

**Transcranial magnetic stimulation of the precuneus enhances memory  
and neural activity in prodromal Alzheimer's disease**

Giacomo Koch, MD<sup>1,2,\*</sup>, Sonia Bonni, PhD<sup>1</sup>, Maria Concetta Pellicciari, PhD<sup>1</sup>, Elias P. Casula, PhD<sup>1</sup>, Matteo Mancini, MSc<sup>3</sup>, Romina Esposito, MSc<sup>1</sup>, Viviana Ponzo, BSc<sup>1</sup>, Silvia Picazio, PhD<sup>1</sup>, Francesco Di Lorenzo, MD<sup>1,4</sup>, Laura Serra, PhD<sup>3</sup>, Caterina Motta, MD<sup>1</sup>, Michele Maiella, MSc<sup>1</sup>, Camillo Marra, MD<sup>5</sup>, Mara Cercignani, PhD<sup>6</sup>, Alessandro Martorana, MD<sup>1,4</sup>, Carlo Caltagirone, MD<sup>1,4</sup>, Marco Bozzali, MD<sup>3,6\*</sup>

1. Non Invasive Brain Stimulation Unit/Department of Behavioral and Clinical Neurology, Santa Lucia Foundation IRCCS, Via Ardeatina 354, 00179, Rome, Italy
2. Stroke Unit, Tor Vergata Policlinic, Viale Oxford 81, 00133, Rome, Italy
3. Neuroimaging Laboratory, Santa Lucia Foundation, IRCCS, Rome, 00179, Italy.
4. Department of Systems Medicine, University of Rome Tor Vergata, Viale Oxford 81, 00133, Rome, Italy
5. Memory Clinic, Neurology Department, Catholic University of Rome, Italy
6. Department of Neuroscience, Brighton and Sussex Medical School, University of Sussex, Brighton BN1 9RR, East Sussex, UK.

**Running title:** Precuneus modulation in AD

\*Corresponding Authors:

Dr. Giacomo Koch, IRCCS Fondazione S. Lucia, Via Ardeatina, 306- 00179-Rome, Italy. Phone number +39 0651501181. E-mail address: g.koch@hsantalucia.it

Dr. Marco Bozzali, University of Sussex, Falmer, Brighton, East Sussex BN1 9RR, UK. Tel.: +44 (0) 1273 873509; Fax: +44 (0) 1273 876721. E-mail address: m.bozzali@bsms.ac.uk



1   **Abstract**

2   Memory loss is one of the first symptoms of typical Alzheimer’s disease (AD), for which there are  
3   no effective therapies available. The precuneus (PC) has been recently emphasized as a key area for  
4   the memory impairment observed in early AD, likely due to disconnection mechanisms within  
5   large-scale networks. Using a multimodal approach we investigated in a two-week, randomized,  
6   sham-controlled, double-blinded trial the effects of high-frequency repetitive transcranial magnetic  
7   stimulation (rTMS) of the PC on cognition, as measured by the Alzheimer Disease Cooperative  
8   Study Preclinical Alzheimer Cognitive Composite in 14 patients with early AD (7 females). TMS  
9   combined with electroencephalography (TMS-EEG) was used to detect changes in brain  
10   connectivity. We found that rTMS of the PC induced a selective improvement in episodic memory,  
11   but not in other cognitive domains. Analysis of TMS-EEG signal revealed an increase of neural  
12   activity in patients’ PC, an enhancement of brain oscillations in the beta band and a modification of  
13   functional connections between the PC and medial frontal areas.  
14   Our findings show that high-frequency rTMS of the PC is a promising, non-invasive treatment for  
15   memory dysfunction in patients at early stages of AD. This clinical improvement is accompanied by  
16   modulation of brain connectivity, consistently with the pathophysiological model of brain  
17   disconnection in AD.

18  
19   **Keywords:** AD, memory, precuneus, transcranial magnetic stimulation

20  
21   **Abbreviations:** Alzheimer’s disease (AD), default mode network (DMN), precuneus (PC),  
22   repetitive transcranial magnetic stimulation (rTMS), magnetic resonance imaging (MRI),  
23   cerebrospinal fluid (CSF), mini mental state examination (MMSE), first dorsal interosseous (FDI),  
24   resting motor threshold (RMT), standard deviation (SD), electroencephalography (EEG), Rey  
25   Auditory Verbal Learning test (RAVLT), Alzheimer Disease Cooperative Study Preclinical  
26   Alzheimer Cognitive Composite (ADCS-PACC), Digit Symbol Substitution Test (DSST), Frontal

1    Assessment Battery (FAB), theta/alpha transition frequency (TF), individual alpha frequency (IAF),  
2    TMS-evoked potentials (TEPs), inter-stimulus interval (ISI), posterior parietal cortex (PPC), global  
3    mean field power (GMFP), event-related spectral perturbation (ERSP), inter-trial coherence (ITC),  
4    standardized low resolution brain electromagnetic tomography (sLORETA), Montreal Neurological  
5    Institute (MNI), long-term potentiation (LTP).

