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ORIGINAL ARTICLE

Transcranial magnetic stimulation as biomarker of excitability in drug development: A randomized, double-blind, placebo-controlled, cross-over study

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Aims: The purpose of this study was to investigate pharmacodynamic effects of drugs targeting cortical excitability using transcranial magnetic stimulation (TMS) combined with electromyography (EMG) and electroencephalography (EEG) in healthy subjects, to further develop TMS outcomes as biomarkers for proof-of-mechanism in early-phase clinical drug development. Antiepileptic drugs presumably modulate cortical excitability. Therefore, we studied effects of levetiracetam, valproic acid and lorazepam on cortical excitability in a double-blind, placebo-controlled, 4-way cross-over study.

Methods: In 16 healthy male subjects, single- and paired-pulse TMS-EMG–EEG measurements were performed predose and 1.5, 7 and 24 hours postdose. Treatment effects on motor-evoked potential, short and long intracortical inhibition and TMS-evoked potential amplitudes, were analysed using a mixed model ANCOVA and cluster-based permutation analysis.

Results: We show that motor-evoked potential amplitudes decreased after administration of levetiracetam (estimated difference [ED] −378.4 μV; 95%CI: −644.3, −112.5 μV; $P < .01$), valproic acid (ED −268.8 μV; 95%CI: −532.9, −4.6 μV; $P = .047$) and lorazepam (ED −330.7 μV; 95%CI: −595.6, −65.8 μV; $P = .02$) when compared with placebo. Long intracortical inhibition was enhanced by levetiracetam (ED −60.3%; 95%CI: −87.1%, −33.5%; $P < .001$) and lorazepam (ED −68.2%; 95% CI: −94.7%, −41.7%; $P < .001$) at a 50-ms interstimulus interval. Levetiracetam increased TMS-evoked potential component N45 ($P = .004$) in a central cluster and decreased N100 ($P < .001$) in a contralateral cluster.

Conclusion: This study shows that levetiracetam, valproic acid and lorazepam decrease cortical excitability, which can be detected using TMS-EMG–EEG in healthy subjects. These findings provide support for the use of TMS excitability measures as

The authors confirm that the PI for this paper is Professor G.J. Groeneveld and that he had direct clinical responsibility for patients.

biomarkers to demonstrate pharmacodynamic effects of drugs that influence cortical excitability.

KEYWORDS

antiepileptic drugs, biomarkers, cortical excitability, levetiracetam, lorazepam, TMS-EEG, TMS-EMG, transcranial magnetic stimulation, valproic acid

1 | INTRODUCTION

Transcranial magnetic stimulation (TMS) is a noninvasive technique that can be used to investigate corticospinal excitability. Stimulation targeted at the motor cortex generates motor-evoked potentials (MEPs) and TMS-evoked potentials (TEPs), that can be quantified by electromyography (EMG)[1] and electroencephalography (EEG),[2,3] respectively. TMS-EMG and TMS-EEG facilitate assessment of different measures of cortical excitability, using a single-pulse (sp) and paired-pulse (pp) stimulation paradigm, of which the latter facilitates assessment of intracortical inhibition.[4,5] This study is intended to broaden and deepen the knowledge about effects of antiepileptic drugs (AEDs) on TMS-EMG–EEG outcomes, to further develop these outcomes as biomarkers for pharmacodynamic effects on cortical excitability. Although TMS-EMG has been widely used to assess the effects of drugs targeted at cortical excitability,[6] the number of research groups investigating pharmacological effects on TEPs is limited.[7–13] There is abundant space for further progress in replicating and extending the current knowledge about cortical excitability and in showing the value of TMS to measure biomarkers for pharmacodynamic effects in early-phase drug development.[7] Before being able to use TMS-related outcomes in clinical drug development with new pharmacological targets, it is of importance to determine the sensitivity of the measurement to detect pharmacological effects in healthy subjects, and the typical effect size of regularly used drugs administered at a dose within the therapeutic range. A reliable biomarker is a valuable investigative tool in clinical drug development, particularly in the development of new pharmacological treatments for diseases with underlying pathology related to cortical excitability, such as epilepsy[14,15] and amyotrophic lateral sclerosis.[16] The goal would be to use TMS-EMG–EEG outcomes as biomarkers for proof-of-mechanism.

Therefore, the primary objective of this study was to evaluate effects of 3 commonly prescribed AEDs (**levetiracetam**, **valproic acid** and **lorazepam**) on cortical excitability in a placebo-controlled, cross-over fashion in healthy subjects. These AEDs are expected to decrease cortical excitability with distinct mechanisms of action. The secondary objective was to evaluate intra- and intersubject variability of cortical excitability measures.

What is already known about this subject

- Pharmacodynamic effects of antiepileptic drugs on transcranial magnetic stimulation (TMS) combined with electromyography (EMG) and have been studied, but studies that assess drug effects on TMS–electroencephalography (EEG) are limited.
- The aim of this study was to show the value of TMS-EMG and TMS-EEG to determine effects of drugs targeting cortical excitability, for the purpose of using them as biomarkers in early-phase clinical drug development.

What this study adds

- Single doses of levetiracetam, valproic acid and lorazepam decrease cortical excitability in healthy subjects.
- The observed intrasubject variability allows the use of TMS-EMG to demonstrate pharmacological effects of compounds that affect cortical neuronal excitability.
- These findings support TMS-EMG–EEG as biomarkers for proof-of-mechanism in the early clinical phase of drug development.

2 | MATERIALS AND METHODS

This study was approved by the Ethics Committee Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, The Netherlands. The trial was executed in accordance to the Declaration of Helsinki at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands, between September 2017 and February 2018. The study is registered in the Dutch Trial Registry (NTR) under NL6638.

2.1 | Subjects

Subjects gave written informed consent. Healthy male subjects aged between 18 and 45 years were recruited using online advertisements and CHDR's subject database. Eligibility was confirmed by a medical screening up to 30 days before the first dose, consisting of evaluation of medical history, physical examination, electrocardiogram, blood chemistry, haematology and urinalysis. Subjects with contraindications according to the TMS safety questionnaire[17] were excluded, as well

as subjects with an abnormal sleeping pattern, (history of) illicit drug or alcohol abuse or a positive test for such substances, nicotine use a month before dosing, or a resting motor threshold (rMT) of >83% of the maximum stimulator output (MSO). Use of medication was prohibited from 14 days prior to the first dose. Use of caffeine was prohibited from 24 hours before dosing.

