Reliability of relaxation properties of knee-extensor muscles induced by transcranial magnetic stimulation

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HIGHLIGHTS

- Transcranial magnetic stimulation (TMS)-induced muscle relaxation demonstrates high within- and between-session reliability in unfatigued and fatigued knee-extensor muscles.
- TMS is a useful technique that researchers can use when investigating changes in muscle relaxation rates both in unfatigued and fatigued knee-extensor muscles.
- In both healthy individuals and patient groups, TMS-induced muscle relaxation could be used for therapy effect monitoring and diagnostic purposes.

ABSTRACT

Transcranial magnetic stimulation (TMS)-induced relaxation rate reflects intrinsic muscle contractile properties by interrupting the drive from the central nervous system during voluntary muscle contractions. To determine the appropriateness of knee-extensor muscle relaxation measurements induced by TMS, this study aimed to establish both the within- and between-session reliability before and after a fatiguing exercise bout. Eighteen participants (9 females, 9 males, age 25 ± 2 years, height 171 ± 9 cm, body mass 68.5 ± 13.5 kg) volunteered to participate in two identical sessions approximately 30 days apart. Maximal and submaximal neuromuscular evaluations were performed with TMS six times before (PRE) and at the end (POST) of a 2-min sustained maximal voluntary isometric contraction. Within- and between-session reliability of PRE values were assessed with intraclass correlation coefficient ($ICC_{2,1}$, relative reliability), repeatability coefficient (absolute reliability), and coefficient of variation (variability). Test-retest reliability of post-exercise muscle relaxation rates was assessed with Bland-Altman plots. For both the absolute and normalized peak relaxation rates and time to peak relaxation, data demonstrated low variability (e.g. coefficient of variation $\leq$ 7.8%) and high reliability (e.g. $ICC_{2,1} \geq 0.963$). Bland-Altman plots showed low systematic errors. These findings establish the reliability of TMS-induced muscle relaxation rates in unfatigued and fatigued knee-extensor muscles, showing that TMS is a useful technique that researchers can use when investigating changes in muscle relaxation rates both in unfatigued and fatigued knee-extensor muscles.

Keywords: fatigue; knee extensors; reliability; transcranial magnetic stimulation; muscle relaxation rate.

1 INTRODUCTION

Transcranial magnetic stimulation (TMS) delivered to the motor cortex is a non-invasive technique that can be used to excite or inhibit different cortical areas of the human brain. When single-pulse TMS of sufficient intensity is delivered to the motor cortex, it induces transient excitation that presents in both the electromyography (EMG) (i.e. motor-evoked potential) and mechanical (force) responses (i.e. twitch or superimposed twitch) of the target muscle, responses that rapidly increase in size during the transition from rest to a weak and then moderate-intensity voluntary contraction [1]. There is a period of near-silence in the EMG following the motor-evoked potential in voluntarily-contracting muscle termed the silent period that is thought to reflect motoneuronal and cortical mechanisms for the first ~100-150 ms and intracortical mechanisms mediated through gamma-aminobutyric acid B-receptor inhibition thereafter [2]. As a result of the withdrawal of voluntary neural drive, muscle fibres that are voluntarily contracting relax and force decreases during the silent period.

Analysis of the rate of muscle relaxation during the silent period induced by TMS delivered to the motor cortex has been proposed because TMS-induced muscle relaxation allows changes in intrinsic muscle contractile properties to be assessed by removing central drive during voluntary contractions. Impairment of muscle relaxation (i.e. myotonia) is involved in a wide spectrum of myotonic disorders [3] and has also been reported in dystonia [4], Parkinson's disease [5], and stroke [6]. Given the potential clinical utility of the TMS-induced muscle relaxation rate [3,7], several studies have investigated the feasibility (i.e. the practicality of TMS to assess muscle relaxation properties) of this technique [7-16]. However, it is also important to determine the reproducibility (i.e. the consistency of TMS-induced muscle relaxation properties) of measuring TMS-induced muscle relaxation rates prior to considering the use of this technique as a tool of

clinical and scientific importance. In spite of this, published reproducibility data are currently largely limited to consecutive trials performed the same day. For example, studies have reported intraclass correlation coefficients greater than 0.9 and coefficient of variations lower than 10% for the peak normalized muscle relaxation rate measured in finger flexors [7], elbow flexors [11], and knee extensors [13]. However, only one study has reported the between-session reliability for normalized peak relaxation rate. Todd et al. (2007) [11] reported an intraclass correlation coefficient of 0.96 and a coefficient of variation of 8.7 ± 3.8% in the elbow flexors. We are not aware of any previous investigation of between-session reliability for normalized peak relaxation rate of the knee extensors. This is an important consideration because findings from a muscle should not be generalized to other muscles [17]. Indeed, evidence shows weaker corticospinal projections to the lower- compared to the upper-limbs [18], mainly due to higher thresholds and smaller amplitude responses of lower limb motoneuron pools and fewer fast corticospinal fibres terminating in the lumbar than in the cervical spinal tract. Furthermore, the knee extensors play a key role during ambulatory, functional, and sporting activities, leading to the use of this muscle group in studies investigating muscle fatigue with TMS [13]. Finally, Todd et al. (2007) [11] used an unequal mix of men and women despite the fact that the peak relaxation rate is markedly faster in men than women [7,9], which may skew subsequent data analysis and reliability scores away from the general population [19]. Furthermore, reliability studies have thus far concentrated on maximal voluntary isometric contractions (MVIC) [7,11,13]. Although MVICs are considered the gold standard for assessing muscle strength [20], maximal effort may not always be desirable or feasible in various disorders. Furthermore, activities of daily living generally require submaximal levels of force production. Therefore, studying TMS-induced muscle relaxation rates during submaximal contractions may overcome many of the limitations of using evaluations based on

MVIC. However, the reliability of TMS-induced muscle relaxation rates during submaximal knee-extensor contractions has not been determined yet. Furthermore, there is a lack of information on the reliability of this technique in the fatigued state, which is important if TMS-induced muscle relaxation rates are to be used to quantify performance fatigability.

Therefore, the aim of the current study was to determine the within- and between-session reliability of measurements of TMS-induced peak relaxation rate in the knee extensors during maximal and submaximal voluntary isometric contractions before and after a fatiguing task.

2 MATERIAL AND METHODS

2.1 Participants

Eighteen university students (9 females, 9 males; age: 25 ± 2 years, height: 171 ± 9 cm, body mass: 68.5 ± 13.5 kg) volunteered for the study. The sample size estimation was based on data from Vernillo, Khassetarash, Millet, Temesi (2021) [13] with an intraclass correlation coefficient of 0.933 for the normalized peak relaxation rate for two consecutive measurements, setting a minimum acceptable value of 0.7, α at 0.05, and β at 0.2 [21]. All participants were informed of the experimental protocol and all associated risks and gave written informed consent prior to participation. Before the start of the protocol, contraindications for TMS were checked [22]. Exclusion criteria for participation were injury to the lower limbs during the previous six months, as well as history of heart disease or hypertension. Participants were instructed to avoid the consumption of caffeine on the day of the experiment and avoid performing any strenuous exercise during the 48 h prior to testing. Footedness was determined by the Revised Waterloo Footedness Questionnaire [23]. Fifteen participants (8 females, 7 males) were right-foot dominant, two participants (2 males) were left-foot dominant, and one participant (1 female) had equal limb

dominance. This study conformed to the standards set by the Declaration of Helsinki, except for registration in a database, and the research was approved by the local Ethics Committee.

