

TMS combined with EEG in genetic generalized epilepsy: A phase II diagnostic accuracy study



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HIGHLIGHTS

- A paired-pulse TMS-EEG protocol with multi-level data analysis is presented.
- The accuracy of TMS-EEG for differentiating genetic generalized epilepsy (GGE) patients from controls is high.
- TMS-EEG differentiates responders from non-responders to antiepileptic drugs in GGE.

ABSTRACT

Objectives: (A) To develop a TMS-EEG stimulation and data analysis protocol in genetic generalized epilepsy (GGE). (B) To investigate the diagnostic accuracy of TMS-EEG in GGE.

Methods: Pilot experiments resulted in the development and optimization of a paired-pulse TMS-EEG protocol at rest, during hyperventilation (HV), and post-HV combined with multi-level data analysis. This protocol was applied in 11 controls (C) and 25 GGE patients (P), further dichotomized into responders to antiepileptic drugs (R, $n = 13$) and non-responders (n-R, $n = 12$). Features ($n = 57$) extracted from TMS-EEG responses after multi-level analysis were given to a feature selection scheme and a Bayesian classifier, and the accuracy of assigning participants into the classes P-C and R-nR was computed.

Results: On the basis of the optimal feature subset, the cross-validated accuracy of TMS-EEG for the classification P-C was 0.86 at rest, 0.81 during HV and 0.92 at post-HV, whereas for R-nR the corresponding figures are 0.80, 0.78 and 0.65, respectively. Applying a fusion approach on all conditions resulted in an accuracy of 0.84 for the classification P-C and 0.76 for the classification R-nR.

Abbreviations: TMS-EEG, TMS combined with EEG; GGE, genetic generalized epilepsies; AEDs, antiepileptic drugs; HV, hyperventilation; EDs, epileptiform discharges; TMS, Transcranial Magnetic Stimulation; TEPs, TMS-evoked potentials; SI, stimulus intensity; MSO, maximum stimulator output; LEThr, lower epileptogenic threshold; UEThr, upper epileptogenic threshold; ISI, inter-stimulus interval; MDS, multi-dimensional scaling.

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Conclusion: TMS–EEG can be used for diagnostic purposes and for assessing the response to antiepileptic drugs.

Significance: TMS–EEG holds significant diagnostic potential in GGE.

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1. Introduction

Epilepsy, the propensity for recurrent, unprovoked epileptic seizures, is one of the commonest serious neurological disorders. It is estimated that approximately 65 million people worldwide suffer from this disease, experiencing significant negative consequences on their physical and mental health, education, ability to work and their overall quality of life (Moshé et al., 2015).

Scalp EEG is the principal laboratory diagnostic test for epilepsy, but suffers from significant limitations. First, it has a fairly low sensitivity, so that a significant proportion of patients (20–45%) remain undiagnosed, leading to delayed treatment (Pillai and Sperling, 2006). Second, it does not predict with sufficient precision who will suffer from recurrent seizures and it cannot be used efficiently as a biomarker for personalized patient management. This may result in unnecessary treatment of those who will remain seizure-free without medication, but also in withdrawal of treatment in those who are bound to suffer further seizures. Finally, EEG does not predict reliably the most effective and well-tolerated pharmaceutical or neuromodulatory interventions. It is clear that a novel biomarker with improved diagnostic and predictive yield, compared to scalp EEG alone, is highly desirable.

In recent years, Transcranial Magnetic Stimulation (TMS) emerged as a novel biomarker with numerous applications in the field of epilepsy (Kimiskidis et al., 2014). Pivotal studies in newly diagnosed and refractory epilepsy suggest that TMS provides evidence of cortical hyperexcitability in a syndrome-specific pattern and may serve as an early predictor of pharmacoresistance in individual patients (Badawy et al., 2007, 2010, 2013). On the other hand, there are limitations to the type of information that can be derived from these studies because they employ the method of TMS–EMG in which responses are recorded exclusively from muscles and stimulation is performed over the primary motor cortex. It is easily conceivable that for the investigation of a disease operating at the cortical level, such as epilepsy, recordings from the cerebral cortex and stimulation over the entire cortical mantle would be far more informative.

The advent of TMS combined with EEG (TMS–EEG) opened up new avenues for the investigation of epilepsy allowing, for the first time in a noninvasive manner, the recording and mapping of neuronal responses induced by TMS at the cortical level, as well as the investigation and modulation of brain connectivity (Ilmoniemi and Kicić, 2010). Recent studies suggest that TMS may result in the induction (Valentin et al., 2008; Kimiskidis et al., 2013, 2015), but also modulation of ictal and interictal epileptiform discharges (EDs) and therefore has significant diagnostic, prognostic and possibly therapeutic potential (Rotenberg, 2010; Kimiskidis, 2016).

Although TMS–EEG is a highly promising method in the field of epilepsy (Rotenberg, 2010), its clinical and research potential remain underutilized. There is a single diagnostic study of TMS–EEG in patients with focal epilepsy (Valentin et al., 2008), concluding that this novel method can reliably identify the epileptogenic zone and may significantly improve the diagnostic approach to epilepsy. No study has investigated the diagnostic potential of TMS–EEG in genetic generalized epilepsies (GGE) so far.

The present paper describes an exploratory TMS–EEG study in GGE with the following objectives. (A) To develop and optimize a TMS–EEG brain stimulation and data analysis protocol in patients with GGE. (B) To investigate the diagnostic accuracy of TMS–EEG in patients with GGE.

2. Methods

2.1. Study design

The study was designed as a phase II diagnostic accuracy study (Gluud and Gluud, 2005; Sackett and Haynes, 2002) aiming: (a) to compare TMS–EEG findings in patients with known disease (GGE) and healthy controls (cross-sectional phase IIa study), and (b) to investigate whether TMS–EEG results in the patient group are related to response to antiepileptic drug (AEDs) treatment (delayed type cross-sectional phase IIb study (Knottnerus and Muris, 2003)). To the latter end, the patient group was dichotomized into a *responder to AEDs subgroup* (patients remaining seizure-free for at least 12 months post-TMS–EEG examination), and a *non-responder to AEDs subgroup* (patients experiencing non-provoked seizures during the post-examination follow-up period) and TMS–EEG findings were compared between the two subgroups (STARD diagram provided in Fig 1).

The reference standard was the diagnosis of two experienced epileptologists who, on the basis of clinical and laboratory data, reached consensus regarding the assignment of a subject in the patient or healthy control group (phase IIa study) and the designation of responder/non-responder status in the patient group (phase IIb study). It should be noted that the epileptologists determining subject status were not involved in the execution of the index test or data analysis. Routine scalp EEG was not employed by itself as a reference standard, due to well-established limitations regarding sensitivity (Krumholz et al., 2007; Valentin et al., 2008).

Sample size calculations were based on the only currently available evidence regarding the diagnostic accuracy of TMS–EEG (Valentin et al., 2008). On the basis of these data, it was estimated that a sample size of 23 patients and 11 controls would provide a sensitivity of 0.91 (minimum sensitivity of 0.65 and minimum specificity of 0.65), with an alpha = 0.05 and beta = 0.10.

2.2. Subjects

Study participants gave written informed consent for the procedures, which were approved by an institutional review board, and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

The study population included a cohort of 25 patients with GGE (11 females; median age 28 years, range 18–43), slightly exceeding the estimated sample size so as to account for lost-to-follow up cases, as well as an age-matched group of 11 healthy controls (6 females, median age 26 years, range 19–47).

The patient group comprised consecutive adult patients with GGE screened and referred from a tertiary Outpatient Epilepsy clinic of a University Hospital on the basis of the following *inclusion criteria*: (a) they passed the TASS questionnaire (Keel et al., 2001), save for the epilepsy related questions, and (b) they had both clinical and EEG features consistent with GGE. All patients had suffered at least two generalized seizures and were started on AEDs prior to study enrollment. *Exclusion criteria* included the presence of CNS disorders other than epilepsy on history or examination, comorbid conditions, EEG evidence of focal abnormalities, slow spike and wave discharges or triphasic patterns, use of centrally acting drugs other than AEDs and history of current or past alcohol or recre-

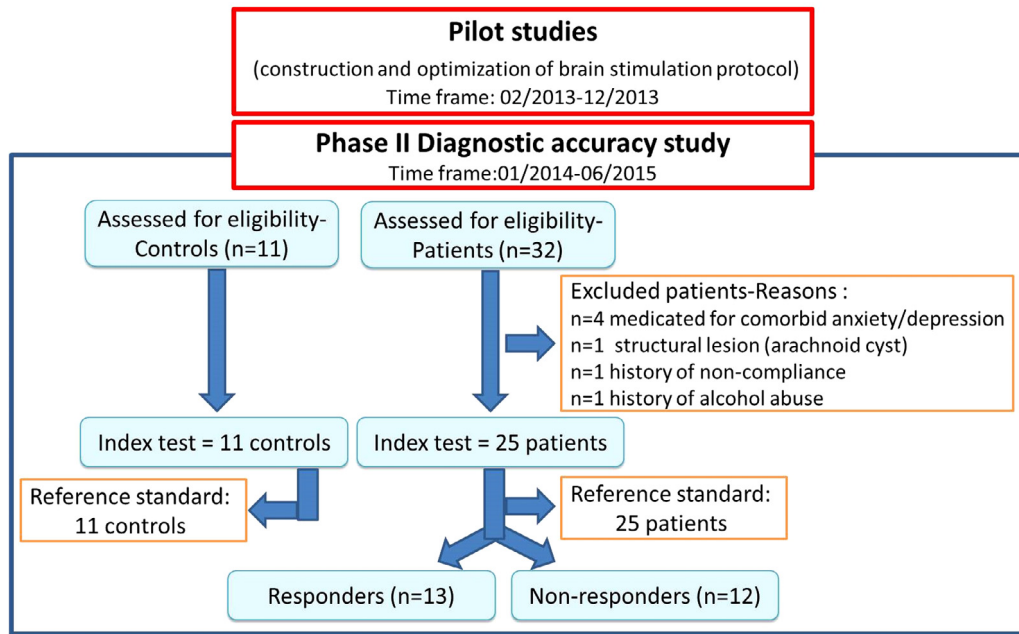


Fig. 1. Flowchart of pilot and diagnostic accuracy studies.