2.2 | Experimental design

This is a randomized, double-blind, double-dummy, placebo-controlled, cross-over study. On 4 visits, subjects received a single dose of levetiracetam 2000 mg (Levetiracetam, oral solution 100 mg/mL, Aurobindo) and placebo capsules; valproic acid 1000 mg (Depakine sugarfree oral solution 200 mg/5 mL, Sanofi-Aventis) and placebo capsules; lorazepam 2 mg (Lorazepam, 2 tablets of 1 mg, Apotex Europe BV) and placebo solution; or placebo solution and placebo capsules. Lorazepam tablets were encapsulated, and matching placebo capsules and solutions were produced. Drug doses were chosen within the therapeutic range. A Williams design was used to balance first-order carry-over effects.[18] The randomization of the treatment order was generated in SAS (version 9.4, SAS Institute Inc., Cary, USA) by a statistician uninvolved with data collection. The randomization remained blinded for all staff, apart from the statistician and the pharmacy preparing the medication. Subjects were enrolled by a blinded physician.

Subjects remained fasted from minimally 8 hours before until 2 hours after dosing. TMS-EMG–EEG measurements were performed before dosing and 1.5, 7 and 24 hours after dosing, based on the pharmacokinetic (PK) profile of the study drugs. The first postdose measurement was performed around time to maximum concentration (T_{max}), the second when an intermediate plasma concentration was expected, and the third at low concentrations. Measurements were performed at approximately the same clock time for all subjects, to minimize potential effects of diurnal variation on TMS outcomes. Samples for PK analysis were drawn directly before all TMS measurements and directly after the measurement at 1.5 hours postdose. Additionally, samples were obtained at 0.5 and 3.5 hours postdose. Between each study visit was a wash-out of at least 7 days. There was a safety follow-up 7–10 days after the last dose.

2.3 | Transcranial magnetic stimulation

Sp- and pp-TMS were applied according to guidelines by Rossi and colleagues,[19] using a MagPro R30 with MagOption stimulator and a MCF-B65 butterfly coil (MagVenture GmbH, Hückelhoven, Germany).

Stimulation was performed at the motor hotspot of the dominant abductor digiti minimi muscle as determined by the Edinburgh Handedness questionnaire.[20] The TMS coil was fixated using a frame at an angle of 45° from the midline, in direct contact with the EEG cap. At the start of each measurement, rMT was determined as the lowest stimulus intensity at which a minimum of 5 out of 10 TMS pulses elicited an MEP with a peak-to-peak amplitude of at least 50 μV.[21,22] Hereafter, 50 single pulses were applied at 120% rMT. This was followed by 50 paired pulses at different interstimulus intervals (ISIs), namely 2, 5, 50, 100, 150, 200, 250 and 300 ms, applied in randomized order (total 400 paired pulses). Conditioning and test pulses were applied at 120% rMT, except for ISIs 2 and 5 ms, where conditioning pulses were applied at an intensity of 80% rMT. The duration between single pulses and pairs of paired pulses was randomized between 3.5 and 4.5 seconds.

2.4 | EMG and EEG acquisition

EMG and EEG were registered simultaneously during TMS stimulation using NeuroCenter software (Clinical Science Systems, Leiden, The Netherlands). EMG was recorded with Ag/AgCl surface electrodes (Blue Sensor N, AMBU, Denmark) on the abductor digiti minimi and corresponding tendon. TEPs were registered using a TMS-compatible 32-leads EEG cap (ANT Neuro, Enschede, The Netherlands) and EEG amplifier (TMSi, Oldenzaal, The Netherlands). The ground electrode, used for both EEG and EMG, was located between Cz and Fpz. Electrode impedances were below 5 kΩ and signals were amplified at a frequency of 2048 Hz. During the TMS measurements, subjects received in-ear headphones with masking noise to minimize auditory evoked potentials.[23] Adapted noise, based on the frequencies of the TMS click, was played at an intensity individualized for each subject with a maximum volume of 95 dB. Masking of auditory components appears to be sufficient as represented by lateralized responses in the topographical plots even at late latencies[24] (Figure S1).

2.5 | Data processing and analysis

The following parameters were extracted from the TMS-EMG data: single pulse rMT (% of MSO) and mean peak-to-peak MEP amplitude (μV); long intracortical inhibition (LICI) at ISIs of 50–300 ms, defined as the percentage ratio of the mean MEP amplitude after the test pulse and the mean MEP amplitude after the conditioning pulse; short intracortical inhibition (SICI) at ISIs of 2 and 5 ms, defined as the percentage ratio of the mean MEP amplitude after the test pulse and the mean amplitude of the unconditioned single-pulse MEPs.

MEP amplitude, SICI and LICI were calculated using in-house written MATLAB (version R2015a, The Mathworks, Natick, MA, USA) scripts. To correct for pre-existing muscle activation, responses were excluded if muscle activity was >50 μV in the 50 ms before each single or conditioning pulse.

TEPs were determined at all 32 EEG leads. EEG responses were analysed in common average montage and were baseline corrected by subtracting the average EEG amplitude 500–50 ms before applying the single or conditioning pulse. Individual EEG trials were defined from 100 ms before until 650 ms after each single or conditioning pulse. Principal component analysis (PCA) was used to reduce artefacts caused by TMS stimulation and muscle activation on the scalp.[25]

The first 4 of 25 principal components were removed, after which individual trials were filtered (fourth order Butterworth bandpass filter; 1–35 Hz) and averaged over 50 repetitions to create the TEP for each EEG lead. Per condition (placebo, levetiracetam, valproic acid and lorazepam) TEP responses after artefact removal are shown for each individual subject in Figures S2–S5.

After all data were collected, review of the blinded TMS-EMG data was performed as per standard operating procedure at CHDR. Measurements with technical errors were removed from analysis.