2.2 Experimental protocol

Each participant visited the laboratory on three different occasions. During the first visit, participants were familiarized with performing maximal and submaximal knee-extensor contractions of the dominant leg with and without TMS. The participant that reported equal limb dominance performed the protocol with the left leg. Data were collected during the second and third visits. Because neuromuscular function of the knee extensors can be influenced by the different phases of the menstrual cycle [24], the first day of menstruation was considered as day 1 of the cycle and women visited the lab on day 15 ± 3 of their menstrual cycle. The two test sessions were separated by 30 ± 5 days for all the participants, regardless of sex. Test sessions were held at the same time of day for each participant to control for within-participant diurnal variation [25]. The fatiguing intervention consisted of a 2-min sustained MVIC. Participants were kept blind to the elapsing time and were both instructed to perform a real MVIC throughout the 2 min and strongly encouraged during the sessions by the investigators. Before each 2-min MVIC (PRE), six neuromuscular evaluations were performed (see "Neuromuscular evaluation" section). At the end of the 2-min MVIC, a single neuromuscular evaluation was performed as an extension of the 2-min MVIC (i.e. the participant was not permitted to relax) (POST).

2.2.1 Force and EMG recordings

Force was measured using a linear strain gauge (S2tech 546QDT, Milan, Italy) calibrated previously and coupled to the chair and the leg with a rigid, noncompliant device. The participants

116 sat upright in a custom-built chair with the hips and knees at 90° of flexion and secured by chest
117 and hips straps.
118 EMG of the *rectus femoris*, *vastus lateralis*, *vastus medialis*, and *biceps femoris* was
119 recorded with pairs of self-adhesive surface electrodes (30 × 22 mm; Ambu Neuroline 715; Ambu
120 A/S, Ballerup, Denmark) in bipolar configuration and reference on the *patella*. Placement of EMG
121 electrodes was on the distal portion of the muscle belly between the anterior superior iliac spine
122 and the superior border of the *patella* for *rectus femoris,* on the distal portion of the muscle belly
123 between the apex of the greater trochanter and the superolateral border of the *patella* for *vastus*
124 *lateralis,* on the distal portion of the muscle belly between the anterior superior iliac spine and the
125 joint space in front of the anterior border of the medial collateral ligament for *vastus medialis*, and
126 on the distal portion of the muscle belly between the ischial tuberosity and the apex of the fibular
127 head for *biceps femoris* [26]. The skin where electrodes were placed was shaved, lightly abraded,
128 and cleaned with isopropyl alcohol in order to achieve a low impedance level (<5 kΩ). Force and
129 EMG signals were analog-to-digitally converted at a sampling rate of 2000 Hz by PowerLab
130 system (16/35, ADInstruments, Bella Vista, Australia) and quad bioamplifier (FE234;
131 ADInstruments) with band-pass filter (10-500 Hz). All data were analyzed offline using Labchart
132 8 software (ADInstruments).
133
134 ***2.2.2 Transcranial magnetic stimulation***
135 The motor cortex was stimulated by a magnetic stimulator (Magstim 200^2; The Magstim Company
136 Ltd, Whitland, UK) with a 110-mm double-cone coil (maximum output of 1.4 T). Single stimuli
137 were delivered to the contralateral motor cortex, producing an induced postero-anterior current.
138 Every centimetre was demarcated from the vertex to 2 cm posterior to the vertex along the nasal-

inion line and 1 cm laterally over the left motor cortex. The optimal coil position was determined by assessing motor-evoked potential responses induced during brief isometric voluntary contractions at 20% MVIC and 50% maximal stimulator output. The optimal coil position was where the largest motor-evoked potentials in the *rectus femoris* were induced [27]. Optimal coil position for the session was marked on a lycra swim cap. Stimulus intensity was determined by stimulus-response curve from responses during brief isometric contractions at 20% MVIC. Four consecutive contractions were performed at 15-s intervals at each of the following randomly ordered stimulus intensities: 20, 30, 40, 50, 60, 70, and 80% maximal stimulator output. If a plateau was not confirmed from these intensities, higher TMS intensities were investigated. Optimal stimulus intensity was defined as the lowest intensity eliciting maximal motor-evoked potential amplitudes with minimal antagonist responses [28]. Mean stimulus intensities were 67 ± 12% and 68 ± 12% (P = 0.892) of maximal stimulator output for the first and second testing sessions, respectively.

2.2.3 Neuromuscular evaluation

For the two testing sessions, the neuromuscular evaluation consisted of six sets of contractions separated by 120 s of rest to minimize neuromuscular fatigue. Each set of contractions involved one brief (2-3 s) MVIC followed by brief contractions at 75 and 50% MVIC. The 75 and 50% submaximal force targets were calculated from the preceding MVIC. Within a set, each contraction was separated by 5 s. The participants contracted to the required force level and once the required force was attained and plateaued, TMS was delivered. Participants were also instructed to recontract as quickly as possible to the pre-stimulus voluntary force [29]. Visual feedback of the force produced was provided to the participants by means of a real-time display on a computer

screen. All the peak forces from the six MVIC trials were within 5% of each other during each of the two testing sessions.

2.3 Data Analysis

The durations of *rectus femoris*, *vastus lateralis*, and *vastus medialis* silent periods were measured by visually inspecting the interval from the TMS stimulus to the return of continuous voluntary EMG [30].

As previously indicated [7-16], muscle relaxation rates were calculated from the decrease in force during the silent period following TMS delivery (Figure 1) and the peak rate of muscle relaxation was calculated as the negative slope over a 10-ms interval (5 ms either side of the steepest instantaneous slope). To account for within- and between-participants' differences in voluntary strength, normalized relaxation rates were calculated by dividing the absolute rates of relaxation by the peak force which preceded the relaxation. This value reflects the relative peak relaxation rate of all knee-extensor muscles that contribute to the measured force (voluntary plus evoked) where the central drive is suppressed by the inhibitory effects of TMS. Time to peak relaxation was also assessed as the time from TMS stimulus until the moment of peak relaxation [9].

****Figure 1 near here****

2.4 Statistical analysis

Data used to assess within-session reliability (PRE) was calculated from the six sets of contractions performed during the first testing session. Data used to assess between-session reliability (PRE)

was calculated from the mean of the six MVICs for each of the two testing sessions. To determine the extent to which the repeated measures varied, within- and between-session reliability was assessed with different approaches both for the absolute and normalized peak relaxation rate, as well as for the time to peak relaxation.

Repeated-measures ANOVAs for session (1 and 2) and neuromuscular evaluation (1 to 6) were used to assess systematic bias in the results. The compound symmetry, or sphericity, was checked using Mauchly's test. When the assumption of sphericity was not met, the significance of F ratios was adjusted using Greenhouse-Geisser (when ε was ≤ 0.75) or Huynh-Feldt (when ε was > 0.75) corrections. When significant main effects or interactions were observed, Bonferroni's test was used for *post hoc* analysis.

To assess variability, the coefficient of variation (CV), and the corresponding 95% confidence interval, was calculated for each participant. The CV refers to the within-participant variation between two measurements [31]. For each participant, CV was calculated as: (standard deviation of the measurements ⁄ mean of the measurements) × 100. To interpret the CV values, Stokes (1985) [32] suggested an analytical goal of ≤ 15%.