ational drug abuse. Following this procedure, 32 patients were screened and 25 were ultimately included in the study. These patients were diagnosed with “juvenile myoclonic epilepsy” ($n = 17$), “juvenile absence epilepsy” ($n = 6$), “GGE with generalized tonic-clonic seizures alone” ($n = 2$). The epilepsy treatment in 16 of these patients was monotherapy with valproate ($n = 10$, median daily dose = 1000 mg, range 500–1500 mg), levetiracetam ($n = 5$, median daily dose = 2000 mg, range 1000–3000 mg) and lamotrigine ($n = 1$, daily dose = 400 mg). In the remaining patients, a combination of levetiracetam and valproate ($n = 3$, median doses = 3000 mg and 2000 mg, ranges = 1000–3000 mg and 1500–2500 mg, respectively), levetiracetam and lamotrigine ($n = 3$, median doses = 250 mg and 3000 mg, ranges = 100–400 mg and 2000–3000 mg, respectively) and levetiracetam, valproate and lamotrigine ($n = 3$, levetiracetam median dose = 3000 mg and range = 1500–4000 mg, valproate dose 1000 mg, lamotrigine dose 200 mg) were used. Healthy subjects were recruited from hospital personnel and university students provided they passed the TASS questionnaire and gave no history of current or past alcohol or recreational drug abuse.

All participating subjects underwent a baseline TMS–EEG examination and thereafter were prospectively followed at regular 3-month visits for at least 12 months with regard to seizure occurrence, defined as the occurrence of seizures in the absence of any possible provocative factor, i.e. missed AED doses, sleep deprivation, intervening illness etc. On the basis of seizure outcome, patients were dichotomized into a *responder to AEDs subgroup* ($n = 13/25$), that is patients remaining seizure-free for at least 12 months post-examination, and a *non-responder to AEDs subgroup* ($n = 12/25$), that is patients experiencing any kind of non-provoked seizures (absences, myoclonias, generalized tonic-clonic seizures) during the follow-up period. The median AED load (defined as the ratio of the actual prescribed daily dose over the average therapeutic daily dose (http://www.whocc.no/atc_ddd_index/)) was 0.80 in the group of responders and 2.49 in the subgroup of non-responders (Wilcoxon rank sum test for equal medians $p = 0.0004$). Illness duration was also significantly different between these two groups (median = 21 years for non-responders and 7 years for responders, Wilcoxon rank sum test for equal medians $p = 0.020$).

2.3. TMS–EEG setup

Recordings were performed as previously described (Kimiskidis et al., 2015) in an electrically-shielded room according to TMS–EEG methodological guidelines (Ilmoniemi and Kicić, 2010). Briefly, all subjects were examined between 8 and 10 am without having been subjected to sleep deprivation and, in the case of GGE patients, at least 72 h after their last seizure. EEG was recorded with a 60-channel TMS-compatible EEG system (eXimia, Nexstim Ltd) which employs a sample-and-hold circuitry so as to eliminate the TMS-related artifact. EEG signals were acquired with a sampling frequency of 1450 Hz and band-pass filtered between 0.1 and 500 Hz.

2.4. Brain stimulation and EEG processing protocol

The essence of all EEG activating procedures in epilepsy is the induction of epileptiform abnormalities. In line with this principle, we hypothesized that the ability of TMS to induce EDs may be a useful means for maximizing the diagnostic and prognostic value of TMS–EEG in epilepsy. On the basis of this criterion (induction of EDs), we selected the stimulation parameters of our protocol in the context of pilot experiments as detailed below (Steps 1–5). At the same time, however, we anticipated that the multi-level analysis of the TMS–EEG data would allow us to define other surrogate markers of ictogenicity, which might further increase the sensitivity of the index test.

With these considerations in mind, the core steps of the employed brain stimulation protocol, crystallized after extensive pilot experimentations ($n = 11$ healthy controls and 10 patients with GGE), were defined as follows:

Step 1. Coil and stimulus location: Circular coil centered over the vertex

Brain stimulation was performed with a Magstim Rapid2 magnetic stimulator (The Magstim Company Ltd) for steps 1,3 and 4 of the pilot studies and a MagPro X100 stimulator (MagVenture A/S, Farum, Denmark) for steps 2 and 5 of the pilot studies and the phase II study. Pilot experiments indicated that stimulation with

a figure of 8 coil (Magstim type 9925) over the motor hand area with increasing stimulus intensities (20–100% maximum stimulator output (MSO)), produced TMS-evoked potentials (TEPs) that were significantly different in GGE patients vs controls (Supplementary Fig. S1) but failed to induce EDs. In contrast, stimulation with a circular 90-mm circular coil (Magstim type P/N 9784-00) manually centered over the vertex was significantly more effective in inducing EDs (in 19 out of 27 stimuli, i.e. 70%) compared to focal stimulation over pericentral (0%) or frontal (0%) areas for identical stimulus intensity (SI) levels (140% Motor Threshold, assessed with an adaptive threshold technique (Awiszus, 2003), (chi-square test, $p < 0.001$). It should be noted that although a circular coil was deemed appropriate for the investigation of the widely distributed epileptogenic networks of GGE, focal coils, preferably combined with neuronavigation techniques, may be better suited for probing the excitability of the spatially restricted networks associated with focal epilepsy.

Step 2. Pulse shape: Biphasic pulses.

In order to explore the significance of pulse waveform for the induction of EDs, we employed a MagPro X100 stimulator (MagVenture A/S, Farum, Denmark), featuring both monophasic and biphasic pulses, in conjunction with a 114-mm circular coil (MC-125). In pilot experiments, a biphasic waveform was significantly more effective compared to a monophasic, in inducing EDs (in 62% vs 0%, respectively) for identical (140% Motor Threshold) SI levels (Fisher's exact test, $p < 0.001$).

Step 3. Stimulus intensity (SI): Optimal SI

Our protocol was designed as an EEG activating procedure aiming at the induction of EDs. Accordingly, instead of using Motor Threshold for calibrating SI, we tracked the optimal SI for eliciting EDs which was determined using the following bracketing method (Kimiskidis et al., 2013). Single stimuli (three for each stimulation level) are delivered at an initial intensity of 40% MSO and increased at 10% steps until an ED is induced (or a maximum, well-tolerated SI level is reached). This initial search provides a rough estimate of the epileptogenic threshold which is further refined by determining the lower (LEThr) and upper (UETHr) epileptogenic threshold. The LEThr is defined as the maximum SI level at which three consecutive stimuli fail to induce EDs and is determined by reducing SI in 1% steps. The optimal SI, employed in the phase II study, corresponds to the LEThr. The UETHr is defined as the minimum SI level at which all three consecutive stimuli produced an ED and is determined by increasing the intensity in 1% steps from the highest level which so far had not resulted in an ED. The search for UETHr was omitted in cases with spontaneous EDs. Mean epileptogenic threshold is the arithmetic mean of LEThr and UETHr. Thereby, a precise estimate of the threshold for eliciting epileptiform discharges in patients with epilepsy can be derived while minimizing the total number of delivered stimuli (Kimiskidis et al., 2015).

In pilot experiments, we explored the validity of this approach by investigating the probability of inducing EDs as a function of stimulation intensity. EDs started to appear above the lower epileptogenic threshold and thereafter the probability of inducing them and the normalized duration (probability of occurrence multiplied by duration (Bittner et al., 2015)) increased in parallel with further increases in stimulation intensity (chi-square test for independence and linear trend, $p < 0.001$ and Friedman test, $p < 0.001$, respectively) (Fig. 2).

More specifically, in a zone of intensities immediately higher to LEThr, TMS induced EDs alternating with normal appearing TEPs in a purely stochastic manner. At even higher intensities, approaching

the mean epileptogenic threshold, TMS-induced EDs appeared quite regularly in a non-random manner.

It should be noted that although LEThr is subthreshold for eliciting EDs in the single-pulse paradigm, during the subsequent manipulations of step 4 (i.e. paired-pulse stimulation), it may result in the provocation of EDs thereby allowing the fine characterization of the severity of the epileptic disorder. All brain stimulation sessions were performed by an experienced neurophysiologist because TMS-induced EDs appear in a graded fashion (Fig. 2) and therefore it is essential to identify them reliably at the lowest SI so as to avoid overstimulation.

Step 4. Inter-stimulus interval (ISI), inter-trial interval and number of stimuli: Paired-pulse TMS (ISI = 250 ms, inter-trial interval = 15 s, $n = 45$ pairs of stimuli in toto and 15 single stimuli).

Pilot studies indicated that paired-stimuli provide better results compared to single pulses with regard to the induction of EDs. For instance, the normalized duration (probability of occurrence multiplied by duration) of TMS-induced EDs was significantly longer with paired stimuli at 4 Hz compared to single-pulse stimulation (Wilcoxon matched-pairs signed-ranks test, $p = 0.029$). In addition, paired pulse stimulation had an improved tolerability profile in comparison to trains of 5 and 10 stimuli and, therefore, was employed in the stimulation protocol.