2.6 | PK analysis

Serum concentrations of levetiracetam were measured by a validated high-performance liquid chromatography diode array detection method at the ISO 15189 certified Clinical Pharmaceutical Laboratory of the Leiden University Medical Center. Serum concentration of valproic acid were measured using an in vitro chemiluminescent microparticle immunoassay (CMIA) using an Abbott Architect system. The lower limit of quantification (LLOQ) was determined at 2.5 μg/mL for levetiracetam and 2 μg/mL for valproic acid. Serum lorazepam concentrations were analysed using an immunoassay at University Medical Center Groningen. The LLOQ was 5.21 ng/mL. All assays were validated in accordance to the EMA bioanalytical method development guideline (all coefficients of variation [CVs] below 15%).

2.7 | Statistical analyses

Treatment effects were analysed up to 7 hours postdose. We predefined that measurements performed at 24 hours after dosing would not be included due to the expected low drug concentrations at this time point but were measured for PK–pharmacodynamic (PKPD) modelling. Due to the exploratory nature of the study, no calculation of sample size was performed. Sample size was based on a previous pharmaco-TMS study in 15 healthy subjects.[9]

Statistical analysis of rMT, MEP and TEP endpoints was performed using a mixed model analysis of covariance (ANCOVA), with treatment, time, period and treatment by time as fixed factors and subject, subject by treatment and subject by time as random factors, and the baseline measurement per period as covariate. Estimated differences between placebo and the AEDs were reported and statistical significance was defined at the 5% level. Analysis of effects on TMS-EMG endpoints was performed in SAS (version 9.4, SAS Institute Inc., Cary, NC, USA).

Statistical analysis of TMS-EEG outcomes was performed using cluster-based permutation analysis (CBPA) incorporating all leads. CBPA was performed in Fieldtrip (Nijmegen, The Netherlands, downloaded 13-08-2015; http://fieldtrip.fcdonders.nl).[26] Comparison of drug *vs*. placebo was performed using dependent samples *t*-tests, for each EEG lead and time sample between 0–300 ms after the test pulse (for both sp- and pp-TMS). To compensate for handedness, topographical plots of left-handed subjects were mirrored. Clusters were formed by *t*-values with a *P*-value <.05, based on neighbouring leads ($n \geq 2$) and adjacent time samples. A permutation test (1500 times) was used to determine significance at the 5% level.[26] Additionally, we applied a Bonferroni correction ($n = 3$) to compensate for multiple testing (3 active conditions). Besides analysing the entire time sample of 300 ms after the test pulse, we applied the same analysis to time periods of interest around the TEP components (N15: 0–20 ms; P30: 20–40 ms; N45: 40–55 ms; P60: 55–80 ms; N100: 80–130 ms; P180: 130–230 ms).

For the purpose of evaluating repeatability, intra- and intersubject variability were calculated, represented by CVs. CVs were calculated within the placebo visit, including measurements up to 7 hours postdose, using estimates of covariance parameters produced by the mixed model analysis. The serum concentration of the AEDs was analysed using a noncompartmental analysis.

2.8 | PKPD analysis

Concentration–effect relationships between MEP amplitude and treatments (including all timepoints up to 24 h postdose) were investigated with nonlinear mixed effects modelling, using PK data linked to the closest available pharmacodynamic measurement in time. Tested PKPD-model structures included intercept (no effect), linear and nonlinear (Emax) relationships, with additional interindividual variability (IIV) and/or between-occasion variability (BOV) for the baseline parameter. Initial analysis was performed in R (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria), where models were compared with an analysis of variance for nested models ($P < .05$) or with the Akaike information criterion (lowest value is favoured) for non-nested models. Treatments for which concentration–effect relationships other than intercept were selected, were also analysed using NONMEM (version 7.4, ICON Development Solutions, Hanover, MD, USA), where models were compared based on drop in objective function value (dOFV>3.84, $P < .05$) for 1 additional parameter for nested models or Akaike information criterion for non-nested models.

2.9 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22.[27–29]

3 | RESULTS

Seventeen subjects were enrolled in the study, of whom 16 completed all study visits (for demographics see Table 1). One subject was excluded after 1 study visit, due to positive illicit drugs testing.

The AEDs and TMS measurements were well tolerated. Individual, and mean ± standard deviation serum concentrations of the AEDs

are shown in Figure 1. Mean maximum concentrations were 45.92 μg/mL (range 32.10–67.60) for levetiracetam, 70.69 μg/mL (range 58.00–86.00) for valproic acid and 19.79 ng/mL (range 14.99–24.84) for lorazepam. Mean serum concentrations per timepoint are also listed in Table S1. Median T_{max} was 0.51 hours for levetiracetam (range 0.5–1.47), and 0.53 hours for valproic acid (range 0.5–2.25), corresponding to the first sampling point. The median T_{max} was 1.91 hours (range 1.37–3.52) for lorazepam, with 10 of 16 subjects showing a lag time of 30 minutes.

TABLE 1 Sample characteristics

$n = 17$	Mean	SD	Median	Range
Age (y)	25	6	24	20–44
Height (cm)	183	8	184	167–194
Weight (kg)	75	13	74	54–109
BMI (kg/m^2)	22	4	21	19–32

BMI, body mass index; SD, standard deviation.

3.1 | Cortical excitability assessed by TMS-EMG

In total, 192 measurements were recorded up to 7 hours postdose. During blinded data review, 6 EMG recordings were excluded because of absence of MEPs (indicating there was no motor hotspot stimulation) or clipping of the EMG signal.

Estimated mean postdose MEP amplitudes were 889.3 μV (placebo), 510.9 μV (levetiracetam), 620.5 μV (valproic acid) and 558.6 μV (lorazepam). All AEDs significantly decreased MEP amplitude after sp-TMS when compared to placebo, with an estimated difference of −378.4 μV (95%CI: −644.3, −112.5; $P < .01$) for levetiracetam, −268.8 μV (95%CI: −532.9, −4.6; $P = .047$) for valproic acid, and −330.7 μV (95%CI: −595.6, −65.8; $P = .02$) for lorazepam, see Figure 2. Intrasubject CV of MEP amplitude was 35%, intersubject CV 84%.

