1.34), with only one subject displaying responses within the timeframe. Delayed responses – presumably due to a longer delay than expected and not to a direct response to the electric or magnetic stimulation – were observed in all the conditions other than TMS; responses were augmented when peripheral electric stimulation was given: TMS = 0.6 ± 0.99; TST = 6.27 ± 5.11; Control = 7.0 ± 5.52 (See Table 1).

In the 2 subjects, for whom F-Waves were measured from the ulnar nerve stimulation site, the minimal latency between the direct response due to the plexus stimulation and the F-wave deriving from the same stimulation area were 4.5 and 4.1 ms.

According to the Benjamini-Hochberg correction for multiple testing, all P values below the threshold of 0.025 remain statistically significant.

## 4. Discussion

The determination of the so-called Motor Threshold (MT) plays a fundamental role in TMS-paradigms. The present study is the first one of a series of studies aiming at assessing the paired pulse stimulation paradigms by means of TST (Caranzano et al., 2017). As the variability of MEPs with conventional TMS is rather high, we wanted to investigate, by means of TST, which is considered to be more sensitive than conventional TMS, if MTs might be lower than usually expected when conventionally measured; in other words, if the concept of MT is method-dependent, and also whether sub-threshold stimulation leads to discharges descending to the spinal cord. Generally speaking, the rationale of this research attempts to contribute to answer the question as to whether inhibition and facilitation, as induced by paired pulse stimulation paradigms, have also a spinal and a peripheral contribution that has not been measured so far.

In specific, we investigated whether sub-threshold TMS consistently evokes discharges of spinal motor neurons. To accomplish this goal, we used the Triple Stimulation Technique (TST) because it corrects for desynchronization effects. The principal finding is that we measured more motor evoked potentials with TST, but those also arise from the peripheral stimulation. Thus, this study fails to support the assumption that TST is more sensitive than TMS when recording electrical discharges from spinal motor neurons at stimulation intensities below the conventionally determined motor threshold. In the case of TST, this is likely due to indirect late spinal responses (i.e. F-waves arising from the stimulation of the brachial plexus at the Erb's point): due to the short response delay that follows proximal stimulation of the brachial plexus, these responses and the direct responses arising from cortical stimulation cannot be discerned.

Studies on TST did not provide such an evidence, because the technique was established by using the maximal stimulator output (MSO) to activate the entire MN pool of a target muscle when stimulating with TMS. This leads to the generation of MEPs with amplitudes corresponding to CMAP of peripheral supramaximal stimulation. In those methodological studies (Magistris et al., 1999, 1998), the authors did not explore formally the threshold for supramaximal stimulation. Consequently, the late spinal responses of peripheral stimulation limit the clinical usefulness of TST in studies on MEPs, when the TST, which is based on the collision technique, is used at sub-threshold stimulation intensities, which do not activate the full MN pool. This limitation needs to be taken into account in future research studies.

The resting Motor threshold is defined as the stimulus strength with a probability of 0.5 of eliciting an MEP with a peak-to-peak amplitude larger than 50 μV at rest (RMT) and 200 μV during contraction (AMT) (Awiszus, 2003; Groppa et al., 2012). The thresholds RMT and AMT are thought to reflect the efficacy of a chain of synapses from pre-synaptic cortical neurons down to the muscle itself (Kobayashi and Pascual-Leone, 2003): membrane excitability of cortical interneurons and corticospinal neurons, the excitability of spinal MNs in the neuromuscular junction, and the one of the muscle itself (Hallett, 2007; Ziemann et al., 1996, 2015). These motor thresholds are commonly used as a reference, but they vary within and between subjects due to methodological reasons, anatomical differences, and various other physiological mechanisms (Wassermann, 2002). We specifically investigated stimulus at 80 % of the RMT in order to be consistent with our previous study and since findings from epidural spinal cord recordings demonstrated the presence of corticospinal volleys after transcranial magnetic stimulation at 80 % of the RMT (Nakamura et al., 1997). Others have also shown, with post-stimulus time histograms of single motor unit recordings, that significant corticospinal excitation can be generated with a single TMS pulse at an intensity of approximately 70–90% of the motor threshold (Lackmy-Vallee et al., 2012). In congruence with these results, we recorded Early- responses in a small amount of trials; even with sub-threshold stimulation in both conditions, TMS and TST.