The ISI of 250 ms was selected on the basis of pilot studies indicating that it is equally effective with intervals of 100, 500 and 1000 ms with regard to the induction of EDs (dependent variable: normalized duration of EDs, Friedman test, $p > 0.05$). Furthermore, previous TMS-EMG studies suggested that an ISI of 250 ms is related to a prominent phase of cortical facilitation in epileptic patients (Badawy et al., 2014). Therefore, one can envisage that an ISI of 250 ms will probably increase the sensitivity of TMS for disclosing cortical hyperexcitability in epilepsy in simultaneous TMS-EMG & TMS-EEG recordings.

Paired-pulse (PP) sessions were performed at rest, during hyperventilation (HV) and immediately post-HV. The PP stimulation required 15 pairs of stimuli in each condition (at rest, during hyperventilation (HV) and immediately post-HV), i.e. 45 pairs of stimuli in toto. Prior to PP sessions, 15 single stimuli at optimal SI were delivered, so as to allow the correction of PP responses as described by Premoli et al. (2014).

For the determination of motor and epileptogenic threshold the precise number of stimuli varied on an individual basis.

Step 5. Sham stimulation.

During the course of stimulation, care was taken to reduce the click noise associated with magnetic stimuli by acoustic protection (headset of Sham noise generator, MagVenture A/S, Farum, Denmark). This strategy is quite effective with regard to acoustic artifacts, but does not eliminate the somatosensory sensation produced by TMS. In order to exclude that any differences in TMS-evoked cortical potentials (TEPs) detected between healthy subjects and epileptic patients are due to differences in the residual acoustic artifacts or sensory stimulation, sham stimulation was performed with the circular coil centered over Cz, but at a distance of 2 cm from the scalp, with a plastic piece intervening between the head and the coil as previously described by Kähkönen et al. (2001). A pilot study indicated that the EEG responses to sham stimulation did not differ between patients and healthy controls (Wilcoxon test for independent samples, $p > 0.05$) and therefore the acoustic/sensory side-effects of TMS were eliminated as a confounder in the analysis.

With the utilized experimental setup, TMS generated stimulus artifacts on the EEG that were removed prior to any further processing and analysis. The main and ubiquitous TMS artifact con-

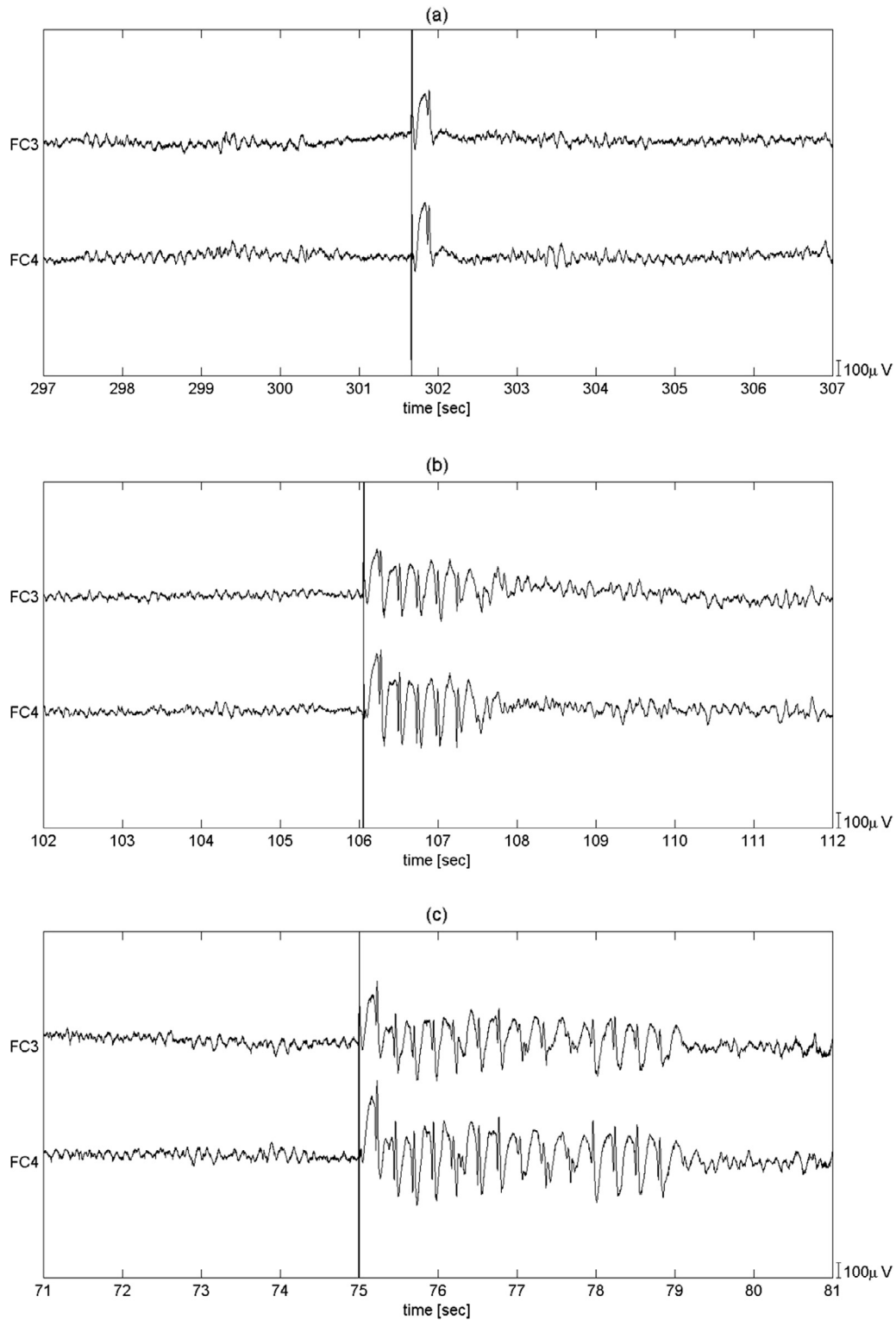


Fig. 2. The graded appearance of TMS-induced EDs with increasing stimulus intensities in a patient with $LE_{Thr} = 79\%$ ($a = 80\%$ MSO demonstrating a spike on the descending slope of N100 wave, $b = 90\%$ MSO, $c = 100\%$ MSO). Raw signals prior to TMS artifact replacement, filtering and re-referencing.

sists of a large amplitude signal deflection starting at the onset of TMS and lasting always less than 20 ms. There is hardly any information in the signal at this interval, and therefore we treat it as a gap and attempt to reconstruct the signal at the gap using a time-dependent weighted average of forward and backward multi-step prediction obtained by a local state space prediction model (for details see Kimiskidis et al. (2013)). TMS at cases caused also base-

line shift and drift, which were sufficiently identified and removed (for details see also Kimiskidis et al. (2013)).

After TMS-induced artifact removal, we apply a bandpass filter (low pass frequency 0.01 Hz and high pass frequency 70 Hz, filter order 60) and downsample to 200 Hz. The EEG data are then re-referenced to infinity (Yao, 2001), a re-referencing scheme that in a recent study (Qin et al., 2010) was found to be more appropriate for

connectivity analysis. Finally, a region of interest (ROI) was defined for further analysis comprising the following channels (F1,FZ,F2,FC3,FC1,FCZ,FC2,FC4,C3,C1, CZ,C2,C4,CP3,CP1,CPZ,CP2,CP4,P1,PZ,P2).

2.5. Data analysis

Data analysis was performed using three hierarchically stratified approaches.

(A) At a descriptive level, raw data were visually assessed for the detection of TMS-induced EDs (defined as interictal and ictal patterns containing generalized, spike and wave complexes with a zero time-lag following magnetic stimulation) or other morphologically abnormal TEPs.

(B) At the Evoked Potential level, data were analyzed in a blinded fashion as to the status of the study subjects using two complementary approaches.

B1. Average response analysis

The measurement of averaged TEPs included the classical TMS-EEG response metrics (latency, amplitude, areas and ratios of the major signal deflections (N30, P60, N100)). In addition, we undertook two further data transformations. First, we normalized TEPs by dividing them by the standard deviation of EEG activity in the 200 ms pre-stimulus baseline and measured the resultant normalized TEPs as above. This transformation was introduced so as to account for inter-individual differences regarding non-brain factors that may affect the amplitude of EEG signals (e.g. differences in skull thickness and anisotropic conductance of skull, dura mater and scalp). Secondly, we subtracted single-pulse TEPs from paired-pulse TEPs (hereafter called *PP correction*), so as to correct the early part of the TEP induced by the second stimulus from the overlapping late part of the first stimulus, as described in the procedure for the quantification of Long-Interval Cortical Inhibition (Premoli et al., 2014).

All measurements were performed using an automated, unsupervised algorithm. This was an important aspect of the study as the full automation of the procedure ensured the reproducibility and objectiveness of all measurements. Briefly, the post-TMS signal was smoothed (zero-phase forward and reverse digital filtering of order 50), so as to eliminate the possibility that fast activities superimposed on the TEP waveform might result in spurious identification of the signal points of interest. Thereafter, the latency of N100 was identified by the global minimum (i.e. extreme negative

value) at the pre-defined time range [85, 200] ms. In the same manner, the latency of P60 was identified by the global maximum in the time range [40, 80] ms and of N30 by the global minimum in [20, 60] ms. The amplitudes of N30, P60, N100 were read from the original (i.e. not smoothed) signal. It should be noted that waveform time ranges have been adapted in the present study to accommodate the enhanced TMS-EEG responses in GGE patients. Similarly, the first negative deflection was termed N30 and probably corresponds to N40 (Bonato et al., 2006) and N45 (Ilmoniemi and Kicić, 2010; Premoli et al., 2014) identified by other authors with different experimental setups.