Estimated mean postdose rMT was 55.3, 55.7, 54.3 and 55.5% of MSO for placebo, levetiracetam, valproic acid and lorazepam, respectively. No significant treatment effects on rMT were detected when

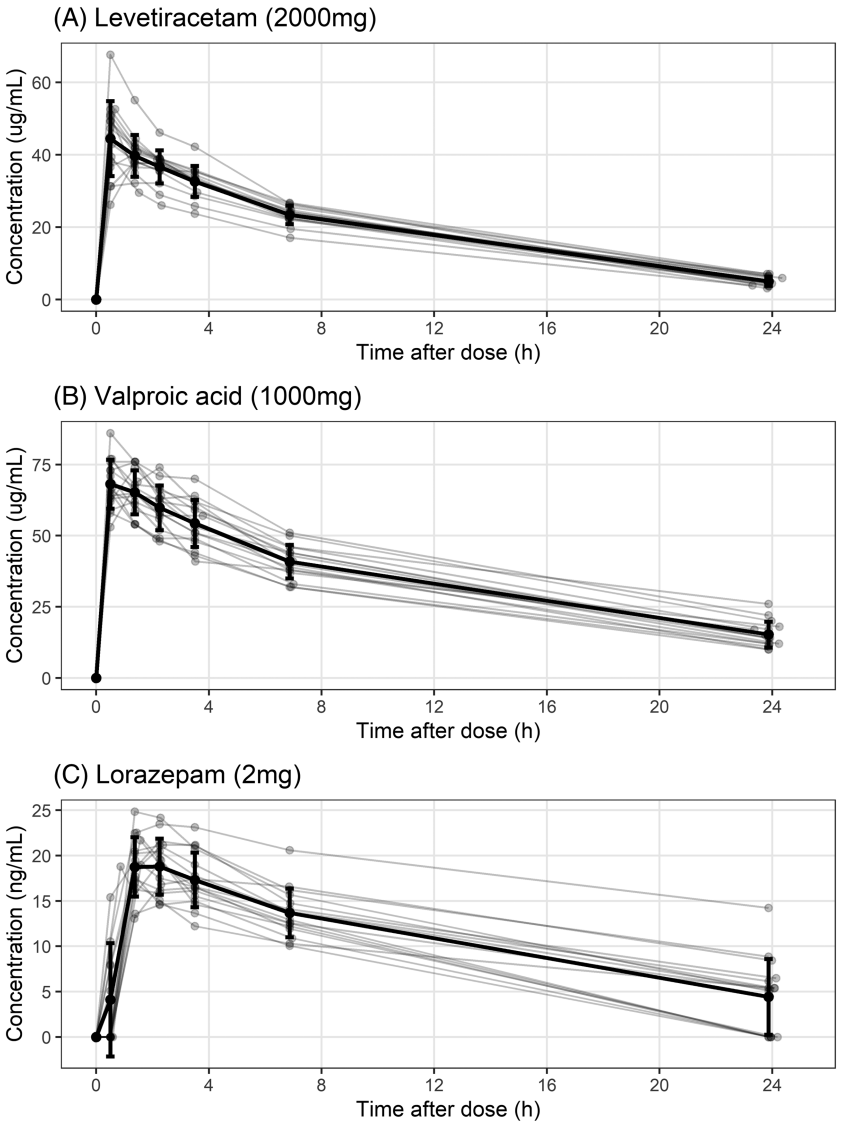

FIGURE 1 Individual and mean ± standard deviation serum concentrations of levetiracetam, valproic acid and lorazepam

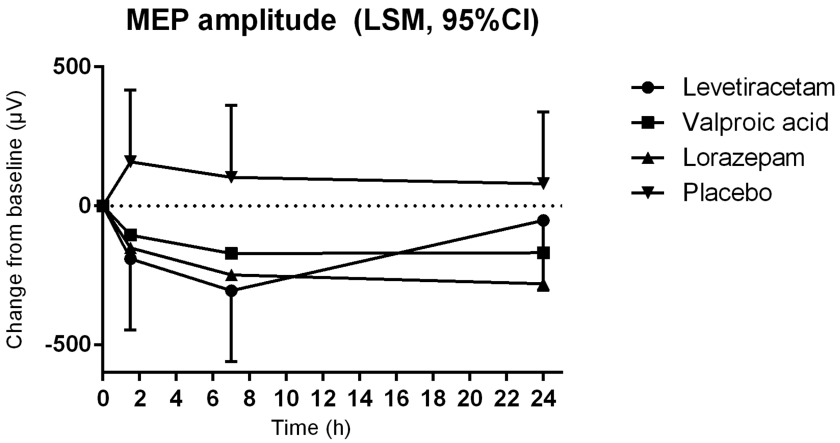

FIGURE 2 Change from baseline of the least square means (LSM) of the motor-evoked potential (MEP) amplitude (μV), using single pulse transcranial magnetic stimulation, for levetiracetam, valproic acid, lorazepam and placebo

TABLE 2 Estimated mean postdose long intracortical inhibition (LICI) and short intracortical inhibition (SICI) (%), up to 7 hours after administration of placebo, levetiracetam, valproic acid and lorazepam, measured using paired-pulse transcranial magnetic stimulation–electromyography at 8 different interstimulus intervals (ISI). Estimated difference of placebo *vs*. treatment (%), with 95% confidence interval (CI) and *P*-value. Intrasubject coefficient of variation (CV; %) and intersubject CV (%) within the placebo treatment visits are listed