6

## 1. Introduction

Alzheimer's disease (AD) typically presents with deficits in learning new information as well as in retrieving old memories (Bäckman et al. 2001). This loss in long-term episodic memory has been referred not only to local damage of the medial temporal lobes, but also to a dysfunction of large-scale networks underlying memory processes. Since the early stages of AD, prominent neuropathological abnormalities (i.e.,  $\beta$ -amyloid plaques and neurofibrillary tangles) are known to affect the posterior cortical regions of the brain, including the precuneus (PC), the posterior cingulate, the retrosplenial, and lateral posterior parietal cortex (PPC) (Buckner et al. 2005). These abnormalities are paralleled by an initial disruption of medial fronto-parietal functional connectivity, as revealed by alterations of the so-called default mode network (DMN), for which the PC is a key node (Buckner et al. 2008; Raichle et al. 2001). The disconnection of the PC precedes (and probably contributes to) the occurrence of regional brain atrophy, which becomes prominent at later disease stages (Gili et al. 2011). Indeed, AD patients often show a reduction of PC cortical thickness accompanied by an abnormal activity during memory task performance, and a decrease in functional connectivity (Chen et al. 2017). This is relevant since the activity of the PC is considered necessary for episodic memory retrieval (Lundstrom et al. 2005; Wagner et al. 2005). Therefore, the PC is a vulnerable region for the transitional stage towards dementia, and might represent an ideal target for tailored interventions aimed at counteracting AD-related memory decline.

So far, the only approved treatment for AD is based on cholinergic and glutamatergic drugs. Yet, these drugs have negligible efficacy on memory deficits, and alternative strategies are needed to improve memory in patients with AD (Canter et al. 2016). Recently, non-invasive brain stimulation methods have been proposed as a novel approach to improve some cognitive performances in patients with dementia (Cotelli et al., 2006; 2008; Ferrucci et al., 2008; Turriziani et al., 2012) and in healthy volunteers (Casula et al., 2017a; Rastogi et al., 2017). These studies provided evidence that repetitive transcranial magnetic stimulation (rTMS) of the dorsolateral prefrontal cortex (DLPFC) may transiently improve language functions, such as naming and

1 sentence comprehension (Cotelli et al., 2011). More recently, other studies applied rTMS over  
2 different brain areas involved in the cognitive decline of AD patients (i.e. right and left DLPFC,  
3 right and left posterior parietal cortex (PPC) associative areas, and Broca and Wernicke language  
4 areas) in combination with adapted cognitive training (Bentwich et al., 2011). These studies showed  
5 promising results in terms of global cognitive functions as indexed by the improvement of the  
6 ADAS-Cog score after 5 weeks of treatment (Nguyen et al., 2017; Rabey et al., 2013). However,  
7 this approach did not show any specific effect on memory functions.

8         Here we tested the hypothesis that rTMS of the precuneus (PC) may be a novel target to  
9 treat memory dysfunction in AD patients. This finds support in recent evidence showing that rTMS  
10 applied to key nodes of the DMN such as the PPC (Wang et al. 2014; Nilakantan et al. 2017) and  
11 PC (Rose et al. 2016; Bonni et al. 2015) improves short and long-term memory functions in healthy  
12 individuals. Moreover, we recently demonstrated that rTMS of the PC exerts its effects not only at  
13 local but also at a network level by modulating the activity of the PC and its connections to other  
14 brain areas (Mancini et al., 2017). Therefore, we hypothesized that high-frequency excitatory rTMS  
15 of the PC might improve long-term memory in patients with AD, by modulating the neural activity  
16 of the PC and its connections with medial parietal and frontal areas. To evaluate the  
17 neurophysiological modifications induced by rTMS and potentially underpinning behavioral  
18 changes, we used single-pulse TMS combined with EEG recordings.