To assess absolute reliability [i.e. the degree to which repeated assessments vary for individuals [31]], and the corresponding 95% confidence interval, the repeatability coefficient (RC) was determined. The RC is the value below which the absolute differences between two subsequent measurements would be expected 95% of the time. It was calculated as: 2.77 × within-participant standard deviation (with 2.77 calculated as: $\sqrt{2} \times 1.96$) [33,34].

To assess relative reliability [i.e. the degree to which individuals maintain their position order in a sample with repeated assessments [31]], and the corresponding 95% confidence interval, two-way random effects, absolute agreement intraclass correlation coefficients ($ICC_{2,1}$) were also

208 calculated [35]. The ICC indicates the error in measurements as a proportion of the total variance
209 in scores [31]. As a general rule, values from 0.7 to 0.8, from 0.8 to 0.9, and > 0.9 were considered
210 questionable, good, and high, respectively [36].

211 For POST, Bland-Altman plots were also generated to establish bias between the first and
212 the second testing session for each participant [37]. To do so, we plotted the difference between
213 the two testing sessions against their mean [37]. Examination of the direction and magnitude of
214 the scatterplot around the zero line provides an approximate indication of the systematic bias and
215 random error, respectively [31]. Confidence intervals defining the limits of agreement were
216 established as an index of random error. The presence of heteroscedasticity in the data sets was
217 objectively assessed by plotting the absolute differences against the mean of the first and the
218 second testing sessions and calculating the correlation coefficient [31]. The presence of
219 heteroscedasticity can lead to the 95% limits of agreement to be underestimated at the higher end
220 of measured values and overestimated at the lower end of measured values [31]. Therefore, if
221 heteroscedasticity was not present, the limits of agreement were calculated as: mean of the first
222 and the second testing sessions ± 1.96 × SD [31,38]. If heteroscedasticity was found to be present,
223 the 95% limits of agreement were calculated as described elsewhere [38].

224 Repeated-measures ANOVAs for session (1 and 2) and contraction intensity (100, 75, 50%
225 MVIC) were used to test differences between PRE and POST. When significant main effects or
226 interactions were observed, Bonferroni's test was used for *post hoc* analysis.

227 Statistical analyses were conducted using IBM™ SPSS™ Statistics (version 28.0.0; IBM
228 Corp., Somers, New York, NY) with the criterion α-level set to 0.05.

229

3 RESULTS

The silent period duration was sufficiently long to include the time of the peak relaxation rate of muscle fibres for all participants during both testing sessions and for all muscles at all contraction intensities (Supplementary Table 1).

The descriptive statistics of the muscle relaxation measures are presented in Supplementary Table 2. Repeated-measures ANOVAs revealed no systematic bias [session × evaluation interaction (all $P \geq 0.466$)].

3.1 Within- and between-session reliability (PRE)

Mean data for muscle relaxation parameters for all contraction intensities during sessions 1 and 2, with the associated CV, RC, and $ICC_{2,1}$ for within and between-session reliability, are presented in Table 1. Absolute and normalized peak relaxation rates and time to peak relaxation rate for all contraction intensities demonstrated high reliability, with CV ≤ 7.8% and $ICC_{2,1} \geq 0.906$.

****Table 1 near here****

3.2 PRE-POST changes

Changes in force measures from PRE to POST for all contraction intensities during sessions 1 and 2 are presented in the Supplementary Figure 1. There was a contraction intensity × time interaction ($P < 0.001$) as force decreased for all contraction intensities (all $P < 0.001$). The decrease in MVIC force from PRE to POST was not influenced by the session (session × contraction intensity × time interaction, $P = 0.925$).

PRE-POST changes in muscle relaxation measures for all contraction intensities during sessions 1 and 2 are presented in Figure 2. Absolute peak relaxation rate showed a contraction intensity × time interaction ($P < 0.001$), decreasing for all contraction intensities (all $P < 0.001$). The decrease in absolute peak relaxation rate from PRE to POST was not influenced by the session (session × contraction intensity × time interaction, $P = 0.944$). Normalized peak relaxation rates showed a contraction intensity × time interaction ($P < 0.001$), decreasing for all contraction intensities (all $P \leq 0.004$). The decrease in normalized peak relaxation rate from PRE to POST was not influenced by the session (session × contraction intensity × time interaction, $P = 0.822$). Time to peak relaxation showed a contraction intensity × time interaction ($P = 0.041$), increasing for all contraction intensities (all $P < 0.001$). The decrease in time to peak relaxation from PRE to POST was not influenced by the session (session × contraction intensity × time interaction, $P = 0.546$).

****Figure 2 near here****

3.3 Reliability of fatigue measurements (POST)

The 95% limits of agreement are illustrated in the Bland-Altman plots (Figure 3). The plots display adequate agreements between the difference in the two testing sessions and the means of the two sessions for absolute and normalized peak relaxation rates and time to peak relaxation rate for all contraction intensities. The slopes of the resulting regression lines were not significantly different from zero (i.e. horizontal to the *x*-axis) (all $r \leq 0.315$, all $P \geq 0.203$), indicating a uniformity of systematic error.

****Figure 3 near here****

4 DISCUSSION

Measuring muscle relaxation rates during maximal and submaximal contraction intensities (i.e. 100, 75, and 50% MVIC) allowed exploration of whether TMS is a reliable technique for determining knee-extensor muscle relaxation rates over a range of medium-to-high contraction intensities before and after a fatiguing exercise task. In the present study, the within- and between-session reliability of TMS-induced muscle relaxation measures was high for the knee-extensor muscles. The findings of this study also showed that muscle relaxation measures had low test-retest reliability bias post-exercise. Therefore, the use of TMS for measuring muscle relaxation is reliable and this technique appears capable of identifying consistent changes in the contractile properties of the knee extensors at different maximal and submaximal contraction intensities after a fatiguing exercise task. As already proposed, this technique reflects the same physiological mechanisms as the relaxation rate from either a single twitch evoked by nerve stimulation [11,13] or voluntary relaxation [7]. However, the advantages are that TMS permits examination of the muscle fibres when the central nervous system is driving voluntary muscle contraction, as well as allowing tracking of fatigue-induced changes in intrinsic KE contractile properties without requiring the interruption of ongoing contractions that can potentially alter the intrinsic muscle contractile properties [11]. Therefore, the current findings, together with previous findings, show that TMS may be more appropriate for measuring muscle relaxation than the resting twitch evoked by femoral nerve stimulation to reveal changes in knee-extensor contractile properties [13].

In both rehabilitation and sports medicine, accurate measurements of unfatigued muscle relaxation may be required to assess the impact of therapeutic interventions or the effects of physical training [7,13]. Therefore, the reliability of TMS-induced knee-extensor muscle

298 relaxation measures must be substantiated before TMS can be used for research or athlete/patient
299 evaluation. All measurements demonstrated high within-session reliability (both absolute and
300 relative) across all contraction intensities and agree well with measurements of TMS-induced
301 muscle relaxation obtained in previous studies for finger flexors [7], elbow flexors [11], and knee
302 extensors [13]. However, the 95% confidence intervals of the ICCs for the normalized peak
303 relaxation rates were wider than those for the absolute peak relaxation rates (Table 2). This
304 indicates that a larger sample size may be needed to attain greater precision of estimates of ICCs
305 for normalized peak relaxation rates [39]. Nevertheless, TMS seems to be a reliable method of
306 characterizing muscle relaxation on a group level.