ISI (ms)		Estimated mean SICI/LICI (%)	Estimated difference with placebo (95% CI), *P*-value	Intrasubject CV	Intersubject CV
2	Placebo	35.7		50%	58%
	Levetiracetam	42.4	6.7 (−6.5, 20.0), $P = .31$		
	Valproic acid	48.5	12.8 (−0.4, 26.0), $P = .06$		
	Lorazepam	47.4	11.7, (−1.5, 24.9), $P = .08$		
5	Placebo	74.0		45%	48%
	Levetiracetam	78.5	4.5 (−20.9, 29.9), $P = .72$		
	Valproic acid	88.7	14.7 (−10.4, 39.8), $P = .24$		
	Lorazepam	90.4	16.4 (−9.1, 41.8), $P = .20$		
50	Placebo	102.9		85%	103%
	Levetiracetam	42.6	***−60.3 (−87.1, −33.5), P < .001***		
	Valproic acid	78.0	−24.9 (−51.2, 1.4), $P = .06$		
	Lorazepam	34.7	***−68.2 (−94.7, −41.7), P < .001***		
100	Placebo	9.9		134%	172%
	Levetiracetam	7.3	−2.6 (−10.9, 5.6), $P = .52$		
	Valproic acid	8.9	−1.0 (−9.2, 7.2), $P = .81$		
	Lorazepam	4.9	−5.0 (−13.3, 3.2), $P = .22$		
150	Placebo	19.9		92%	121%
	Levetiracetam	21.0	1.1 (−11.2, 13.5), $P = .86$		
	Valproic acid	18.1	−1.8 (−14.0, 10.4), $P = .77$		
	Lorazepam	14.7	−5.2 (−17.6, 7.2), $P = .40$		
200	Placebo	64.4		38%	60%
	Levetiracetam	70.6	6.2 (−10.3, 22.8), $P = .45$		
	Valproic acid	63.4	−1.0 (−17.6, 15.6), $P = .91$		
	Lorazepam	56.8	−7.6 (−24.1, 8.8), $P = .36$		
250	Placebo	64.7		47%	45%
	Levetiracetam	73.8	9.0 (−8.5, 26.6), $P = .31$		
	Valproic acid	77.7	13.0 (−4.3, 30.3), $P = .14$		
	Lorazepam	78.9	14.2 (−3.3, 31.6), $P = .11$		

(Continues)

TABLE 2 (Continued)

ISI (ms)		Estimated mean SICI/LICI (%)	Estimated difference with placebo (95% CI), *P*-value	Intrasubject CV	Intersubject CV
300	Placebo	55.3		34%	49%
	Levetiracetam	54.4	−0.9 (−12.7, 10.9), $P = .88$		
	Valproic acid	52.9	−2.4 (−14.0, 9.3), $P = .68$		
	Lorazepam	66.6	11.3 (−0.4, 23.1), $P = .06$		

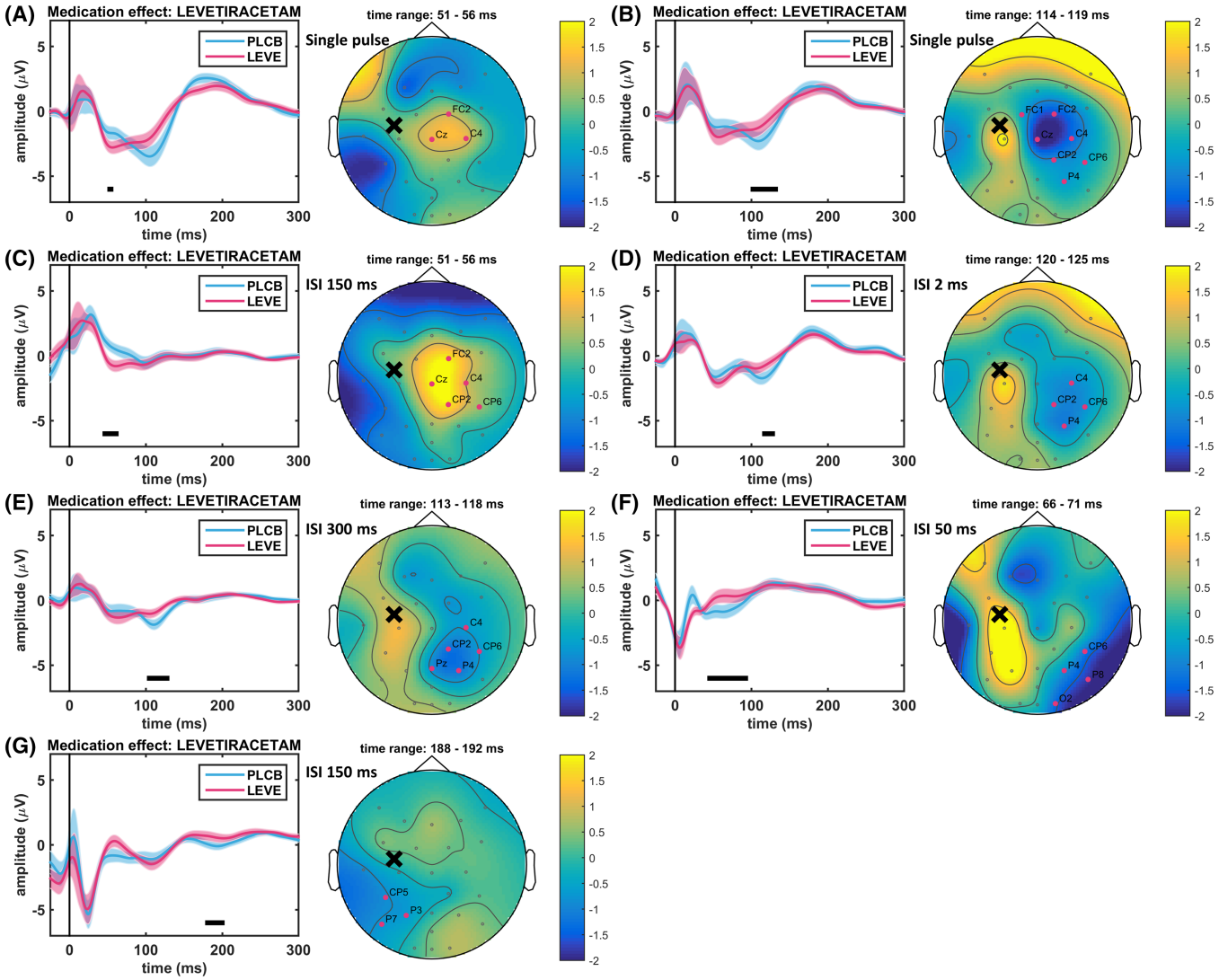

FIGURE 3 Significant clusters found using cluster-based permutation analysis of TEPs, comparing placebo (PLCB; in blue) to levetiracetam (LEVE; in red). (A) single pulse (N45 cluster), (B) single pulse (N100 cluster), (C) ISI 150 ms (N45/P60 cluster), (D) ISI 2 ms (N100 cluster), (E) ISI 300 ms (N100 cluster), (F) ISI 50 ms (N45/P60/N100 cluster) and (G) ISI 150 ms (P180 cluster). For each cluster the grand average (mean ± standard error of the mean) over all significant electrodes is presented, as well as the difference in topographical distribution at the time of the cluster. The colours of the topographical plot of the cortex show the increase or decrease of amplitude (μV) of the response. The black cross represents the stimulation site, the red dots significant electrodes and the thick black bar below the average TEP response corresponds to the time interval with significant differences

compared to placebo, with estimated differences of 0.4% for levetiracetam (95%CI: −1.1%, 1.9%; $P = .61$), −1.0% for valproic acid (95%CI: −2.5%, 0.5%; $P = .19$) and 0.2% for lorazepam (95% CI: −1.3%, 1.7%; $P = .78$). Intrasubject CV of rMT was 4%, intersubject CV 14%.