B2. Single trial analysis

In order to extract additional information from TMS-EEG responses, the instantaneous *Signal Energy profiles* were calculated and further analyzed, using visual data mining techniques. The approach contributed to the understanding of single-trial responses by analyzing the TMS-related temporal patterning in a principled manner. Since a preliminary stage of analysis, based on standard spectral representation, showed that TMS mostly affected the brain activity within the δ -band, we decided to investigate the associated temporal patterning in greater detail. To this end a response profile was constructed for each subject separately with the following steps: (A) Each single-trial segment, from every sensor, was first filtered within [0.5–4] Hz frequency range (by applying a 3rd order Butterworth filter in zero-phase mode). By squaring the obtained signal values, we obtained estimates of instantaneous signal power. (B) The derived time-courses were then averaged across sensors and trials. In this way a single time-dependent energy-related activation was assigned to each subject. (C) The derived δ -band profiles were compared (group-level analysis) across subjects, so as to detect putative activation differences between controls and patients and also between responders and non-responders.

To perform a detailed analysis of TMS-induced energy profiles that extends beyond averaging, we resorted to a well-developed framework for studying and characterizing evoked-response waveforms. This includes a distance preserving technique in conjunction with an intelligible visualization scheme (Laskaris et al., 2003, 2013). In a nutshell, analyzed profiles were first compared in pairwise mode, using the “cosine-distance” (i.e. one minus cosine similarity) as dissimilarity measure. The dissimilarities were tabulated in a $[N \times N]$ distance-matrix D (with N denoting the number of

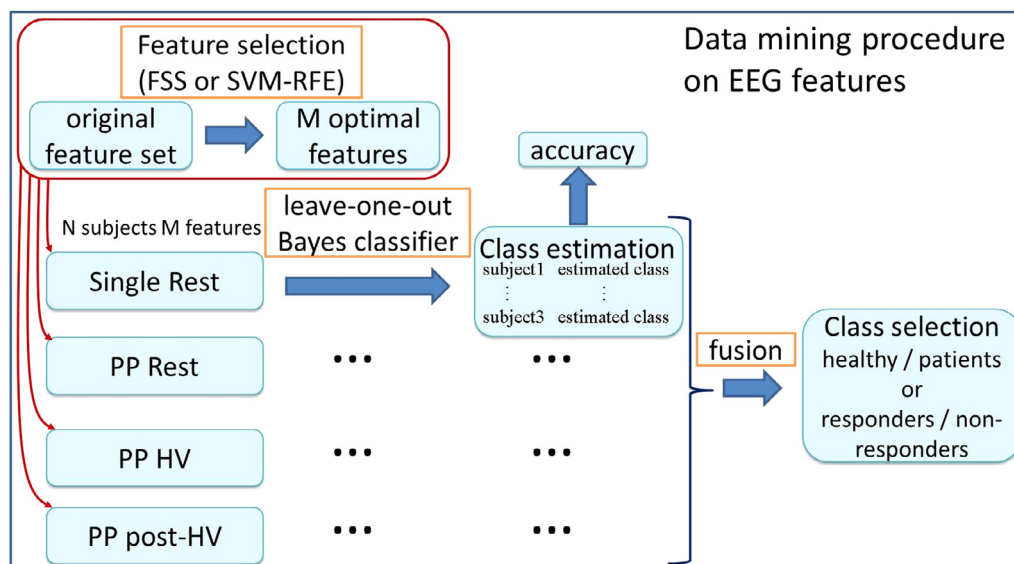


Fig. 3. Flow chart of the data mining procedure applied to the post-TMS EEG features.

profiles and hence the number of subjects) that was then fed to multidimensional scaling (MDS) algorithm. This resulted to a mapping $Y_{[N \times 2]} = \text{MDS}(D)$, in which profiles were represented as points in a 2D coordinated space. The obtained scatter-plot reflected the waveform variability and, by appending the group-labels, was finally turned to a semantic map (Laskaris and Ioannides, 2002). This map can be thought of as the summarizing “spectrum” of waveform variations. It further facilitated the selection of prototypical profiles that portray naturally the main morphological characteristics of each group.

(C) Data mining procedure

Fig. 3 displays the flow chart of the data mining procedure we followed in order to classify patients and healthy subjects as well as responders and non-responders.

The input data to the procedure are the four datasets containing the post-TMS EEG features for all subjects at each of the four conditions, denoted as “Single Rest”, “PPRest”, “PP HV” and “PPpostHV”, respectively. For each dataset two feature selection methods are applied, the FSS wrapper (Tsimpiris and Kugiumtzis, 2012) and the SVM-RFE embedded method (Mundra and Rajapakse, 2010), each finding a small feature subset that improves classification accuracy for each of the two classification tasks (i.e., patients vs healthy subjects and responders vs non-responders). For the classification, the Bayes classifier is used (Duda et al., 2001). The classification accuracy is computed on the basis of leave one out cross validation using the small feature subset provided by

each of the two feature selection algorithms. The above procedure assigns a class label (1: patient, 0: healthy, or 1: responder, 0: non-responder) at each subject and for each of the four datasets. The final class selection at each subject on the basis of all four datasets is done using a fusion process (Ross and Jain, 2003). The fusion assigns the one class (patient or non-responder) if its frequency in the four datasets exceeds a given threshold.

3. Results

The brain stimulation procedure was well tolerated. There were no significant adverse events although paired stimuli at high SIs were occasionally described as uncomfortable, by patients and healthy subjects alike. In general, TMS-evoked EDs (vide infra) went unnoticed by patients, although at times they were described as resembling their habitual absences. No convulsive seizures occurred during any TMS sessions.

In a routine 5-min EEG preceding the TMS session (performed in order to guide the epileptogenic threshold hunting procedure), 2 patients (1 of them non-responder) had spontaneous EDs and another 4 (three of them non-responders) demonstrated non-specific abnormalities. The rest of the patient group and all control subjects had normal findings. The above findings correspond to a sensitivity of 0.24 and specificity of 1.0 for differentiating epileptic patients from healthy controls, and sensitivity of 0.33 and specificity of 0.84 for differentiating responders from non-responders.

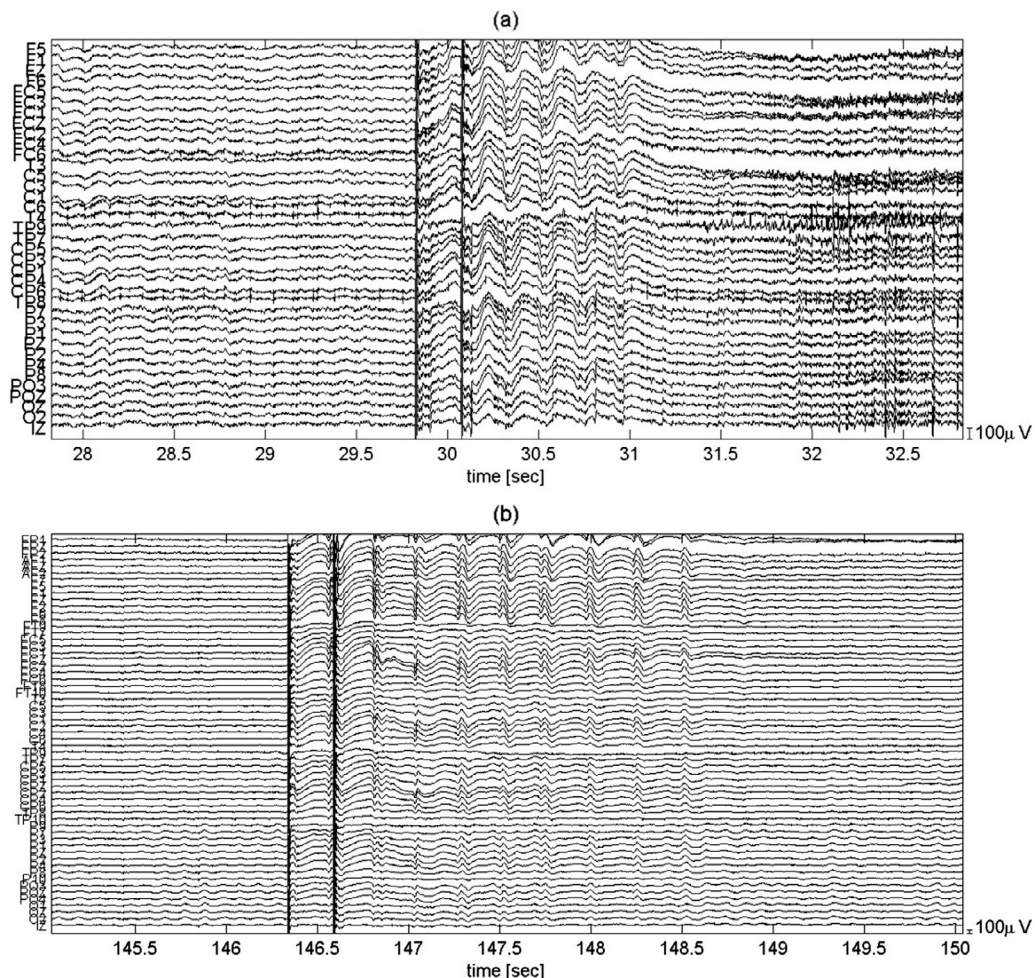


Fig. 4. Abnormal TMS-EEG findings in patients with GGE. (a) A burst of repetitive slow waves. (b) A generalized, bisynchronous 3 Hz SW discharge. Unfiltered signal with negativity plotted as an upward deflection. This polarity convention is followed in all subsequent figures.

3.1. Descriptive level

At the descriptive level, we observed that in healthy participants, in all responders to AEDs (13/25) and in six out of 12 non-responders, TMS failed to induce EDs.