307 The results in the current study also showed that TMS-induced knee-extensor muscle
308 relaxation measures are reliable between testing sessions. Findings from this study showed that
309 test-retest reliability (both absolute and relative) was high for all TMS-induced knee-extensor
310 muscle relaxation measures across all contraction intensities. Surprisingly, to the best of our
311 knowledge, there are no test-retest reliability studies of TMS-induced muscle relaxation.
312 Therefore, it is not possible to make meaningful comparisons, illustrating the need for more
313 research in this area. Nevertheless, the results of this study suggest that if standardized procedures
314 are adopted for TMS muscle relaxation data collection and analysis, then the re-test reliability is
315 high and any change between sessions may be attributed to real changes. This study suggests that
316 TMS may be useful for monitoring longitudinal changes in knee-extensor muscle relaxation
317 measures in individuals. High levels of between-session reliability are important for studies
318 involving an intervention, such as a rehabilitation program, where the investigators attempt to
319 monitor changes in a particular muscle group (e.g. knee extensors). Information will then provide
320 researchers with an indication of the size of differences required to observe significant differences

between sessions, paving the way for investigating the effects of various acute and chronic interventions with confidence.

Establishing the reliability of TMS as a tool for the adequate assessment of knee-extensor muscle relaxation measurement changes with fatigue is an important pre-requisite before TMS can be adopted as a standard measure of fatigue-induced changes in muscle relaxation. Indeed, the potential day-to-day change in fatigue levels may make it difficult to assess the effects of different fatiguing interventions due to the potential for low day-to-day reproducibility. Therefore, to gain a better understanding of the reliability of muscle relaxation measures post-exercise, Bland-Altman plots [37] were produced and 95% limit of agreements calculated for each measure and contraction intensity. In this way, we were able to characterize the absolute reliabilities of these measures with fatigue and illustrate systematic bias, if present, between session 1 and session 2. Overall, muscle relaxation measures showed low systematic errors and the slope of the regression lines fitted to the data in Figure 4 were also not significantly different from zero (all $P \geq 0.203$). Therefore, it can be concluded that the small amount of systematic error between the two sessions is consistent and independent of the size of the measured values [38]. To the best of our knowledge, no other studies have reported 95% limit of agreements for muscle relaxation measurements, illustrating the need for more research in this area. Nevertheless, TMS-induced knee-extensor muscle relaxation measures are reliable in the presence of muscle fatigue.

In conclusion, the use of TMS to characterize knee-extensor muscle relaxation rates has high within- and between-session reliability. Therefore, the ability of this technique to determine a real change in knee-extensor muscle relaxation rates is good both on a group level and on an individual level. The current findings suggest that TMS can be used with confidence by researchers when investigating changes in muscle relaxation rates over a range of medium-to-high contraction

intensities before and after a fatiguing task. The findings have wide implications for the use of TMS as a technique to assess muscle relaxation rates in both healthy individuals and patient groups.

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CONFLICTS OF INTEREST

All authors declared no competing interests.

REFERENCES

1. Kischka U, Fajfr R, Fellenberg T, Hess CW. Facilitation of motor evoked potentials from magnetic brain stimulation in man: a comparative study of different target muscles. *J Clin Neurophysiol.* 1993; 10(4):505-512.

2. Skarabot J, Mesquita RNO, Brownstein CG, Ansdell P. Myths and Methodologies: How loud is the story told by the transcranial magnetic stimulation-evoked silent period? *Exp Physiol.* 2019; 104(5):635-642.

3. Kleine BU, Stegeman DF. Stimulating motor wisdom. *J Appl Physiol.* 2007; 102(5):1737-1738.

4. Buccolieri A, Avanzino L, Marinelli L, Trompetto C, Marchese R, Abbruzzese G. Muscle relaxation is impaired in dystonia: a reaction time study. *Mov Disord.* 2004; 19(6):681-687.

5. Corcos DM, Chen CM, Quinn NP, McAuley J, Rothwell JC. Strength in Parkinson's disease: relationship to rate of force generation and clinical status. *Ann Neurol.* 1996; 39(1):79-88.

6. Seo NJ, Fischer HW, Bogey RA, Rymer WZ, Kamper DG. Effect of a serotonin antagonist on delay in grip muscle relaxation for persons with chronic hemiparetic stroke. *Clin Neurophysiol.* 2011; 122(4):796-802.

7. Molenaar JP, Voermans NC, de Jong LA, Stegeman DF, Doorduin J, van Engelen BG. Repeatability and reliability of muscle relaxation properties induced by motor cortical stimulation. *J Appl Physiol.* 2018; 124(6):1597-1604.

8. McNeil CJ, Bredius MS, Molenaar JP, Gandevia SC. The influence of motor cortical stimulus intensity on the relaxation rate of human lower limb muscles. *Exp Brain Res.* 2013; 228:235-242.

9. Molenaar JP, McNeil CJ, Bredius MS, Gandevia SC. Effects of aging and sex on voluntary activation and peak relaxation rate of human elbow flexors studied with motor cortical stimulation. *Age.* 2013; 35(4):1327-1337.

10. Todd G, Butler JE, Taylor JL, Gandevia SC. Hyperthermia: a failure of the motor cortex and the muscle. *J Physiol.* 2005; 563(Pt 2):621-631.

11. Todd G, Taylor JL, Butler JE, Martin PG, Gorman RB, Gandevia SC. Use of motor cortex stimulation to measure simultaneously the changes in dynamic muscle properties and voluntary activation in human muscles. *J Appl Physiol.* 2007; 102(5):1756-1766.

12. Yacyshyn AF, Nettleton J, Power GA, Jakobi JM, McNeil CJ. The effect of muscle length on transcranial magnetic stimulation-induced relaxation rate in the plantar flexors. *Physiol Rep.* 2017; 5(18): e13442.

13. Vernillo G, Khassetarash A, Millet GY, Temesi J. Use of transcranial magnetic stimulation to assess relaxation rates in unfatigued and fatigued knee-extensor muscles. *Exp Brain Res.* 2021; 239(1):205-216.

14. Hunter SK, Butler JE, Todd G, Gandevia SC, Taylor JL. Supraspinal fatigue does not explain the sex difference in muscle fatigue of maximal contractions. *J Appl Physiol.* 2006; 101(4):1036-1044.

15. Hunter SK, Todd G, Butler JE, Gandevia SC, Taylor JL. Recovery from supraspinal fatigue is slowed in old adults after fatiguing maximal isometric contractions. *J Appl Physiol.* 2008; 105(4):1199-1209.

16. Sundberg CW, Kuplic A, Hassanlouei H, Hunter SK. Mechanisms for the age-related increase in fatigability of the knee extensors in old and very old adults. *J Appl Physiol (1985).* 2018; 125(1):146-158.

17. Vernillo G, Temesi J, Martin M, Millet GY. Mechanisms of fatigue and recovery in upper versus lower limbs in men. *Med Sci Sports Exerc.* 2018; 50(2):334-343.

18. Brouwer B, Ashby P. Corticospinal projections to upper and lower limb spinal motoneurons in man. *Electroencephalogr Clin Neurophysiol.* 1990; 76(6):509-519.