Levetiracetam and lorazepam both significantly enhanced LICI compared to placebo at ISI 50 ms (i.e. the percentage ratio decreased, indicating more intracortical inhibition). No significant effects on LICI were detected at the other ISIs, nor on SICI. Results and CVs for SICI and LICI are listed in Table 2.

3.2 | Cortical excitability assessed by TMS-EEG

Of 192 TMS-EEG recordings, 3 were excluded after blinded data review of the EMG data, because of absence of MEPs.

For sp-TMS, levetiracetam significantly increased the N45 compared to placebo ($P = .004$) in a centrally located cluster (Figure 3A). Furthermore, levetiracetam significantly decreased the N100 in a contralateral centroparietal cluster ($P < .001$; Figure 3B).

In line with the results for sp-TMS, levetiracetam significantly increased the N45 and P60 in a similar centroparietal cluster at ISI

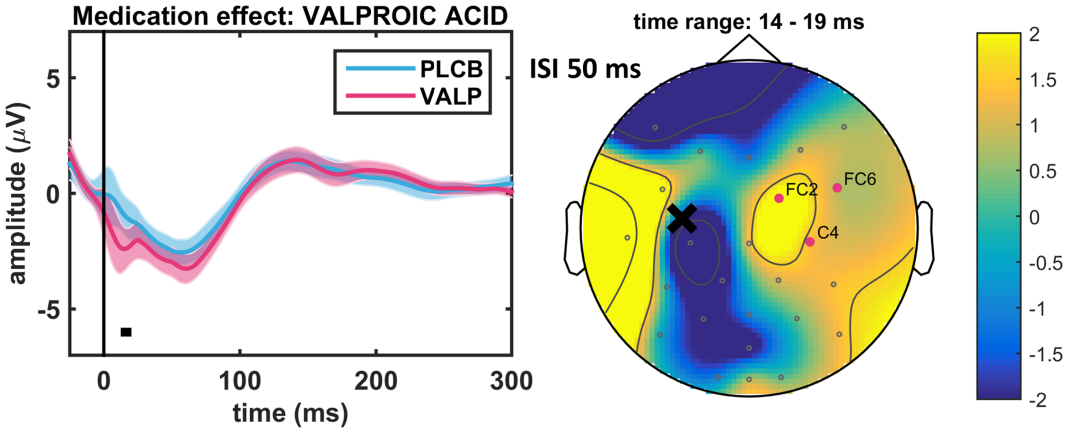

FIGURE 4 Significant N15 cluster comparing paired pulse transcranial magnetic stimulation-evoked potentials of placebo (PLCB; in blue) with valproic acid (VALP; in red) for ISI 50 ms. The grand average (mean ± standard error of the mean) over all significant electrodes is presented, as well as the difference in topographical distribution at the time of the cluster. The colours of the topographical plot of the cortex show the increase or decrease of amplitude (μV) of the response. The black cross represents the stimulation site, the red dots significant electrodes and the thick black bar below the average transcranial magnetic stimulation-evoked potential response corresponds to the time interval with significant differences

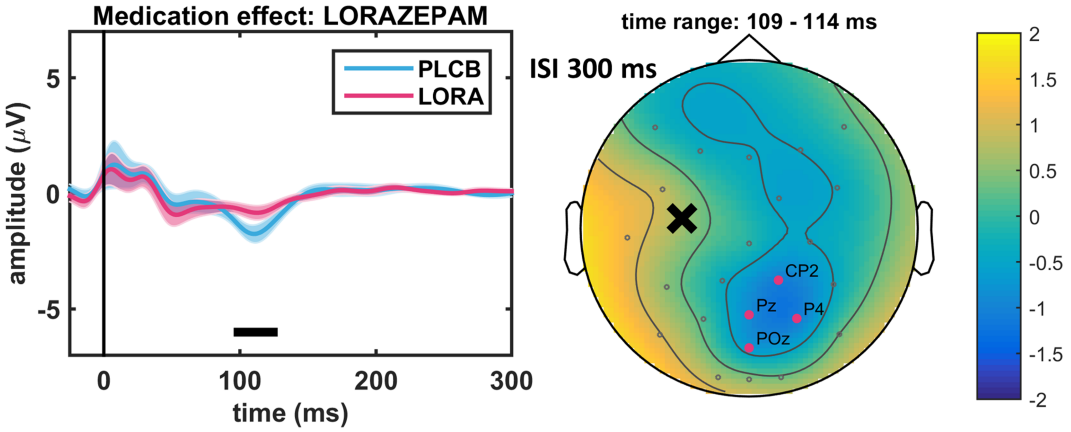

FIGURE 5 Significant N100 cluster comparing paired-pulse transcranial magnetic stimulation-evoked potentials of placebo (PLCB; in blue) with lorazepam (LORA; in red) for ISI 300 ms. The grand average (mean ± standard error of the mean) over all significant electrodes is presented, as well as the difference in topographical distribution at the time of the cluster. The colours of the topographical plot of the cortex show the increase or decrease of amplitude (μV) of the response. The black cross represents the stimulation site, the red dots significant electrodes and the thick black bar below the average transcranial magnetic stimulation-evoked potential response corresponds to the time interval with significant differences

150 ms ($P < .001$ and $P = .004$, respectively; Figure 3C). In addition, we found that levetiracetam significantly decreased N100 clusters at ISIs 2 and 300 ms ($P = .003$ and $P = .003$, respectively; Figure 3D and E), these clusters are comparable to the N100 cluster found using sp-TMS. Furthermore, we found a significant N45, P60 and N100 cluster ($P = .004$, $P < .001$ and $P = .004$, respectively) at ISI 50 ms (Figure 3F). A significant P180 cluster ($P = .006$) was detected at ISI 150 ms (Figure 3G).