## 2. Materials and Methods

### 2.1 Experimental Design and participants

The current study was approved by the Ethical Committee of Santa Lucia Foundation (Protocol number: CE/PROG.465). Written informed consent was obtained from all patients before entering the study. Neuronavigated rTMS was used to stimulate the PC of AD patients for two weeks in a sham-controlled crossover design. Thirty patients, admitted to the Specialist Memory Clinics of “Tor Vergata” University (Rome, Italy) and Catholic University of Rome (Rome, Italy) between January 2014 and June 2016 for complaining of memory symptoms, were screened for the current study. Patients’ recruitment was performed according to current diagnostic criteria for prodromal AD (Dubois et al. 2016), referring to the early symptomatic phase of AD, characterized by episodic memory loss in the presence of AD pathology as supported by CSF or imaging biomarker evidence. All AD patients had to be cognitively intact before the occurrence of cognitive impairment. Subjective memory impairment, corroborated by an informed caregiver had to occur at least 12 months earlier. After the first visit, patients underwent, for diagnostic purposes, a complete clinical investigation, including medical history and neurological examination, a complete blood screening, a neuropsychiatric evaluation, brain MRI scanning, and an extensive neuropsychological assessment exploring all cognitive domains (Table 1). Lumbar puncture was performed in all patients to confirm the typical CSF profile of AD pathology, i.e., reduced concentrations of amyloid  $\beta_{1-42}$ , increased levels of total- and phosphorylated-tau. Major systemic and psychiatric disorders, other neurological conditions, and signs of concomitant cerebrovascular disease on MRI scans were carefully investigated and excluded in all patients. Patients who agreed to participate (N=14; see Table 2) were randomly assigned to rTMS or sham as their first experimental arm belonging. The order of administration of either intervention (i.e., rTMS or sham) was counterbalanced across all patients. rTMS (or sham) was applied daily, at the same day-time, in a 10 session course, Monday to Friday. A two-week washout interval was applied before patients were crossed over to either experimental arm for two more weeks. Neuropsychological and neurophysiological evaluations, the

latter performed by combining TMS and EEG, were performed on the Monday morning before and after each of the two week-treatment (rTMS or sham). All researchers performing patients' evaluations were blind to their experimental arm belonging. Details of the study design are summarized in Figure 1A.

## 2.2 Intervention - Repetitive TMS

rTMS was carried out using a Magstim Rapid<sup>2</sup> magnetic biphasic stimulator connected with a figure-of-eight coil with a 70-mm diameter (Magstim Company, Whitland, UK) that generates 2.2 T as maximum output. Each daily stimulation session consisted of 42-sec trains delivered at 20 Hz spaced-out by 28 seconds of no stimulation (total number of stimuli: 1600). The entire session lasted approximately 20 minutes. Intensity of stimulation was set at 100% of the resting motor threshold (RMT), defined as the lowest intensity producing MEPs of >50 µV in at least five out of 10 trials in the relaxed first dorsal interosseous (FDI) muscle of the right hand (Rossini et al. 2015). RMT was assessed over the optimal stimulus site to elicit MEPs in the right FDI, termed "motor hotspot", identified by positioning the coil approximately over the central sulcus and moving it on the scalp by 0.5 cm steps on left M1.

During the rTMS treatment, the coil was positioned over the PC and constantly monitored using the Softaxic neuronavigation system (EMS, Bologna, Italy) coupled with a Polaris Vicra infrared camera (NDI, Waterloo, Canada). Individual T1-weighted MRI volumes were used as anatomical reference (Figure 1B). Since the coil-to-cortex distance directly affects the magnitude of magnetic stimulation, for each patient we calculated a distance-adjusted MT (AdjMT).  $AdjMT = MT + m \times (D_{siteX} - DMI)$ , where *AdjMT* is the adjusted MT in % of stimulator output, *MT* is the unadjusted MT in % of stimulator output, *DMI* is the distance between the scalp and M1 hotspot, *D<sub>SiteX</sub>* is the distance between the scalp and a second cortical region (SiteX), and *m* is the distance-effect gradient. This procedure provides a more accurate index of cortical excitability and improves the efficacy of MT-calibrated TMS (Stokes et al. 2005). Coil orientation was parallel to



the midline with the handle pointing downward. For sham treatment, stimulation was applied using the same parameters with the sham coil positioned to the scalp in correspondence to the target area. For each patient, a source estimation on pre-processed TMS-EEG data was run at the beginning of each treatment session to confirm the correct anatomical targeting for rTMS.