In four non-responders, TMS evoked morphologically abnormal responses (i.e. repetitive slow waves or complexes with spiky components) (Fig. 4a) that did not satisfy the defining criteria of EDs. In the remaining two patients, TMS produced generalized, bilaterally synchronous, symmetrical and regular 3-Hz spike-and-wave discharges (Fig. 4b).

As previously mentioned with regard to single stimuli (Step 3 of Brain Stimulation Protocol), at a given stimulus intensity (i.e. LEThr) TMS-induced EDs appeared in a stochastic manner alternating randomly with normal appearing TEPs. The probability of inducing them was not significantly different among different states (i.e. resting state (53%) vs HV (77%) vs post-HV (67%), chi-square test, $p > 0.05$).

The morphology of TMS-induced EDs varied substantially even when all stimulation parameters remained constant. On certain occasions, a low amplitude spike, representing the minimal degree of ictogenicity, appeared on the descending slope of the N100 wave and was time-locked to the initial magnetic stimulus with a mean latency of 239.2 ms (range 237.0–243.8 ms). This spike was localized in frontal and central areas and was clearly identified as a high-frequency blob in the ERSF occurring approximately 240 ms post-TMS (Fig. 5). Occasionally, a second spike, time-locked to the first one, appeared in the descending slope of the second N100 wave.

In other cases, TMS induced generalized spike and wave discharges (Fig. 6). As previously mentioned, the total duration of these phenomena is dependent on the stimulation intensity. It should be emphasized, however, that even within a particular

stimulus intensity level (i.e. LEThr) substantial variation of ED duration was evident. For instance, at 100% MSO the median duration of EDs ($n = 42$) was 4275 ms with an interquartile range of 2389 ms.

The dominant frequency of EDs was 3–4 Hz, as was the case for spontaneous EDs. From a topographical point of view, EDs appeared initially in frontal areas and, with increasing stimulation intensities, gradually spread over central and parietal areas retaining frontal maxima. The topographical features of TMS-induced EDs resembled the topography of spontaneous ones (Fig. 5 and 6 and Supplementary Figs. S2 and S3). The similar (yet not identical) frequency spectra and topographical distribution of TMS-induced and spontaneous EDs indicates that TMS activated the endogenous epileptogenic networks of these patients.

3.2. Evoked potential level

At the Evoked Potential level, data were analyzed both as average response as well as single trials.

3.2.1. Average response analysis

Average and single trial responses of adequate quality for further analysis were recorded in all healthy subjects and in 91 of 100 recordings of the 25 epilepsy patients in the four conditions (the inadequate quality recordings were one for PP rest, 3 for PP HV and 5 for PP postHV).

In the resting state, the grand-average TMS-evoked response in patients and controls consisted of three major deflections following the first TMS stimulus (N30a, P60a, N100a) with peak latencies of approximately 35 ms, 56 ms and 149 ms in the control group and 30 ms, 57 ms and 137 ms in the patient group, respectively (Fig. 7).

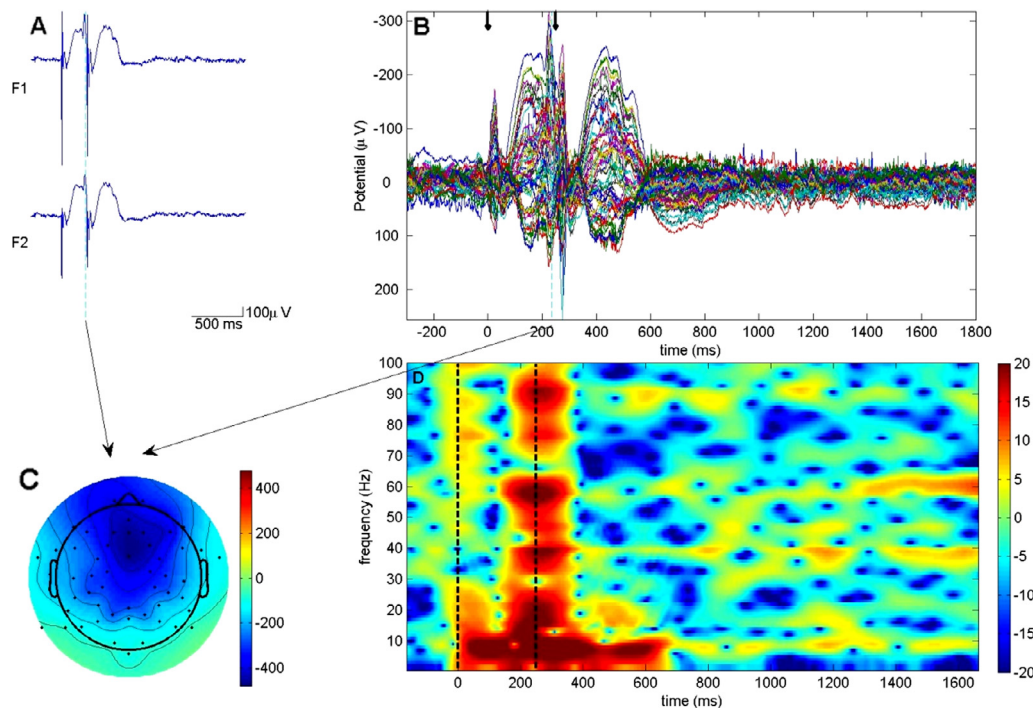


Fig. 5. (A) TMS induces a spike on the descending slope of N100 at a latency of 235 ms post-stimulus. (B and C) Display the corresponding topoplotted and 55-channel butterfly plot (five channels with artifacts omitted). (D) ERSF image for channel F1. Red and blue colors denote increased and decreased power, respectively, in comparison with pre-TMS baseline on a power scale of -20 to $+20$ dB. The x-axis indicates time with TMS stimulation at $t = 0$ ms and the y-axis represents the frequency spectrum (0–100 Hz). The topoplotted in C is at the time of the spike at 235 ms post-stimulus and it is associated with a significant power increase in high frequencies in D. In B–D, the data are first referenced to infinity. Downward arrows in B and dotted lines in D indicate replaced TMS stimuli. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

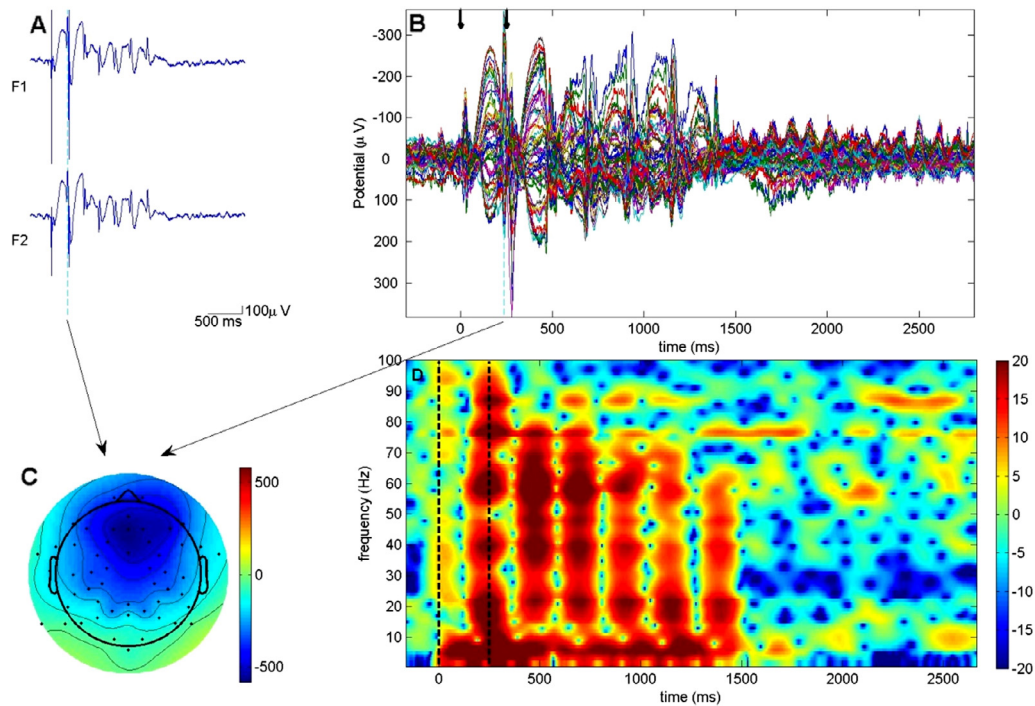


Fig. 6. (A) A TMS-induced ED. (B–D) Display the corresponding 55-channel butterfly plot, ERSP and topoplots as in Fig. 5. The ERSP displays oscillatory power increases and decreases of higher and lower frequencies corresponding to successive spike and wave discharges.

Following the second stimulus, two major deflections were consistently identified (P60b, N100b), with latencies of 57 ms and 164 ms in the control group and 58 ms and 152 ms in the patient group, respectively (Fig. 7). Although N30b was occasionally evident, in many cases it was obscured by the trailing slope of N100a. Some representative features (latencies, amplitudes and areas) of these components are summarized in Table 1.