19. Tammemagi MC, Frank JW, Leblanc M, Artsob H, Streiner DL. Methodological issues in assessing reproducibility--a comparative study of various indices of reproducibility applied to repeat ELISA serologic tests for Lyme disease. *J Clin Epidemiol.* 1995; 48(9):1123-1132.

20. Nuzzo JL, Taylor JL, Gandevia SC. CORP: Measurement of upper and lower limb muscle strength and voluntary activation. *J Appl Physiol (1985).* 2019; 126(3):513-543.

21. Walter SD, Eliasziw M, Donner A. Sample size and optimal designs for reliability studies. *Stat Med.* 1998; 17(1):101-110.

22. Rossi S, Hallett M, Rossini PM, Pascual-Leone A. Screening questionnaire before TMS: an update. *Clin Neurophysiol.* 2011; 122(8):1686.

23. Elias LJ, Bryden MP. Footedness is a better predictor of language lateralisation than handedness. *Laterality.* 1998; 3(1):41-51.

24. Ansdell P, Brownstein CG, Skarabot J, et al. Menstrual cycle-associated modulations in neuromuscular function and fatigability of the knee extensors in eumenorrheic women. *J Appl Physiol.* 2019; 126(6):1701-1712.

25. Ly JQM, Gaggioni G, Chellappa SL, et al. Circadian regulation of human cortical excitability. *Nat Commun.* 2016; 7:11828.

26. Botter A, Oprandi G, Lanfranco F, Allasia S, Maffiuletti NA, Minetto MA. Atlas of the muscle motor points for the lower limb: implications for electrical stimulation procedures and electrode positioning. *Eur J Appl Physiol.* 2011; 111(10):2461-2471.

27. Temesi J, Vernillo G, Martin M, Krüger RL, McNeil CJ, Millet GY. Sustained maximal voluntary contractions elicit different neurophysiological responses in upper- and lower-limb muscles in men. *Neuroscience.* 2019; 422:88-98.

28. Temesi J, Gruet M, Rupp T, Verges S, Millet GY. Resting and active motor thresholds versus stimulus-response curves to determine transcranial magnetic stimulation intensity in quadriceps femoris. *J Neuroeng Rehabil.* 2014; 11:40.

29. Mathis J, de Quervain D, Hess CW. Dependence of the transcranially induced silent period on the 'instruction set' and the individual reaction time. *Electroencephalogr Clin Neurophysiol.* 1998; 109(5):426-435.

436 30. Taylor JL, Butler JE, Allen GM, Gandevia SC. Changes in motor cortical excitability
437 during human muscle fatigue. *J Physiol.* 1996; 490:519-528.

438 31. Atkinson G, Nevill AM. Statistical methods for assessing measurement error (reliability)
439 in variables relevant to sports medicine. *Sports Med.* 1998; 26(4):217-238.

440 32. Stokes M. Reliability and repeatability of methods for measuring muscle in physiotherapy.
441 *Physiother Pract* 1985; 1(2):71-76.

442 33. Beckerman H, Roebroeck ME, Lankhorst GJ, Becher JG, Bezemer PD, Verbeek AL.
443 Smallest real difference, a link between reproducibility and responsiveness. *Qual Life Res.* 2001;
444 10(7):571-578.

445 34. Vaz S, Falkmer T, Passmore AE, Parsons R, Andreou P. The case for using the repeatability
446 coefficient when calculating test-retest reliability. *PLoS One.* 2013; 8(9):e73990.

447 35. McGraw KO, Wong SP. Forming inferences about some intraclass correlation coefficients.
448 *Psychol Methods.* 1996; 1(1):30-46.

449 36. Vincent J. *Statistics in kinesiology - 5th edition*, Champain (IL), Human Kinetics Books;
450 2021.

451 37. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods
452 of clinical measurement. *Lancet.* 1986; 1(8476):307-310.

453 38. Bland JM, Altman DG. Measuring agreement in method comparison studies. *Stat Methods*
454 *Med Res.* 1999; 8(2):135-160.

455 39. Atkinson G, Nevill AM. Selected issues in the design and analysis of sport performance
456 research. *J Sports Sci.* 2001; 19(10):811-827.
457

FIGURE AND TABLE LEGENDS

Figure 1. (Panel A) Overlaid raw traces of knee-extensor force during the six neuromuscular evaluations consisting of maximal (100% MVIC) and submaximal (75 and 50% MVIC) voluntary isometric contractions before the 2-min sustained maximal contraction. (Panel B) Peak muscle relaxation rates before (top three traces) and at the end (bottom three traces) of the 2-min sustained MVIC at different contraction intensities. Data were recorded from a single participant (30-year-old male). Arrows indicate timing of the motor cortical stimuli. Circles indicate the time of peak relaxation.

Figure 2. Mean and individual changes in muscle relaxation measures during maximal (100% MVIC) and submaximal (75 and 50% MVIC) voluntary isometric contractions for both session 1 and session 2. Neuromuscular function evaluations were performed before (PRE) and after (POST) the 2-min sustained MVIC as an extension of the 2-min MVIC. Repeated-measures ANOVA showed that absolute peak relaxation rate, normalized peak relaxation rate and time to peak relaxation decreased for all contraction intensities between PRE and POST (all $P \leq 0.004$). Standard deviations were omitted for clarity.

Figure 3. Bland-Altman plots for test-retest reliability of muscle relaxation measures post-exercise. The differences between the two testing sessions (*y*-axes) are plotted against the mean of the two testing sessions (*x*-axes) for absolute and normalized peak relaxation rates, as well as time to peak relaxation during maximal (100% MVIC) and submaximal (75 and 50% MVIC) voluntary isometric contractions. For each panel, the middle horizontal dashed line represents the mean difference between the two testing sessions (systematic bias) and the upper and lower dashed lines

represent the 95% limits of agreements (random error, as ± 1.96 × standard deviation). The solid line represents the slope of the regression.

Table 1. Reliability of muscle relaxation induced by transcranial magnetic stimulation during PRE maximal (100% MVIC) and submaximal (75 and 50% MVIC) voluntary isometric contractions. Values are means (95% confidence interval).

Supplementary Table 1. Comparison of the time to peak relaxation and duration of the silent period induced by transcranial magnetic stimulation during maximal (100% MVIC) and submaximal (75 and 50% MVIC) voluntary isometric contractions before (PRE) and as an extension of the 2-min sustained MVIC (POST). Repeated-measures ANOVA showed that silent periods for *rectus femoris*, *vastus lateralis* and *vastus medialis* increased for all contraction intensities (all $P < 0.001$). Values are means ± standard deviations.

Supplementary Table 2. Descriptive statistics for muscle relaxation induced by transcranial magnetic stimulation during maximal (100% MVIC) and submaximal (75 and 50% MVIC) voluntary isometric contractions at PRE. Values are means ± standard deviations or *P* values.

Supplementary Figure 1. Mean and individual changes in voluntary isometric contraction force during maximal (100% MVIC) and submaximal (75 and 50% MVIC) voluntary isometric contractions for both session 1 and session 2. Neuromuscular function evaluations were performed before (PRE) and after (POST) the 2-min sustained MVIC as an extension of the 2-min MVIC.