### *2.3 Cognitive Evaluations*

To evaluate the behavioural effects of the rTMS, we employed a battery of tests assessing long-term episodic memory, executive functions, attention, and global cognition, according to the Alzheimer Disease Cooperative Study Preclinical Alzheimer Cognitive Composite (ADCS-PACC) (Donohue et al. 2014). This battery is a well-known clinical instrument with high sensitivity to cognitive decline in prodromal and mild dementia, and with a sufficient range to detect early decline in preclinical disease stages. The battery includes: 1) the immediate and delayed total recall score from the Rey Auditory Verbal Learning test (RAVLT), to evaluate long-term episodic memory; 2) the Digit Symbol Substitution Test (DSST) from the Wechsler Adult Intelligence Scale–Revised, to evaluate response speed, sustained attention, visual-spatial skills, and set-shifting; 3) the Mini Mental State Examination (MMSE), to evaluate global cognition, and 4) the Frontal Assessment Battery (FAB), to evaluate executive functions.

### *2.4 EEG recordings*

EEG was performed using a TMS-compatible EEG equipment (BrainAmp 32MRplus, BrainProducts GmbH, Munich, Germany), and was continuously recorded from 29 scalp sites positioned according to the 10-20 International System. TMS-compatible Ag/AgCl pellet electrodes were mounted on an elastic cap, while additional electrodes were used as ground and reference. Horizontal and vertical eye movements were detected by recording the electrooculogram (EOG) to off-line reject trials showing ocular artifacts. The ground electrode was positioned in AFz, while the reference one was positioned on the nose tip. The EEG and EOG signals were band-pass filtered at

0.1-1000 Hz and digitized at a sampling rate of 5 kHz. Skin/electrode impedance was maintained below 5 k $\Omega$ .

#### *Resting EEG*

Resting EEG was recorded before each TMS/EEG session for 3 minutes with open eyes. As a first step, data were downsampled to 1000 Hz and band-pass filtered between 1 and 80 Hz (Butterworth zero phase filters). A 50 Hz notch filter was also applied to reduce noise from electrical sources. Identification and removal of artifacts (muscle activity, eye movements and blink-related activity) was made with independent component analysis (INFOMAX-ICA). Then, signal was segmented in 2-sec epochs. Power density was estimated by means of the Fast Fourier Transform (10% Hanning-window) from 4 to 45 Hz, with a frequency resolution of 0.5 Hz. For each participant, frequency bands were established based on two subjective anchor frequencies, which is the theta/alpha transition frequency (TF) and the individual alpha frequency (IAF) peak (Klimesch, 1997). The TF represents the minimum power in the alpha frequency range, whereas the IAF represents the frequency with the maximum power peak within the alpha range (7-12 Hz). Based on TF and IAF, we estimated the frequency band range for each participant, as follows: delta, from TF-4 to TF-2; theta, from TF-2 to TF; low alpha, from TF to IAF; high alpha, from IAF to IAF+2. For the estimation of individual beta and gamma frequencies we detected three peaks in the frequency range from IAF+2 to 45 Hz, these peaks were named beta1, beta2 and gamma peak. Low beta range was computed from IAF+2 to the lower spectral power between beta1 and beta2 peak; high beta range was computed from beta 1 to the lower spectra value power between beta 2 and gamma peak; gamma range was computed from beta 2 to 45 Hz. The mean band power was then obtained by averaging the power values of all the single-trial epochs for each participant.

#### *TMS/EEG*

1 To evaluate the neurophysiological modifications induced by rTMS and potentially underpinning  
2 behavioral changes, we used single-pulse TMS combined with EEG recordings. During all  
3 neurophysiological evaluations, patients were seated on a dedicated, comfortable armchair in a  
4 soundproof room. Each neurophysiological assessment began with a TMS-EEG session to evaluate  
5 the cortical excitability and the oscillatory activity evoked by single-pulse TMS. Single-pulse TMS  
6 was carried out using a Magstim Rapid<sup>2</sup> magnetic biphasic stimulator connected with a figure-of-  
7 eight coil with a 50-mm diameter (Magstim Company, Whitland, UK). Each TMS-EEG session  
8 consisted of 80 single pulses applied at a random inter-stimulus interval (ISI) of 2-4sec. **TMS-EEG**  
9 **was applied over the PC, which was the target of the rTMS intervention, and over the left posterior**  
10 **parietal cortex (l-PPC). Importantly, we chose to stimulate the l-PPC as a control site for its**  
11 **proximity to the PC, since we want to assess the spatial specificity of our rTMS intervention.** The  
12 order of stimulation of either area was counterbalanced across patients. The intensity of stimulation  
13 of single-pulse TMS was set at 90% of the AdjMT (PC:  $57.8 \pm 3.1$ ; l-PPC:  $54.2 \pm 2.6$ ). A short break  
14 was run between TMS-EEG stimulation of either site. The coil was differently oriented respect to  
15 the mid-sagittal axis of the patient's head, for each stimulation site: parallel over the PC, at 15° over  
16 l-PPC, with the handle pointing backward. The coil position was constantly monitored using the  
17 Softaxic neuronavigation system, to ensure a high degree of reproducibility across  
18 neurophysiological assessments.