While to some extent not surprising, the presence of such responses raise questions regarding the underlying mechanisms for the conditioning effects of subthreshold stimuli in paired-pulse TMS paradigms (Reis et al., 2008). Paired pulse paradigms are a well-established method to study the cortical excitability whereby a sub-threshold stimulus conditions the subsequent test stimulus. Nakamura et al. (1997) and Di Lazzaro et al. (2006) studied the effect of the conditioning stimulus with direct epidural recordings of descending spinal cord volleys. They demonstrated that the subthreshold conditioning stimulus facilitates descending waves at an inter-stimulus interval of 25 ms, but not at 10 or 15 ms, despite the facilitation of the MEP. ICF at these brief intervals might be mediated by spinal interactions. The presence of some Early responses following such stimuli with TMS and TST, as we observed here, reinforces the hypothesis of involved spinal interactions. Moreover, the presence of such responses might explain part of the variability seen in paired-pulse paradigms. Previously, using TST to study intra-cortical inhibition and intra-cortical facilitation, we have seen such a variability as well (Caranzano et al., 2017).

MEPs are extremely variable and different factors contribute to their variability. Besides the TMS intensity and polarity, these factors include intrinsic fluctuations in excitability of pyramidal cells and spinal motoneurons (MNs), heterogeneous MN properties, the number of activated MNs in the cortex and in the spinal cord as well as the timing of their discharges (Devanne et al., 1997; Groppa et al., 2012; Lackmy and Marchand-Pauvert, 2010; Rösler et al., 2002). When full activation of the MN-pool is achieved, the desynchronization of the descending volleys contribute to the variability with about one third of the MEP's amplitude and can be corrected by means of TST (Rösler et al., 2002). However, as we show here, the usefulness of the technique is limited by the supra-maximal peripheral stimulation that generate Delayed responses. To a large extent, when using TST, these Delayed responses, by being of a small size, may not greatly influence the MEPs, nevertheless they may explain part of their variability. On the other hand, and most importantly, our study shows the particular influence of Delayed responses on MEPs when small amplitude responses are studied (i.e. the ones used to define the motor threshold: 50 μV).

Among the possible causes for Delayed responses, indirect late spinal responses, i.e. F-waves, may play a role. The F-waves result from the antidromic volley of the supra-maximal stimulation of the brachial plexus and are presumed to appear with a delay of approximately 4 ms following the direct M-response. The absence of differences between the TST and the control condition, which consists only of peripheral stimulations, points to a contribution of these late spinal responses to both the intermediate and the delayed responses.

On the other hand, subthreshold cortical magnetic stimulation may not affect these late spinal responses (Inghilleri et al. (2003). Inghelleri and colleagues did neither detect changes in the frequency nor in the amplitude of F-waves following a sub-threshold cortical magnetic stimulation. But suprathreshold (120 % RMT) TMS increases both the amplitude and the frequency of the elicited F-waves.

Besides late spinal responses, repetitive spinal MN motoneuron discharges (repMNDs) after cortical stimulation may also trigger Delayed responses. The presence of such repetitive discharges have been explored in the past with the TST and an extended TST-protocol based on a collision technique (Z'Graggen et al., 2005). These repMNDs were elicited with supra-threshold stimulation and correlate with the stimulation intensity and the voluntary muscle contraction but were not found at subthreshold stimulation intensity as of here 80 % and at rest. Thus, repMNDs with subthreshold paired pulse stimulation paradigms are not expected. On the other hand, with supra-maximal TST, the influence of repMNDs is negligible as they are of small amplitude (Magistris et al., 1998).

We conclude that TST is not more sensitive than TMS in detecting discharges of spinal MNs at subthreshold stimulation intensities. In addition, sub-threshold TST is limited by the effects of the peripheral stimulation. Thus, the TST is useful to assess the integrity of the MN pool and their corticospinal projections, but at lower stimulation intensities such as in paired-pulse stimulation paradigms to explore the cortical physiology, the confounding effects of peripheral stimulation need to be taken into account.

## Declaration of Competing Interest

The authors report no declarations of interest.

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