During HV, the amplitude of all components increased in the group as a whole (i.e. control subjects and patients combined). This effect marginally persisted after normalization of the data (i.e. taking into account the amplitude increase in baseline EEG activity typically associated with HV) and returned to resting values during the post-HV period. In particular, the N100a and N100b amplitude increased from rest to HV (also after PP correction) with higher

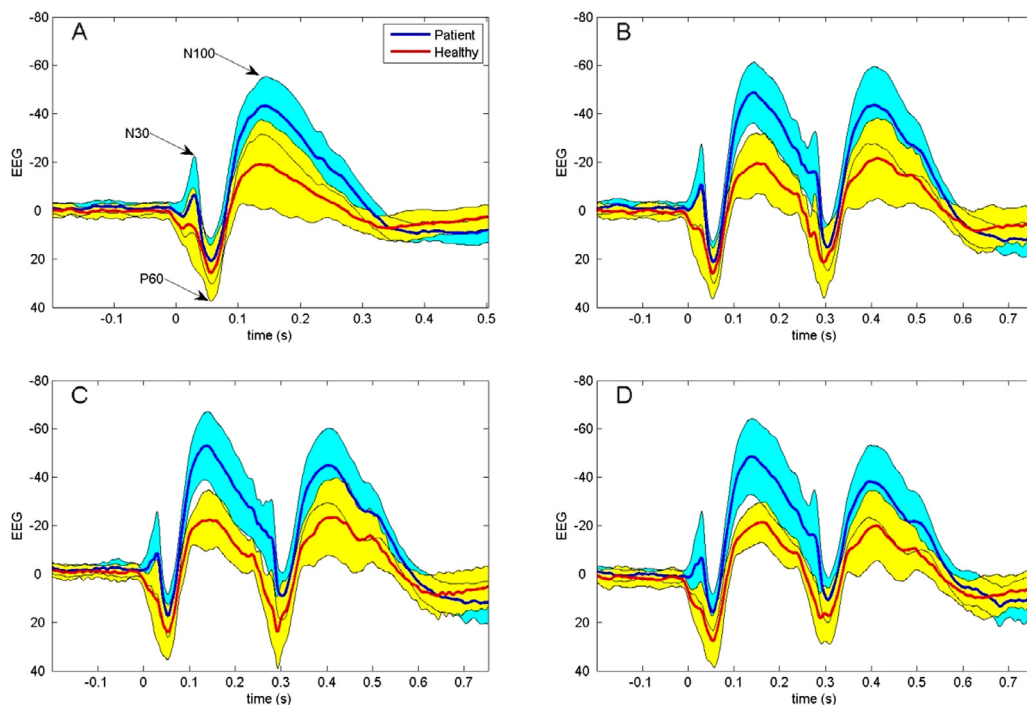


Fig. 7. Grand-average waveforms of TEPs in patients (cyan band indicating SEM) and controls (yellow band, SEM) in response to single stimuli (A) and paired stimuli at rest (B), during HV (C) and post-HV (D). Stimulus artifacts at 0 and 250 ms were removed and replaced by a gap-filling algorithm as described in the text. N30, P60, N100 are evident in both TEPs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Descriptive statistics (median and interquartile range in parentheses) of some representative TMS–EEG features for the four conditions, and for each of the classes of healthy (first row), responders (second row) and non-responders (third row) within each condition. In the acronyms of the features, ‘a’ and ‘b’ stand for the response to the first and second TMS, respectively, ‘Lat’ stands for latency, ‘Amp.Dif’ for difference in amplitude of a given peak from the following peak, and ‘area’ for the area under the curve from the first to the second given point. Asterisks (*) denote statistically significant differences between responders and non-responders.

	N30a Lat (ms)	N30a Amp.Dif (μV)	N100a Lat (ms)	N100a Amp (μV)	P60a-N100a Area (μV xms)	N100b Lat (ms)	N100b Amp.Cor (μV)	P60b-N100b Area.Cor (μV xms)
Single	35.17(16.55) 31.72(6.90) 30.00(7.59)	7.32(40.74) 17.24(14.78)* 46.58(45.87)	137.93(41.03) 129.66(24.66) 132.76(46.90)	15.05(20.70) 34.71(37.05) 60.37(48.31)	1.92(1.93) 2.09(2.62) 5.05(3.98)			
PP resting	34.83(13.79) 30.34(1.72) 29.66(6.90)	15.07(30.79) 19.57(13.85) 44.82(56.52)	148.97(33.62) 135.52(21.38) 145.17(38.62)	19.87(13.70) 32.77(55.03)* 63.51(48.92)	2.10(2.52) 2.58(2.49) 4.81(4.47)	164.14(39.48) 142.41(51.38) 152.41(24.83)	24.62(22.52) 27.59(52.60) 57.11(85.74)	2.56(3.16) 2.13(3.69) 7.14(6.85)
PP HV	30.34(11.21) 30.00(2.76) 30.34(6.55)	12.14(27.87) 21.89(19.65) 36.81(66.04)	135.17(35.00) 127.59(22.76) 119.31(41.38)	20.10(14.51) 45.41(51.69) 53.02(63.05)	2.75(2.26) 2.07(3.47) 3.28(5.42)	171.03(39.66) 132.41(63.45) 144.48(35.86)	27.29(28.73) 46.16(52.59) 48.09(70.32)	1.53(3.49) 1.93(2.21) 4.97(5.98)
PP post-HV	29.66(12.07) 31.03(5.34) 30.00(5.86)	32.86(38.47) 17.36(18.34) 37.37(59.00)	155.86(25.86) 131.38(22.07) 141.72(44.83)	27.28(18.00) 24.81(29.94)* 54.36(53.84)	3.58(2.85) 1.83(1.95)* 4.11(4.50)	151.03(31.90) 143.79(37.93) 150.34(22.76)	26.64(31.42) 30.52(45.80) 43.74(39.61)	2.32(2.91) 2.05(0.94) 4.20(5.69)

statistical significance after the second stimulus (Friedman test adjusting for subjects, $p = 0.016$ for first stimulus, $p = 0.002$ for second stimulus without PP correction and $p = 0.001$ with PP correction; post hoc Bonferroni corrected comparisons using Wilcoxon signed rank test for zero median gave statistical significance only for rest – HV at first stimulus and for both rest – HV and HV – postHV at the second stimulus, with higher statistical significance with PP correction).

In the patient group (and the subgroup of non-responders in particular), the N30a, N100a and N100b were higher, compared to controls, during the resting state, HV and post-HV (Table 1). However, these differences did not reach statistical significance (for the particular features presented in Table 1) due to large inter-subject variability and small sample sizes.

In order to assess the effect of clinical factors, such as AED load and illness duration, on TMS–EEG responses, we repeated the statistical comparison for each TMS–EEG feature of responders vs non-responders with analysis of covariance (ANCOVA). It should

be noted that both AED load and illness duration differed significantly between the two groups (see Section 2.2). The results of the analysis showed that, when AED load emerged as a statistically significant covariate, the differences in the corresponding feature values between the two groups tended to accentuate. For instance, in the PP rest condition, accounting for the effect of AED load, statistically significant differences were established for N30aAmpDif, P60a-N100aArea and N100bAmpCor and further accentuated for N100aAmp. In contrast, disease duration did not exert a significant effect as a covariate. Similar results were obtained in the other three conditions.

3.2.2. Single trial analysis

Regarding the time-varying Signal Energy within δ -band, the three group-averaged profiles are shown in the top-panel of Fig. 8. Their over-plotting is indicative of differences within the first 600 ms after the TMS-pulse. The stimulation results in increased activation in patients, and in particular the non-

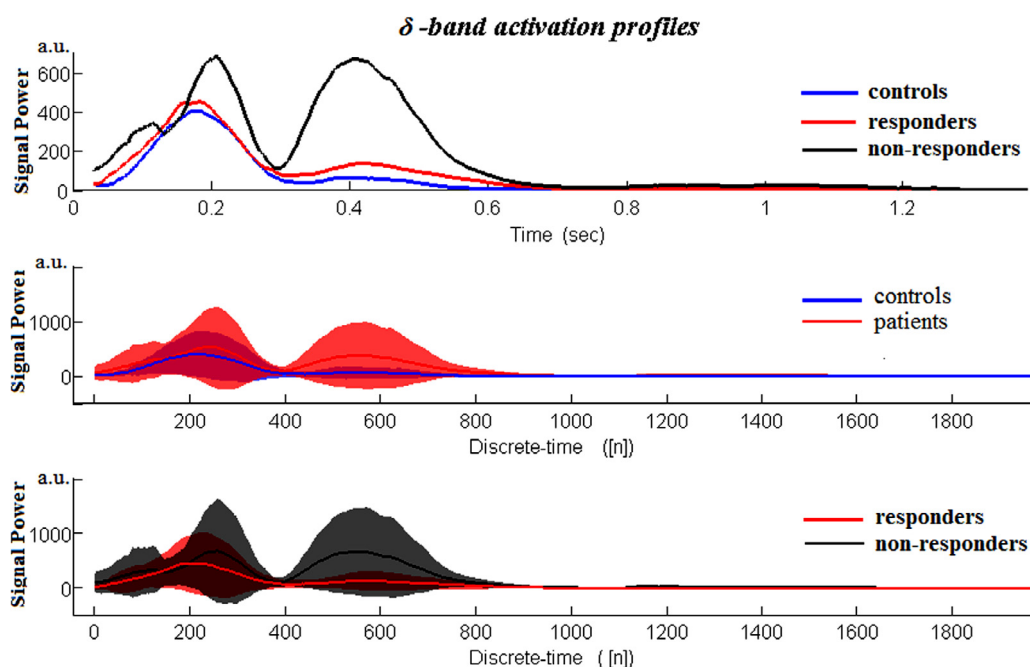


Fig. 8. Top: Group-averaged profiles of time-dependent signal power in δ -band. Middle: Latency-dependent range of values, estimated as mean \pm SD, for patients (with responders and non-responders taken together). Bottom: Latency-dependent range of values for the two subgroups of patients.

responders. To avoid any misinterpretation (that may be caused via looking at the averaged data without considering the inter-subject variations), we have also included graphics reflecting the range of values at each time point.