19 TMS-EEG data were preprocessed offline using Brain Vision Analyzer (Brain Products  
20 GmbH, Munich, Germany). TMS-EEG data were analyzed offline with Brain Vision Analyzer  
21 (Brain Products GmbH, Munich, Germany) and EEGLAB toolbox running in a MATLAB  
22 environment (MathWorks Inc., Natick, USA; Delorme and Makeig, 2004). As a first step, a cubic  
23 interpolation from 1 ms before to 10 ms after the TMS pulse was applied to remove the TMS  
24 artifact. Afterwards, the signal was downsampled and filtered as on the resting EEG data.  
25 Physiological and TMS-related artefactual components were detected using INFOMAX-ICA and  
26 removed basing on their scalp distribution, frequency, timing and amplitude (Casula et al., 2017b).

1 Data were then segmented into epochs starting 1 s before the TMS pulse and ending 1 s after it.  
 2 Afterwards, all the epochs were visually inspected and those with excessively noisy EEG were  
 3 excluded from the analysis, resulting  $68.7 \pm 2.9$  in the pre-rTMS condition,  $68.9 \pm 2.9$  in the post-  
 4 rTMS,  $70.7 \pm 2$  in the pre-sham,  $70.1 \pm 2.1$  in the post-sham, stimulating the PC. For l-PPC  
 5 stimulation, we used  $76.5 \pm 1.1$  trials in the pre-real condition,  $76.1 \pm 1.9$  in the post-real,  $76.4 \pm 0.8$  in  
 6 the pre-sham,  $76.9 \pm 1.4$  in the post-sham.

7 Two sets of outcome measures were obtained, assessing, respectively, cortical excitability  
 8 (using a spatio/temporal-domain analysis) and cortical oscillatory activity (using a time/frequency-  
 9 domain analysis). To evaluate changes in cortical excitability, we evaluated both, global and local  
 10 cortical responses evoked by TMS. The global response was assessed, for each patient and each  
 11 stimulation site (PC and l-PPC), by measuring the first five peaks (10-35 ms for P1; 36-62 ms for  
 12 P2; 63-90 ms for P3; 91-165 ms for P4 and 166-250 ms for P5) of the global mean field power  
 13 (GMFP) waveform within the 300 msec following the TMS pulse. Local responses were measured  
 14 by the TMS-evoked potentials (TEPs) waveform at each electrode within the five temporal  
 15 windows used for GMFP analysis.

16 To evaluate changes in the oscillatory domain, we performed a time/frequency  
 17 decomposition based on Morlet wavelet (parameters  $c=3$ ; 41 linear 1 Hz steps from 4 to 45 Hz), and  
 18 then we computed event-related spectral perturbation (ERSP) and inter-trial coherence (ITC). ERSP  
 19 is a measure of event-related changes in spectral power over time in a certain frequency range  
 20 computed as:

$$TRSP(f, t) = \frac{1}{n} \sum_{k=1}^n |F_k(f, t)|^2$$

21 Where, for n trials, the spectral estimate F was computed at trial k, at frequency f and time t.  
 22 ITC is a measure of the partial or exact synchronization of activity at a particular latency and  
 23 frequency to an experimental event to which EEG data trials are time locked (i.e. TMS pulse),  
 24 computed as:

$$ITC(f, t) = \frac{1}{n} \sum_{k=1}^n \frac{F_k(f, t)}{|F_k(f, t)|}$$

ITC coefficient takes value between 0 and 1, where 0 represents absence of synchronization between EEG data and the time-locked event and 1 represents their perfect synchronization. Analysis of ERSP values was conducted over five regions of interest (ROI), each one comprising three electrodes: frontal ROI (F3, Fz, F4); central ROI (C3, Cz, C4); parietal ROI (P3, Pz, P4); left ROI (F7, T7, P7) and right ROI (F8, T8, P8). To minimize the effect of possible artifacts occurring at the time of stimulation, the frequency values were averaged over a 20-250 ms time window from the TMS pulse, corresponding to the timing of the oscillatory activity. The spectral power was computed in the frequency ranges between 4-7 Hz (theta), 8-13 Hz (alpha), 14-30 Hz (beta) and 31-45 Hz (gamma).