503 Repeated-measures ANOVA showed that force decreased for all contraction intensities between
504 PRE and POST (all $P < 0.001$). Standard deviations were omitted for clarity.
505

506 Figure 1
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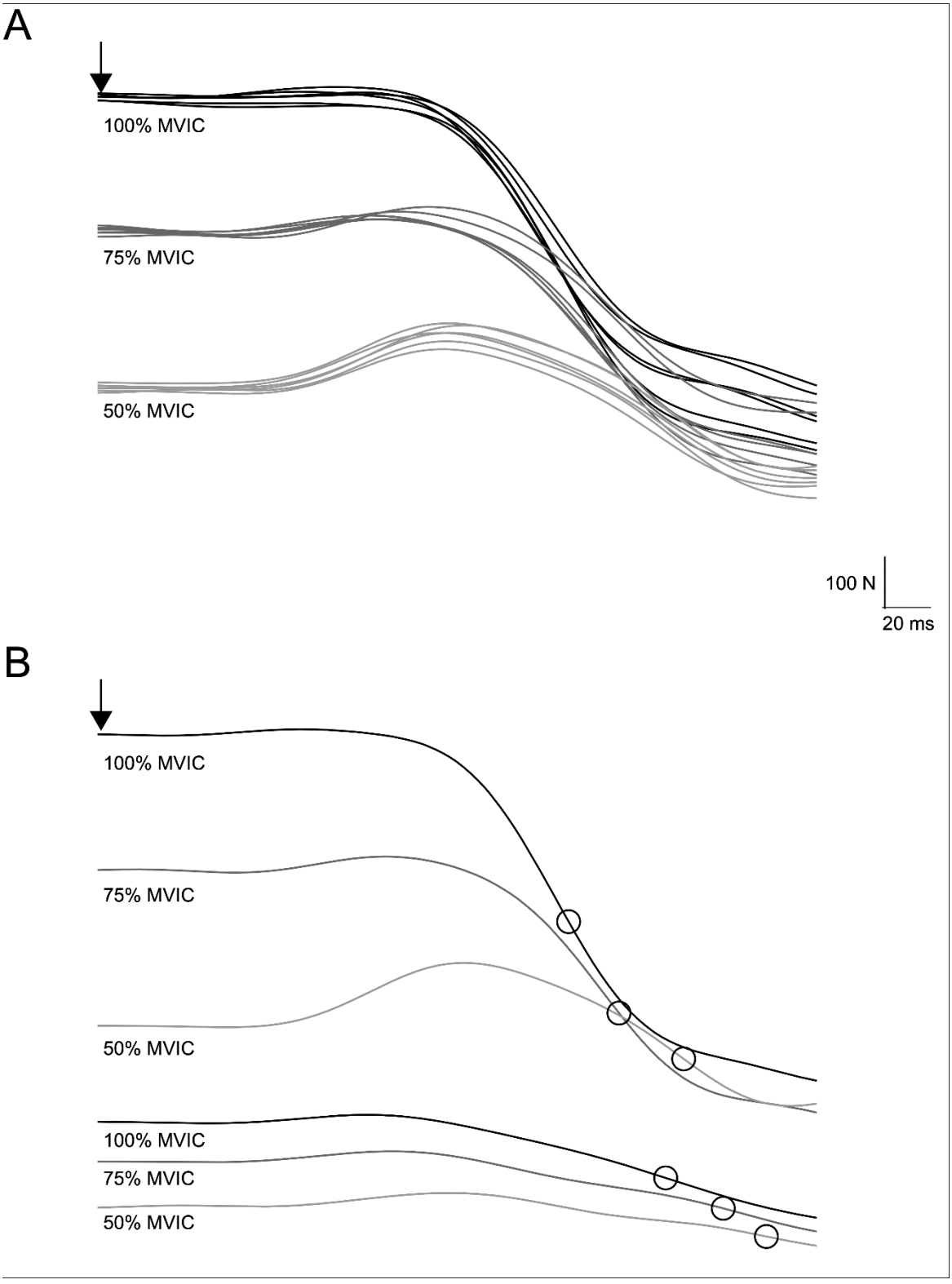

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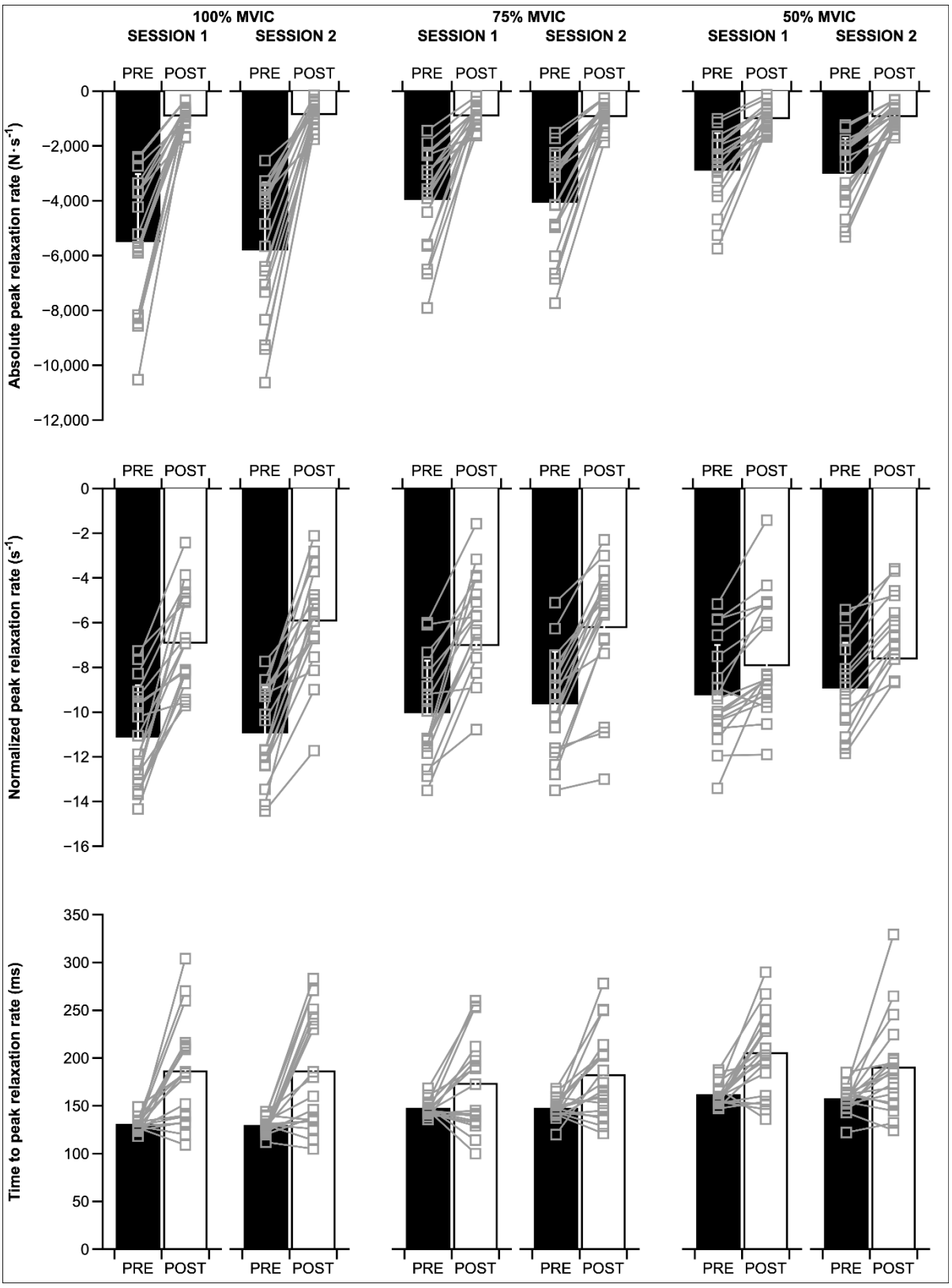