Valproic acid significantly increased the N15 amplitude ($P = .005$) at ISI 50 ms in a contralateral cluster (Figure 4).

Lorazepam significantly decreased the N100 ($P = .001$) at ISI 300 ms in a contralateral parietal cluster (Figure 5).

3.3 | Concentration–effect relationship of MEP amplitude

A concentration–effect relationship between MEP amplitude and levetiracetam could be characterized with a proportional effect on baseline, described with an Emax-equation ($P < .001$ compared to intercept only). IIV and BOV on the baseline parameter was log-normally distributed and statistically preferred over either IIV or BOV alone, although shrinkage for BOV was moderate (25 and 33%). Parameters were estimated with small uncertainty (i.e. relative standard error, RSE < 50%), except for EC50 which was estimated with an RSE of 90.38%. Inclusion of IIV for EC50 or EMAX did not improve the model in terms of OFV nor RSE%. Residual unexplained variability was best described with a proportional error structure. Model parameters from analysis in NONMEM are listed in Table S2 and a model simulation for the typical individual is shown in Figure S6. Based on available data, no concentration–effect relationships could be found for valproic acid and lorazepam on MEP amplitude.

4 | DISCUSSION

In this study, the effects of 3 AEDs on cortical excitability were assessed using sp- and pp-TMS-EMG and TMS-EEG. All drugs decreased cortical excitability. Levetiracetam, valproic acid and lorazepam all significantly decreased MEP amplitude. Additionally, levetiracetam and lorazepam enhanced LICI at ISI 50 ms. Levetiracetam affected the amplitude of TEP components N45 and N100 in EEG clusters after sp-TMS.

The mechanism through which these drugs inhibit cortical excitability differ, which is reflected by the distinguishing fingerprints that were detected on TEP components. This finding gives new insights into pharmacological effects on TEPs, in addition to the existing literature.[30] In this discussion, we compared our results to placebo-controlled trials, to facilitate accurate comparison to our results.

4.1 | TMS as a pharmacodynamic biomarker

We assessed the variability of TMS-EMG and the feasibility of TMS-EMG–EEG for the purpose of using it in early-phase clinical drug development. The main goal would be to use TMS-EMG–EEG outcomes as pharmacodynamic biomarkers for proof-of-mechanism of novel compounds that modulate cortical excitability. In our opinion, TMS-EMG and TMS-EEG are suitable to be used for this purpose. This is supported by our observed significant effects of single doses of 3 AEDs with different mechanisms of action, in a sample size that is typically used in early-phase proof-of-mechanism-like drug studies. Moreover, TMS-EMG–EEG embodies certain qualities that are favourable to pharmacodynamic biomarkers: the method is noninvasive and relatively quick to perform, which allows for multiple measurements at different drug concentrations.

Although it should be noted that the variability of the outcome parameters is relatively high, the effect size was large enough to generate significant results in a small number of subjects. Therefore, we consider TMS-EMG–EEG outcomes as useful biomarkers for proof-of-mechanism of new compounds. In our opinion, TMS-EMG can be used in Phase 1 dose escalation study designs, to evaluate target engagement and to aid in dose finding for further studies. Because the intersubject variability of TMS-EMG was higher than the intrasubject variability, we would propose to use TMS to demonstrate pharmacological effects in a cross-over rather than a parallel study design.

As an exploratory outcome of this study, we have evaluated the concentration–effect relationship between the study drugs and MEP amplitude. A significant PKPD relationship was detected for levetiracetam, but not for valproic acid and lorazepam. It should be noted that the design of our study was not ideal for the assessment of PKPD relationships, because the concentration range observed in this study is relatively small and the number of post-dose measurements is limited. This is also demonstrated by the high uncertainty around the estimated EC50 parameter for levetiracetam. Whether TMS-EMG–EEG, despite the high variability of the outcomes, can be used for evaluation of concentration–effect relations therefore remains to be confirmed in future studies. Administration of multiple dose levels of the same compound can inform this concentration–effect relationship across a wider range of concentrations, which would lower the parameter uncertainty currently observed in the model.

4.2 | Effects of levetiracetam

Levetiracetam targets **synaptic vesical glycoprotein SV2a**, which decreases central neurotransmitter release[31] and therefore theoretically decreases cortical excitability. We showed a significant decrease of MEP amplitude induced by levetiracetam, indicating reduced excitability, in line with previously reported results.[32] Other studies showed a nonsignificant decrease of MEP amplitude after administration of levetiracetam[33,34] and brivaracetam, an AED with a closely related mechanism of action.[7]

With use of CBPA of sp-TEPs, our study demonstrated that levetiracetam increased the amplitude of the N45 component in a central cluster and decreased the N100 amplitude contralateral to

the stimulation site. The decrease in N100 is consistent with changes caused by brivaracetam.[7] The increase in N45 is also in line with literature.[9] We found the effect in a contralateral cluster, whereas the N45 component showed widespread negativity in the study by Premoli *et al*., with the maximum effect in the ipsilateral hemisphere.[9]

To our knowledge, our study is the first to evaluate effects of levetiracetam on paired pulse TMS-EEG. Interestingly, the effect we observe on the N100 cluster following sp-TMS, is very similar in shape and localization to the significant N100 clusters detected at ISIs 2 and 300 ms.