## 2.5 Statistical Analysis

To assess the effect of rTMS on patients' performance at RAVLT, repeated-measures ANOVAs with treatment (rTMS or sham) and time (pre- or post-treatment) as factors were performed. The same test was used to assess changes in the patients' performance at all tests included in the ADCS-PACC, to ensure that the improvement induced by rTMS was specific for episodic memory.

To assess the rTMS effect on the spectral power of resting EEG we used a repeated-measures ANOVAs with factors treatment (rTMS and sham) and time (pre- and post-treatment) separately conducted for each frequency band (delta, theta, low alfa, high alfa, low beta, high beta and gamma). Effects on amplitude of GMFP peaks (global response) were measured with a repeated-measures ANOVA with factors treatment (rTMS and sham), time (pre- and post-treatment) and peak (P1, P2, P3, P4, P5) performed separately for the two stimulation sites (PC or l-PPC).

To assess the treatment effects on local cortical response over the PC and the l-PPC, we used multiple dependent t-tests comparing TEPs waveform at each electrode within the five temporal

1 windows used for GMFP analysis. Non-parametric, cluster-based permutation statistics was  
2 conducted to correct for multiple comparisons (Maris et al. 2007). This method performs a non-  
3 parametric statistical test by calculating Monte Carlo estimate of the significance probabilities from  
4 two surrogate distributions constructed by randomly permuting the two original conditions data for  
5 3000 times (Casula et al., 2017c). The clusters for permutation analysis were defined as the two (or  
6 more) neighboring electrodes in which the t-value at a given time point exceeded a threshold of  
7  $P < 0.05$ .

8 Repeated-measures ANOVA with factors region-of-interest (frontal, central, parietal, left,  
9 right), treatment (rTMS or sham) and time (pre- or post-treatment) were performed to evaluate the  
10 rTMS effects on oscillatory activity (ERSP), separately for each frequency band. Analysis of ITC  
11 was conducted on the beta-band activity (14-30 Hz) considering the electrode over the stimulation  
12 site, i.e., Pz for the PC and P3 for l-PPC. Repeated-measures ANOVA with treatment (rTMS or  
13 sham) and time (pre- or post-treatment) as factors was performed to evaluate the effects of rTMS on  
14 ITC beta-band activity.

15 For all the ANOVA procedures, the Huynh–Feldt  $\epsilon$  correction factor was applied where  
16 appropriate to compensate for effects of non-sphericity. Bonferroni’s correction was applied to  
17 post-hoc comparisons.

## 19 2.6 Source analysis

20 In order to confirm the targeting of the stimulation, as a preliminary analysis, we used source  
21 estimation on the pre-processed TMS-EEG data at the beginning of the treatment for each subject.  
22 Briefly, using BrainStorm software we estimated the activity of the dipoles underlying the brain  
23 electrical activity, by means of the ICBM152 template (Tadel et al., 2011). Since this kind of  
24 inverse problem presents an infinite number of dipole patterns that could generate the same  
25 electrical activity registered by the electrodes, we used minimum norm imaging to approach the  
26 forward modelling. As a preliminary step, this method finds a cortical current source density

1 distribution that can approximate the data, and then defines the solution with the minimum energy,  
2 using source covariance. We modelled one dipole at each grid point, normally oriented to the  
3 cortical surface. We then used the standardized low-resolution brain electromagnetic tomography  
4 (sLORETA) measure to normalize the current density map at each point (Pascual-Marqui et al.,  
5 2002). The estimated source distribution was averaged across the patients (Supplementary Movie 1)  
6 and the PC was selected as region of interest using the definition of the Desikan-Killiany atlas  
7 (Desikan et al., 2006).

8

## 9 *2.7 Behavioral-neurophysiological correlations*

10 In order to investigate possible relationships between clinical and neurophysiological outcomes, we  
11 tested correlations using the Pearson's coefficient (two-tailed) between the modulations observed in  
12 the RAVLT and in the GMFP.

1    **3. Results**

2    Fourteen patients with AD (F/M=7/7) took part in the study, which was conducted between January  
3    2014 and June 2016. Demographic and clinical characteristics of the cohort are summarized in  
4    Table 1. They all completed the protocol successfully, attending all experimental sessions.