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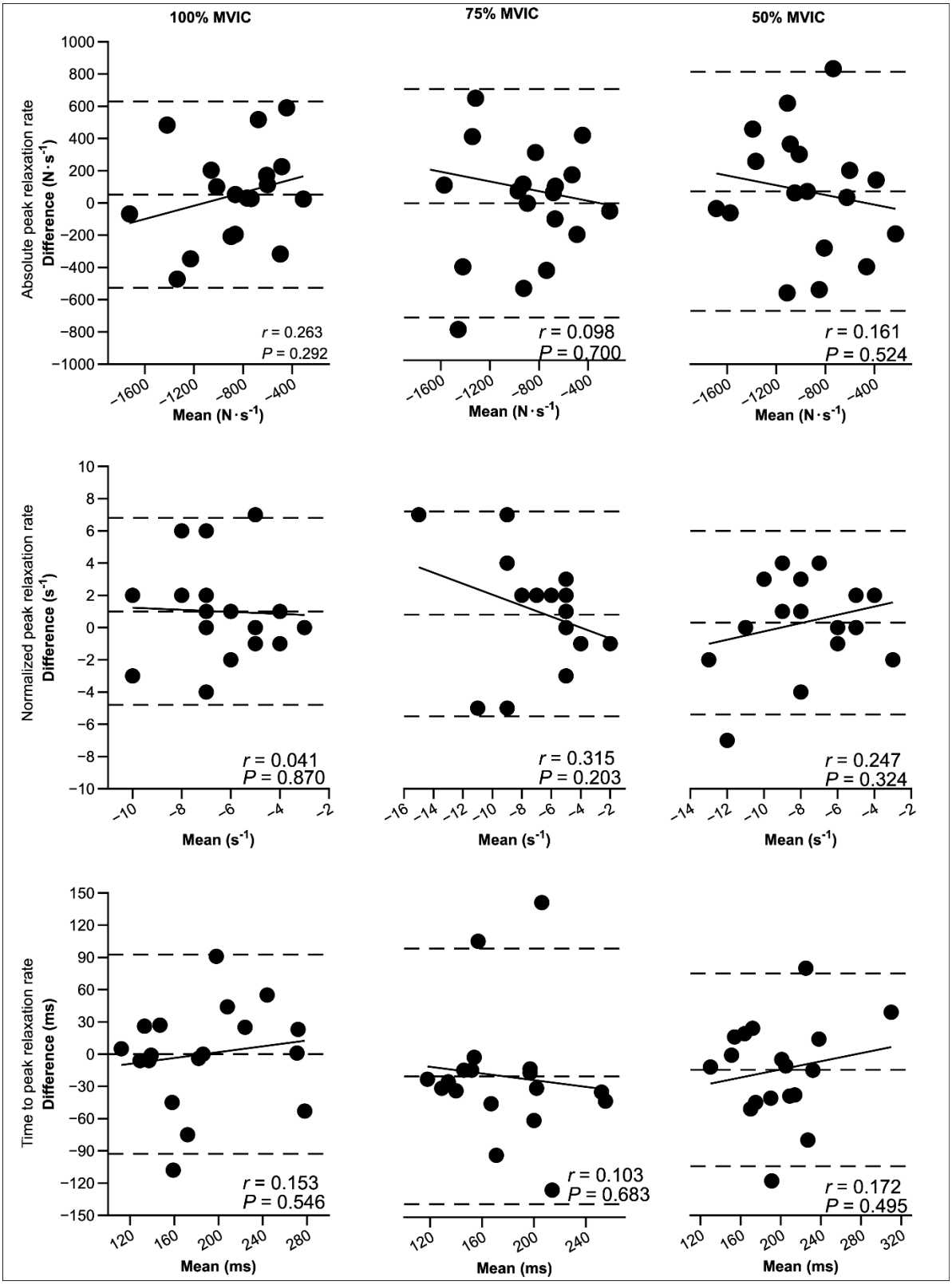

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513 **Table 1.** Reliability of muscle relaxation induced by transcranial magnetic stimulation during PRE maximal (100% MVIC) and
514 submaximal (75 and 50% MVIC) voluntary isometric contractions. Values are means (95% confidence interval).

Parameter	Within-session reliability			Between-session reliability		
	RC	CV	$ICC_{2,1}$	RC	CV	$ICC_{2,1}$
100% MVIC						
Absolute peak relaxation rate	1059 (796–1322) $N \cdot s^{-1}$	7.5 (6.1–8.9) %	0.995 (0.990–0.998)	833 (662–1004) $N \cdot s^{-1}$	5.9 (4.8–6.9) %	0.997 (0.994–0.999)
Normalized peak relaxation rate	1.7 (1.4–2.0) s^{-1}	5.7 (4.5–6.9) %	0.989 (0.979–0.995)	1.6 (1.2–2.0) s^{-1}	5.4 (4.0–6.8) %	0.985 (0.971–0.994)
Time to peak relaxation	11.5 (9.1–13.8) ms	3.2 (2.6–3.8) %	0.922 (0.850–0.967)	8.0 (6.5–9.5) ms	2.2 (1.9–2.6) %	0.963 (0.929–0.984)
75% MVIC						
Absolute peak relaxation rate	802 (638–965) $N \cdot s^{-1}$	7.8 (6.4–9.3) %	0.995 (0.990–0.998)	705 (528–882) $N \cdot s^{-1}$	6.6 (5.3–7.9) %	0.996 (0.992–0.998)
Normalized peak relaxation rate	1.5 (1.3–1.8) s^{-1}	6.2 (4.5–7.9) %	0.990 (0.980–0.996)	1.4 (1.1–1.7) s^{-1}	5.6 (4.2–7.1) %	0.990 (0.980–0.996)
Time to peak relaxation	15.0 (12.0–18.0) ms	3.7 (3.0–4.3) %	0.906 (0.817–0.960)	9.9 (8.6–11.2) ms	2.4 (2.1–2.8) %	0.967 (0.935–0.986)
50% MVIC						
Absolute peak relaxation rate	518 (424–612) $N \cdot s^{-1}$	7.5 (5.5–9.5) %	0.995 (0.990–0.998)	420 (310–531) $N \cdot s^{-1}$	5.8 (4.3–7.3) %	0.997 (0.994–0.999)
Normalized peak relaxation rate	1.6 (1.3–1.9) s^{-1}	7.1 (5.0–9.3) %	0.990 (0.980–0.996)	1.2 (1.0–1.5) s^{-1}	5.5 (3.9–7.1) %	0.992 (0.984–0.996)
Time to peak relaxation	13.8 (11.3–16.3) ms	3.1 (2.5–3.6) %	0.966 (0.935–0.986)	9.9 (7.7–12.0) ms	2.3 (1.8–2.8) %	0.978 (0.958–0.991)

515 RC, repeatability coefficient; CV, coefficient of variation; $ICC_{2,1}$, two-way random effects, absolute agreement intraclass correlation
516 coefficient.
517
518

519 **Supplementary Table 1.** Comparison of the time to peak relaxation and duration of the silent
520 period induced by transcranial magnetic stimulation during maximal (100% MVIC) and
521 submaximal (75 and 50% MVIC) voluntary isometric contractions before (PRE) and as an
522 extension of the 2-min sustained MVIC (POST). Repeated-measures ANOVA showed that silent
523 periods for *rectus femoris*, *vastus lateralis* and *vastus medialis* increased for all contraction
524 intensities (all $P < 0.001$). Values are means ± standard deviations.