There is substantial evidence that the N45 component represents γ-aminobutyric acid-A (**$GABA_A$) receptor** mediated inhibition, whereas **$GABA_B$ receptor** activity is reflected by the N100 component.[10] Our findings on the N45 component may therefore provide further indication that levetiracetam indirectly affects $GABA_A$ergic inhibition.[9,35] The effect of levetiracetam on N100 in the contralateral hemisphere may be caused by inhibition of cortico-cortical connections, as previously suggested for brivaracetam.[7]

4.3 | Effects of valproic acid

The antiepileptic mechanism of action of valproic acid has not been completely clarified. It induces inhibition through the increase of GABA availability. Furthermore, valproic acid blocks **voltage-gated sodium channels**, affects neuronal potassium and calcium regulation, and inhibits N-methyl-D-aspartate (NMDA) transmission.[36]

To our knowledge this is the first study to report that valproic acid decreased MEP amplitude in healthy subjects. A previous study did not report an effect on MEP amplitude, but this study was not placebo-controlled.[37] The effect on MEP amplitude confirms that valproic acid decreases cortical excitability, as can be expected based on the mechanism of action.

To the best of our knowledge, no previous studies using TMS-EEG were performed to investigate the effect of valproic acid in healthy subjects. Using TMS-EEG, we detected a significant N15 cluster at ISI 50 ms. Interestingly, considering the proposed mechanisms of action of the drug, our results indicate that valproic acid does not induce the same effect on the N45 and N100 components as lorazepam and other positive allosteric modulators (PAMs) of $GABA_A$ receptors.[10] The effect also does not bear resemblance to the effect of sodium channel blockers, such as lamotrigine, which increased N45 and decreased P180,[9] nor NMDA-receptor antagonists, such as dextromethorphan which increases the N45 component.[13] The effects of valproic acid on TEPs will need to be repeated to confirm if the effects on N15 can be reproduced.

4.4 | Effects of lorazepam

Lorazepam is a $GABA_A$ receptor PAM that stimulates GABAergic inhibition.[38] Our study demonstrated a decrease in MEP amplitude by lorazepam, in line with previous findings on stimulus response curves,[39,40] indicating reduced excitability. LICI at ISI 50 ms was enhanced by lorazepam, which is associated with $GABA_B$ receptor mediated inhibition,[41] similar to the N100 component. No effect on SICI was detected, corresponding to results of other studies.[39,42]

Previous studies using sp-TMS showed effects of other $GABA_A$-PAMs on N45 and N100, leading to the hypothesis that the N45 component is correlated to $GABA_A$ receptor mediated inhibition.[10] It is therefore unexpected that we did not replicate these findings with lorazepam, which may be explained by a smaller number of subjects and relatively large variability in the measurements, indicating that our study is possibly underpowered for demonstrating this effect. In our study, lorazepam did induce a significant cluster with a decrease of N100 using pp-TMS (ISI 300 ms).

4.5 | Recommended statistical analysis in placebo controlled TMS trials

Previous studies often assessed drug effects on cortical excitability by comparing predose and postdose outcomes in treatment and placebo condition separately. However, in a placebo-controlled trial, a more appropriate analysis would be to compare the treatment effect to placebo.[43] In the current study, an ANCOVA was used, because it can provide a comparison between treatment and placebo, using the baseline measurement per period as covariate. This analysis takes into account the intersubject variability by introducing a random subject effect, while the intrasubject variability is given by the residual error term. Time effects, such as diurnal variation, are taken into account by including time effect in the model, while the subject by time interaction allows for different time effect between subjects. Similarly, the subject by treatment interaction included in the model allows for different treatment effect across subjects. Finally, potential pretreatment differences are corrected by including the baseline as covariate. Due to these advantages over predose *vs*. postdose comparison, we recommend direct comparison of treatment *vs*. placebo in future placebo-controlled pharmaco-TMS studies.

4.6 | Possible limitations

The use of a 32-lead EEG cap, as opposed to 64 leads, could have impacted results and could explain why certain treatment-induced EEG clusters detected in previous studies were not confirmed in this study. A cluster, consisting of a minimum of 3 leads with significant signal changes in the same direction, covers a relatively larger area using 32-lead EEG and therefore needs to be more extensive than with 64-lead EEG.

Single trial PCA was applied to reduce artefacts caused by TMS stimulation and muscle activation on the scalp. PCA has shown to be an effective method to reduce both artefacts simultaneously, as seen for example in subjects 4, 5 and 8 in Figures S2–S5. However, in others (e.g. subjects 2, 6 and 11) the final TEP is still contaminated by residual artefacts. Since no consensus has been reached within the TMS-EEG community on a common *gold standard* analysis approach,

numerous alternative artefact rejection methods exist with each their own advantages and disadvantages.[44] As the final TEP is most likely to be largely influenced by the applied preprocessing pipeline,[45] we chose to use PCA making our results comparable with previous own findings, although this method may not always perform optimally.

Although not a limitation itself, it should be noted that the stimulation intensity was adjusted prior to each TMS session, based on the rMT at the start of each measurement. This is in contrast to some previous studies, such as a study analysing levetiracetam effects on TMS-EEG.[9] We chose this approach to make sure that stimulation intensity was always related to the rMT, and that changes in rMT (e.g., due to drug effects) would not lead to subthreshold stimulation. Importantly, we did not observe a significant change in rMT and therefore this should not have impacted the comparison of the results of our study to previous studies.

5 | CONCLUSIONS

The aim of this study was to show the value of TMS-EMG and TMS-EEG in determining effects of drugs targeting cortical excitability, for the purpose of developing these measurements as pharmacodynamic biomarkers for use in early-phase clinical drug development. Pharmacodynamic effects on TMS-EMG have been intensively studied, but studies that assess drug effects on TMS-EEG are limited. Therefore, we investigated the sensitivity of TMS-EMG–EEG to detect effects of 3 commonly prescribed AEDs on cortical excitability in a double-blind, placebo-controlled, 4-way cross-over study in healthy subjects. Our study shows that a single dose of levetiracetam, valproic acid and lorazepam decreases cortical excitability, as expected from antiepileptic drugs. These findings support the development of TMS-EMG and TMS-EEG as suitable biomarkers for proof-of-mechanism of new treatments in the early clinical phase.

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COMPETING INTERESTS

Michel J.A.M. van Putten is co-founder of Clinical Science Systems, Leiden, The Netherlands. All other authors declare no conflict of interest.

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PATIENT CONSENT

Subjects gave written informed consent.

CLINICAL TRIAL REGISTRATION

The study is registered in the Dutch Trial Registry (NTR) under NL6638.

DATA AVAILABILITY STATEMENT

Data and scripts that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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