6    *3.1 Source analysis*

7    The source analysis of TMS-evoked EEG activity showed that the response was spatially  
8    distributed along the wall of the medial superior parietal cortex bilaterally, corresponding to the PC  
9    (Movie 1) (Bzdok et al. 2015). This confirmed, in all participants, a correct anatomical  
10   identification of the stimulation site (Figure 1C). The Montreal Neurological Institute (MNI)  
11   coordinates of the stimulation site averaged across participants were [x,y,z]=[0,-65±2,37±9]. The  
12   mean scalp-to-cortex distance for the PC was 27±0.3 mm, and the AdjMT for rTMS was 60.8±3.1%  
13   of maximum stimulator output.

15   *3.2 Cognitive evaluation*

16   ANOVA on the performance at Delayed Recall of the Rey Auditory Verbal learning Test showed a  
17   significant time × treatment interaction [F(1,13)=5.98; p=0.029] (Figure 2). Post-hoc analysis  
18   revealed a significant improvement at the test performance after rTMS (pre vs. post, 2.42±0.8 vs.  
19   3.14±0.8). No significant effects were detected after sham stimulation (post vs. pre, 2.86±0.7  
20   vs. 2.50±0.8). No significant effects were observed on patients' executive functions, attention or  
21   global cognition (all ps>0.05, table 3).

Comment [Cla1]: Credo sia SEM

Comment [Cla2]: SEM

Comment [Cla3]: Questo credo sia SEM (standard error mean) e non SD perché in Table 3 Sd è 2.7 o 2.9

Comment [Cla4]: SEM

23   *3.3 Resting EEG*

24   ANOVAs performed on the mean spectral power for each frequency band, did not reveal any  
25   significant main effect of rTMS (all ps>0.05), time (all ps>0.05), nor any significant interaction  
26   between the two factors (all ps>0.05).



### 3.4 TMS-evoked cortical activity

ANOVA performed on mean TMS-evoked activity evaluated over PC revealed a significant  $rTMS \times \text{time} \times \text{peak}$  interaction [ $F(4.52)=5.74$ ;  $p=0.0006$ ], due to a significant increase of P3 after real  $rTMS$  treatment (Figure 3a). Post-hoc analysis showed for P3 a significant difference between pre and post real  $rTMS$  treatment ( $p=0.008$ ), and between post real and post sham ( $p=0.005$ ). For all peaks, no significant differences were observed between baseline of each  $rTMS$  treatment (all  $ps>0.05$ ) and between pre and post sham  $rTMS$  ( $p>0.05$ , Figure 3b). No significant effects were found when stimulating l-PPC indicating that the  $rTMS$  protocols was unable to induce a general effects on cortical excitability of surrounding cortical sites (Figure 3c,d). When stimulating the PC, the cluster-based analysis did not reveal any significant difference between the baselines of the two groups (i.e. pre-sham  $rTMS$  vs. pre-real TMS; Monte Carlo  $p>0.05$ ). As revealed by TEPs and GMFP amplitude, PC- $rTMS$  produced a significant increase of TMS-evoked activity from PC over a specific time window, i.e. from 60 to 90 ms after TMS over two distinct cluster of electrodes: one frontal, comprising Fz, FC2 and F4 electrodes; and one parieto-occipital, comprising Pz, O1 and O2 (Monte-Carlo  $p<0.01$ ). No difference was detected after sham stimulation, nor after stimulation of l-PPC (Monte-Carlo  $p>0.05$ ).

### 3.5 TMS-evoked oscillatory activity

ANOVA performed on mean ERSP values evaluated over PC revealed a significant  $ROI \times rTMS \times \text{time}$  interaction when beta oscillatory activity was evaluated [ $F(4.52)=3.093$ ;  $p=0.023$ ]. Post-hoc analysis showed a significant increase of beta activity over the parietal ROI in the post-  $rTMS$  condition compared to pre- ( $3.565\pm0.37$  vs.  $2.257\pm0.46$   $\mu V$ ;  $p=0.021$ ) (Figure 4a). No effect was revealed in the sham  $rTMS$  condition (Figure 4b), nor between the baselines of the two conditions (pre-real  $rTMS$  vs. pre-sham  $rTMS$ ;  $p>0.05$ ). ANOVA did not reveal any significant differences in the other frequency bands (all  $ps>0.05$ ).

1 ANOVA performed on mean ITC values evaluated over PC revealed a significant rTMS  $\times$  time  
2 interaction when beta oscillatory activity was evaluated [ $F(1,13)=11.36$ ;  $p=0.005$ ]. Post-hoc  
3 analysis showed a significant increase of beta activity after rTMS condition compared to pre-  
4 ( $0.0006\pm0.01$  vs.  $0.007\pm0.01$ ;  $p=0.002$ ) (Figure 4c). Again, no significant effects were observed  
5 after sham treatment (Figure 4d) nor when stimulating the l-PPC (Figure 4e-h).