Variable	**Session 1**		**Session 2**	
	PRE	**POST**	**PRE**	**POST**
100% MVIC				
Time to peak relaxation (ms)	130 ± 7	186 ± 55	129 ± 8	186 ± 61
Rectus femoris silent period (ms)	263 ± 35	330 ± 88	253 ± 46	320 ± 59
Vastus lateralis silent period (ms)	263 ± 36	331 ± 92	254 ± 46	322 ± 59
Vastus medialis silent period (ms)	267 ± 35	338 ± 91	258 ± 47	326 ± 59
75% MVIC				
Time to peak relaxation (ms)	147 ± 8	173 ± 50	147 ± 11	182 ± 45
Rectus femoris silent period (ms)	262 ± 33	301 ± 33	247 ± 46	297 ± 60
Vastus lateralis silent period (ms)	263 ± 33	304 ± 32	248 ± 46	298 ± 60
Vastus medialis silent period (ms)	267 ± 33	310 ± 28	253 ± 46	302 ± 61
50% MVIC				
Time to peak relaxation (ms)	161 ± 11	205 ± 44	157 ± 13	190 ± 51
Rectus femoris silent period (ms)	255 ± 33	296 ± 38	247 ± 45	290 ± 49

Vastus lateralis silent period (ms)	256 ± 33	297 ± 37	248 ± 45	291 ± 50
Vastus medialis silent period (ms)	261 ± 33	301 ± 38	253 ± 45	296 ± 49

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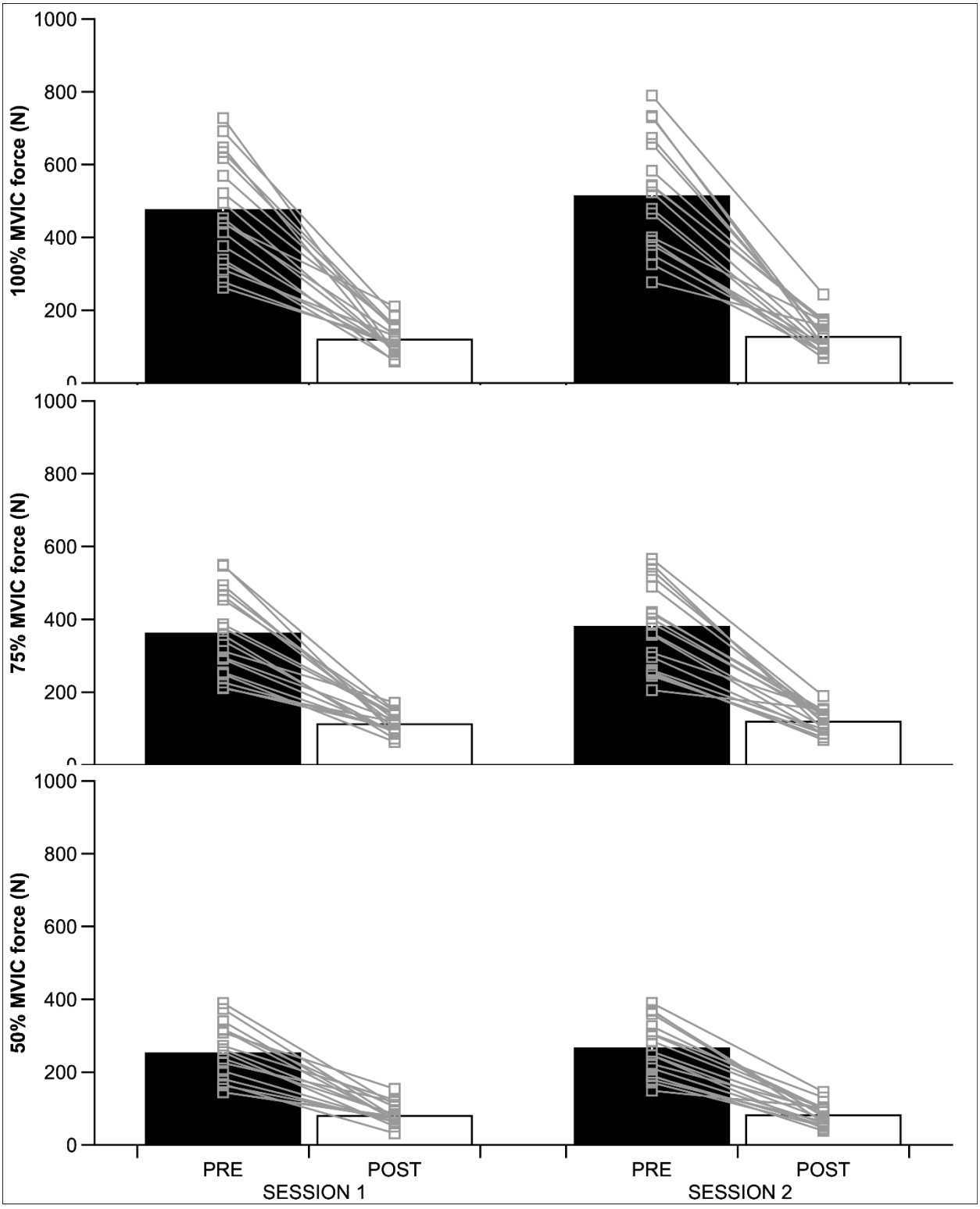
Supplementary Table 2. Descriptive statistics for muscle relaxation induced by transcranial magnetic stimulation during maximal (100% MVIC) and submaximal (75 and 50% MVIC) voluntary isometric contractions at PRE. Values are means ± standard deviations or *P* values.

Variable	Session 1	Session 2	Session × contraction interaction
100% MVIC			
Absolute peak relaxation rate	-5472 ± 2444 N·s^{-1}	-5781 ± 2451 N·s^{-1}	0.466
Normalized peak relaxation rate	-11.1 ± 2.3 s^{-1}	-10.9 ± 2.0 s^{-1}	0.614
Time to peak relaxation rate	130 ± 7 ms	129 ± 8 ms	0.891
75% MVIC			
Absolute peak relaxation rate	-3936 ± 1834 N·s^{-1}	-4042 ± 1859 N·s^{-1}	0.969
Normalized peak relaxation rate	-10.0 ± 2.3 s^{-1}	-9.6 ± 2.3 s^{-1}	0.747
Time to peak relaxation rate	147 ± 8 ms	147 ± 11 ms	0.827
50% MVIC			
Absolute peak relaxation rate	-2867 ± 1335 N·s^{-1}	-2978 ± 1273 N·s^{-1}	0.630
Normalized peak relaxation rate	-9.2 ± 2.2 s^{-1}	-8.9 ± 2.0 s^{-1}	0.843
Time to peak relaxation rate	161 ± 11 ms	157 ± 13 ms	0.672

532 Supplementary Figure 1



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