The overlap between healthy controls and patients (both subgroups taken together) is provided in the middle panel, while the overlap between responders and non-responder is given at the bottom. In view of all the graphs together, Fig. 8 provides some evidence about stronger response in non-responders. This difference is present around 200 ms and enhanced around 400 ms. It may be interpreted as reflecting a higher excitable medium in the case of non-responders. However, a more elaborate study of these profiles was considered necessary, since high between-group overlaps were evident. For this reason, the semantic map (Fig. 9) was constructed, so as to unfold the variations in the TMS-induced activation profiles.

By traversing the semantic map from the right most end (where profiles of the non-responders are mostly gathered) to the left, we selected several profiles from each group. These profiles (after being normalized independently to unity norm) have been displayed in Fig. 9b. The visual contrast between the selected profiles (mainly between controls and non-responders) clearly indicates that a prolonged or delayed activation (around 400 ms) is the main distinctive characteristic of the non-responders. Apart from this observation, the overall layout of the semantic map in Fig. 9a is suggestive of a sufficient separability among groups, that is occasionally disrupted by the profiles of isolated subjects (like control subject 11).

3.3. Feature selection

Many of the 57 features considered for feature selection have overlapping and complementary information. Thus, the features

in the optimal feature subset providing maximal accuracy values varied among different conditions, indicating that the employed multi-level analysis is a relevant approach.

On the basis of the optimal feature subset, the cross-validated accuracy of paired pulse TMS–EEG for differentiating GGE patients from controls was 0.86 at rest with the subset containing only the amplitude of N100a (N100aAmp) and the P60a amplitude normalized by the baseline (P60aAmpBS), 0.81 during HV using the same features, and 0.92 post-HV using P60aAmpBS and the amplitude difference N30a–P60a normalized by the baseline (N30aAmpDifBS) (Table 2). The cross-validated accuracy for differentiating responders from non-responders and controls was 0.80 at rest with the subset containing only the two coordinates of the 2D semantic map of energy profiles, 0.78 during HV using only the P60b–N100b area (P60bN100bArea), and 0.65 post-HV using a larger subset of N30aAmpDif, P60aN100aArea, P60bAmpBS and N100bLatCor. All accuracy levels were statistically significant. The highest accuracy for the patient – healthy classification was achieved with post-HV and for the non-responders – responders with PP rest. Single pulse TMS–EEG was associated with lower accuracy values compared to PP in both classification tasks.

The fusion of the classified cases at each condition provided the overall accuracy of the data mining procedure based on the post TMS–EEG features. The rule for fusion was defined according to the purported use of the classification results, and we considered three scenarios: (a) balanced performance in sensitivity and specificity, (b) highest sensitivity, and (c) highest specificity. With an appropriate percentage threshold and a corresponding decision rule we could always detect correctly a patient as such (sensitivity 1.00), a control as such (specificity 1.00), or achieve a balanced percentage of correctly identified patients and controls (Table 3). In all these three scenarios an overall accuracy at the level of 0.85 was attained. Searching for appropriate thresholds for the

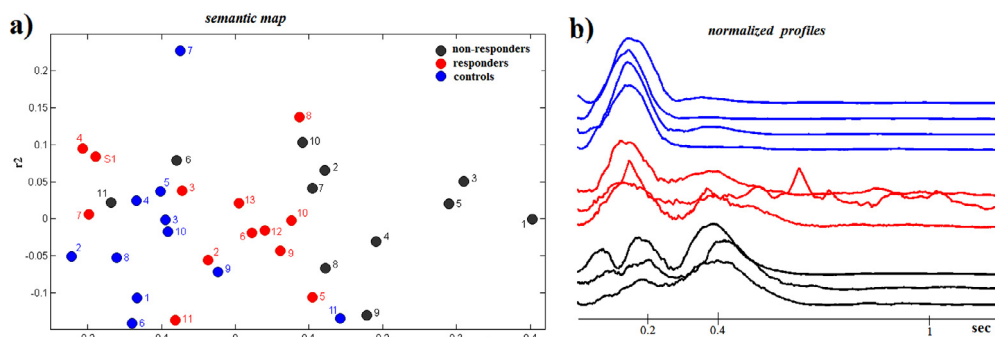








Fig. 9. (a) Low dimensional embedding of the time-dependent signal-power profiles. (b) Selected profiles from each of the three groups (colors indicate the groups and labels the identity of subjects). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2

The performance indices of accuracy (Acc), sensitivity (Sens), specificity (Spec), negative predictive value (NPV) and positive predictive value (PPV), obtained from leave-one-out cross-validated classification on the basis of the selected optimal subset of post-TMS–EEG features. The results are for two classification tasks, patients vs healthy (P–H), and non-responders vs responders (nR–R), and the four conditions.

Classes	Condition	Acc	Sens	Spec	NPV	PPV
P–H	Single rest	0.67	0.60	0.82	0.47	0.88
	PP rest	0.86	0.85	0.89	0.73	0.94
	PP HV	0.81	0.78	0.89	0.67	0.93
	PP postHV	0.92	1.00	0.71	1.00	0.90
R–nR	Single rest	0.68	0.58	0.77	0.67	0.70
	PP rest	0.80	0.78	0.82	0.82	0.78
	PP HV	0.78	0.63	0.90	0.75	0.83
	PP postHV	0.65	0.63	0.67	0.67	0.63

Table 3
The performance indices of accuracy (Acc), sensitivity (Sens) and specificity (Spec) obtained from fusion of the estimated classes, patients vs healthy (P–H) or non-responders vs responders (nR–R), according to the percentage threshold and decision rule on the four conditions for assigning the “positive class” (patient or non-responder).

		Thres	Decision rule	Acc	Sens	Spec
P–H	Balanced	50%		0.84	0.86	0.82
	Highest sens	25%		0.88	1.00	0.64
	Highest spec	75%		0.84	0.76	1.00
nR–R	Balanced	50%		0.76	0.80	0.73
	Highest sens	50%		0.76	0.80	0.73
	Highest spec	75%		0.81	0.70	0.91

differentiation of non-responders and responders, we could not obtain a sensitivity or specificity of 1.00, and the correct detection of non-responders (sensitivity) could not improve over 0.8 even after allowing for the “positive class” (patient or non-responder) to be detected in only one of the four conditions. The overall accuracy in all three scenarios was at the level of 0.8. We report both the decision rule and percentage threshold because for epilepsy patients 9% of recordings were not evaluable.

4. Discussion

The present exploratory TMS–EEG study was designed with a dual objective: first, to develop, test and validate a brain stimulation and data analysis protocol suitable for investigating cortical excitability in patients with GGE; second, to explore the diagnostic potential of this protocol in a cohort of GGE patients. Our preliminary data indicate that the presented TMS–EEG protocol differentiates with high accuracy epileptic patients from healthy controls and may also be of use in assessing the response to antiepileptic drugs.

The overarching criterion in the construction of our stimulation protocol was the comparative ability of various TMS paradigms to induce EDs. Accordingly, before embarking on the discussion of the results of the phase II study, a brief comment on this important aspect of our protocol is warranted.

4.1. TMS-induced EDs

The brain stimulation protocol that was ultimately employed in the present study evoked EDs in two out of 25 epileptic subjects (8%) and produced morphologically abnormal TEPs in additional four patients. Importantly, the topographical and spectral features of TMS-induced EDs were similar (but not identical) to spontaneous IEDs. Although these observations are based on a small number of subjects and require verification in larger cohorts, it appears that TMS-induced EDs are subject-specific and reflect the activation of the endogenous epileptogenic networks of GGE patients. The incidence of TMS-induced EDs in our cohort was lower than previously reported in focal epilepsy (Valentin et al., 2008). This finding may relate to different epilepsy type (focal vs generalized), but also differences in disease severity between the two cohorts. In the present study, 13 out of 25 patients were seizure-free under antiepileptic medication, whereas in the study by Valentin et al. all patients suffered from pharmacoresistant epilepsy. It should be also noted that we employed a strict definition of TMS-induced EDs (i.e. generalized spike and wave complexes with a *zero time-lag* following magnetic stimulation) so as to avoid the caveat of characterizing spontaneous IEDs occurring in the early post-stimulation period as TMS-induced phenomena.

On the other hand, previous single and paired-pulse TMS–EMG and TMS–EEG studies in patients with GGE did not report the induction of ictal or interictal phenomena. There are various explanations for this discrepancy. First, it should be emphasized that the vast majority of TMS-induced EDs (particularly those of short duration) were unnoticed by the patients and did not result in overt behavioral manifestations. Accordingly, they would remain unnoticed in the context of TMS–EMG studies in GGE patients. In addition, and even more importantly, TMS-induced EDs occurred exclusively when the particular stimulating protocol was employed (i.e. employing a circular coil at specific intensities (LEThr or higher) in a paired pulse paradigm). Other stimulating protocols (using single stimuli with a figure of 8 coil at lower intensities, for instance 120% MT) that are routinely used in relevant TMS studies were ineffective in that respect. Therefore, the reason that TMS-induced EDs were observed in our cohort is apparently related to the specific TMS paradigm that was employed.

One can only speculate about the mechanism whereby TMS at high stimulation intensities results in the generation of generalized spike-and-wave (SW) discharges. Experimental studies in a thalamic slice preparation (Blumenfeld and McCormick, 2000) demonstrated that stimulation with single electrical pulses resulted in spontaneous oscillations at 8–10 Hz, resembling normal sleep spindles. In contrast, high frequency stimulations in the “corticothalamic” pathway provoked intense burst firing at 3–4 Hz in the entire network, reminiscent of typical spike-wave seizures. It is tempting to speculate that magnetic stimuli at high intensities, which increase the firing of pyramidal neurons (as evidenced by the increase in the number and amplitude of I- and D-waves (di Lazzaro et al., 1998)), may induce a similar effect. Clearly, the issue of how TMS induces a transition from normal rhythms to SW discharges needs to be further addressed in appropriate experimental studies.

4.2. Phase II study

The principal finding of the phase II study is that TMS–EEG responses in patients with epilepsy, particularly in the subgroup of non-responders to antiepileptic drugs, are characterized by increased amplitudes and enhanced signal energy, compared to healthy controls. It could be argued that these results were confounded by the use of AEDs. All patients were chronically medicated with diverse antiepileptic drug regimens at study enrollment and these centrally acting drugs exert significant effects on various TMS measures of cortical excitability (Ziemann et al., 2015). This possibility, however, is unlikely for a number of reasons. First, an analysis of covariance did not identify AEDs as a factor implicated in the differentiation of responders from non-responders. To the contrary, the inclusion of AED loads as a covariate of the TMS–EEG features increases the discrimination of responders and non-responders for certain TMS–EEG features. Sec-

ond, on the basis of the existing TMS–EMG literature (Ziemann et al., 2015), one would expect AEDs to “normalize” the TMS–EEG signatures of hyperexcitability associated with epilepsy. Accordingly, if AEDs were the principal factor for the differentiation of these two classes, non-responders (who had significantly higher AED loads) would be expected to lie closer to control subjects compared to responders, which is clearly not the case. Illness duration was found not to be a significant covariate of TMS–EEG features and therefore has no significant effect for the discrimination of responders and non-responders.

From a *pathophysiological point of view*, our findings indicate that in GGE patients the cortical and subcortical networks mediating TMS–EEG responses may be viewed as a “hyper-excitable medium”. TMS–EEG responses in patients with epilepsy, particularly in the subgroup of non-responders to antiepileptic drugs, were characterized by increased amplitudes and enhanced signal energy, compared to healthy controls. Similar findings were previously reported in GGE by del Felice et al. (2011) who observed an increased amplitude of late TMS–EEG responses in patients with JME following sleep deprivation. The mechanisms underlying the augmented responses to TMS in patients with GGE are unknown. According to current thinking, N100, the most prominent and reproducible TEP potential, reflects the activity of GABA-B receptors (Premoli et al., 2014), whereas earlier TEP components are probably associated with short-latency EPSPs and GABA-A receptor IPSPs (Rogasch and Fitzgerald, 2013). In trying to relate our findings to previous TMS studies in the field of epilepsy, it is worth noting that N100 is the substrate of cortical silent period (SP), an inhibitory phenomenon of the motor system also mediated by GABA-B receptors (Farzan et al., 2013). A number of TMS–EMG studies concluded that SP duration is significantly increased in patients with GGE (Cincotta et al., 2015), a finding that is certainly in line with the increased amplitude of N100 observed in the present study.

The physiological implications of the enhanced N100 waveforms in patients with GGE are not clear. Bender et al. (2005) viewed N100 as “an *in vivo* model to assess thalamo-cortical inhibitory processes in human subjects”. These authors suggested that N100 may be interpreted as a “wave” response to an externally generated “spike” essentially preventing the TMS-induced synchronized neuronal discharge from generalization by recruitment of intra-cortical and thalamo-cortical inhibitory mechanisms. It might be speculated that a similar mechanism may be operant in patients with GGE and underlie the enhanced TMS–EEG responses in these subjects.

Safety issues are clearly of paramount importance in order for TMS–EEG to enter clinical practice as a diagnostic and prognostic tool. In the present study, no convulsions occurred as a result of brain stimulation and other reported adverse effects were of minor importance. This favorable profile should be ascribed to two methodological factors. First, the employment of a bracketing technique for the definition of the epileptogenic threshold which limited the total number of delivered stimuli thereby avoiding unnecessary over-stimulation. The second, and even more important, factor was the real-time registration of EEG responses to brain stimulation which enables the on-line modification of stimulation parameters or even the termination of a session, thereby greatly improving the safety of TMS applications in epilepsy. On the other hand, it is clear that the preliminary assessment of the safety aspects of this method needs to be validated in larger patient cohorts with varying degrees of disease severity.

What are the *clinical implications* of our findings? In the everyday management of patients with epilepsy, EEGs are routinely performed in order to assess response to antiepileptic drug treatment. This widely adopted practice, however, is based on insecure evidence. Some studies, particularly in patients with GGE, suggest

that the continuing presence of interictal epileptiform discharges (IEDs) in the resting EEG is associated with the occurrence of clinical seizures and may prove useful, in certain cases, in assessing therapeutic response (Miller and Blume, 1993). However, other retrospective and prospective studies refute this view and conclude that the presence of IEDs in the follow-up EEGs of patients with epilepsy is not correlated with seizure frequency (Steinhoff et al., 2013; Selvitelli et al., 2010). It is clear that the important issue of a biomarker predicting response to antiepileptic drug treatment in clinical practice remains currently unresolved.

Our data suggest that the combination of EEG with TMS may serve as a diagnostic and possibly prognostic biomarker in epilepsy. The cross-validated diagnostic accuracy of TMS–EEG in the present study was high reaching values of 0.92 for differentiating GGE patients from controls and 0.80 for differentiating responders from non-responders to antiepileptic drugs. Overall, these values indicate excellent and moderate diagnostic performance, respectively. It is important to note that features providing maximal accuracy values varied among different conditions (rest, HV and post-HV), and were derived from both average and single trial analysis. This observation proves that a multi-level analysis improves diagnostic accuracy and therefore is a clinically relevant approach. Moreover, we found that combining the classification of patients and controls, as well as non-responders and responders, at the four conditions (single pulse at rest and pair pulse at rest, HV and post-HV) we could derive fusion rules that allow for optimizing the rate of true positives (patient or non-responders) or negatives (healthy or responders).

A direct comparison between TMS–EEG and the conventional EEG performance and reading was clearly not an objective of the current study. However, it could be argued that the objective, operator-independent analysis of TMS–EEG data is an important advantage compared to the subjective interpretation that remains the cornerstone of EEG reading in clinical practice. It is well known that the reported diagnostic and prognostic value of EEG in epilepsy varies widely among different studies. One reason for this discrepancy relates to the different threshold for declaring abnormalities during EEG readings (Gilbert et al., 2003). In a recent meta-analysis of EEG test performance, differences of readers' thresholds for classifying an EEG as positive accounted for 37% of the variance in EEG diagnostic accuracy and this variation influenced the ability of the EEG to predict seizure risk on an individual basis. It is clear that the emergence of an EEG tool that might increase the diagnostic and prognostic power of EEG, for instance by the objective, operator-independent analysis of EEG data, might be clinically useful. Our data raise the possibility that TMS–EEG may prove to be a useful technique in that respect.

The present study suffers from certain *limitations*. First of all, it should be stressed that as a result of the current study design (i.e. the fact that study participants were already under treatment prior to study enrollment), it cannot be concluded that TMS–EEG can predict antiepileptic drug efficacy. The single TMS–EEG recording of the presented protocol may be regarded as a print-out of cortical excitability comprising two distinct components—essentially a “trait” and a “state” component of a patient's epilepsy. The former reflects the ictality (ictal strength) of the epileptic disorder while the latter includes the counterbalancing effect of AEDs as well as other exogenous and endogenous factors (hormonal influences, sleep deprivation, etc.). It is clear that further studies are warranted to dissect the TMS–EEG print-out of GGE patients into its constituent components by investigating drug-naïve patients, on one hand, and performing proper pharmacological TMS–EEG studies, on the other. Secondly, TMS induced EDs only in a minority of patients; therefore, it is essential to optimize stimulation paradigms so as to increase the sensitivity of the technique. Thirdly, although convulsions did not occur in our study, this possibility

cannot be totally ruled out. In order to address this issue, one can envisage the development of algorithms allowing the detection of seizures, and the delivery of TMS stimuli in a closed-loop mode so as to abort electrographic and/or clinical events (Kugiumtzis and Kimiskidis, 2015). Alternatively, one might explore the diagnostic accuracy of TMS–EEG using a similar stimulation and data analysis protocol, but employing intensities that are subthreshold for eliciting EDs. Finally, it should be noted that the present study employed a large diameter circular coil which resulted in bi-hemispheric stimulation and ultimately succeeded in inducing EDs. At the same time, however, this approach, is associated with disadvantages. First, it results inadvertently in the stimulation of motor cortex bilaterally, thereby producing upper limb contractions which limit the tolerability of the method. Second, the employed fixed-diameter coil does not allow the delivery of spatially-tailored individualized E-fields and therefore the minimal necessary extent of the stimulation field in order to induce EDs cannot be precisely delineated. Emerging but yet to be developed technologies may allow the adjustment of the spatial extent of the induced E-field to achieve diagnostically relevant results while avoiding excessive cortical stimulation. This technological advance is expected to improve significantly the tolerability of the method.

What are the *future perspectives*? First of all, the present results should be externally validated and extended to other patient cohorts (e.g. patients with a suspected first epileptic seizure or seizure-free patients prior to AED withdrawal). Another important issue is the design and execution of repeatability studies (Lioumis et al., 2009), so as to define precisely the measurement error of TMS–EEG features in patients with epilepsy, which is a prerequisite for the application of the method at an individualized level. These exploratory studies, if positive, may ultimately lead to a multi-center phase III study so as to crystallize the diagnostic and prognostic potential of TMS–EEG in epilepsy.

In conclusion, the results of the present exploratory phase II study indicate that TMS–EEG can be used for diagnostic purposes as well as to stratify the severity of GGE. On the basis of these data, it is concluded that further testing of this promising technique is warranted.

Conflict of interest statement

The authors report no conflicts of interest relevant to the content of the present article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.clinph.2016.11.013>.

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