6

### 7 *3.6 Behavioral-neurophysiological correlations*

8 Analysis of relationships between clinical and neurophysiological data did not reveal any significant  
9 correlation (all  $p>0.05$ ).

#### 4. Discussion

We evaluated here the usefulness of rTMS in modifying selectively the cognitive performance of patients with typical AD at early clinical stages. In our patient sample, we demonstrated a significant beneficial effect of this intervention in improving episodic memory. Our neurophysiological data suggest that this improvement is underpinned by changes in cortical activity of the PC and its connectivity with frontal areas.

rTMS induced an average increase of 0.8 recalled items (36%) at RAVLT (delayed recall). These results provide the first evidence that rTMS could be used as a non-pharmacological intervention to counteract memory loss in AD. The study design was rigorous, with rTMS being sham-controlled in a cross-over design. AD patients, as well as experimenters performing evaluations were blind to the treatment condition at any time of the study – thus minimizing the risk of observing a placebo effect. Despite the sample was relatively small, these results are robust, and could provide the basis for planning a clinical trial with a between-group design aimed at evaluating the potential beneficial effects of rTMS of the PC in slowing cognitive decline when applied during a longer period (i.e. six months).

In all AD patients, rTMS delivery on the PC was strictly verified in terms of anatomical localization by a source reconstruction analysis of TMS-evoked EEG activity, using a neuronavigation system. Critically, cognitive evaluations were constantly paralleled by TMS-EEG monitoring, thus allowing a concomitant assessment of the clinical effect of rTMS alongside with information on neurophysiological modulation of the brain. Taken altogether, these findings provide novel evidence that non-invasive treatment of network dysfunction, through stimulation of the PC, represents an effective strategy to enhance long-term memory in AD.

The rTMS-induced improvement of long-term memory reinforces the notion that PC is directly involved in memory dysfunction in prodromal AD (Lundstrom et al. 2005). With this regard, recent models of long-term memory showed that, in healthy conditions, the encoding of episodic memory is associated with reduced PC activity, while the retrieval is associated with

1 increased PC activity (Daselaar et al. 2009). This interaction, which has been termed “the  
2 encoding/retrieval flip” (Huijibers et al. 2012), is reduced in elderly adults with amyloid pathology  
3 (Vannini et al. 2012). Moreover, memory recall is associated with greater activity in medial regions  
4 of the DMN in both, healthy subjects and AD patients (Dhanjal et al. 2014). It is therefore plausible  
5 that the excitatory rTMS protocol we applied here could have reinforced this memory-related  
6 cortical mechanism by increasing PC activity.

7       From a neurobiological perspective, rTMS could have induced clinical improvement by  
8 promoting changes in synaptic plasticity, the most important biological mechanism for learning and  
9 memory. Long-term potentiation (LTP) is considered as a main neurophysiological correlate of  
10 these cognitive functions (Bliss and Lømo 1973). We recently demonstrated that AD patients show  
11 a disruption in LTP-like cortical plasticity since early clinical stages (Koch et al. 2012; Di Lorenzo  
12 et al. 2016). In this context, high-frequency rTMS might have induced LTP-like cortical plasticity  
13 within the PC of our cohort of AD patients. Consistent with this hypothesis, TMS-EEG analysis  
14 revealed a specific increase of PC neural activity. This enhancement was evident not only at a local  
15 but also at a network level. Indeed, changes in neural activity were located over two distinct clusters  
16 of electrodes: one corresponding to the site of stimulation (PC), one corresponding to the medial  
17 frontal cortex, suggesting that rTMS induced relevant modulations over a medial parieto-frontal  
18 circuit. Interestingly, the topography of this EEG network resembles the anatomical distribution of  
19 the DMN, as identified by functional MRI (Buckner et al. 2008; Raichle et al. 2001). We also found  
20 that rTMS induced an enhancement of TMS-evoked beta activity, both in terms of power and phase  
21 synchronization, focused over the medial parietal electrodes underlying the site of rTMS delivery.  
22 Since we applied rTMS at 20 Hz, a frequency that falls within the range of beta oscillations, our  
23 results could be explained by a possible long-lasting entrainment of beta-rhythm induced by rTMS  
24 (Rosanova et al. 2009). In this perspective, our findings are in agreement with models proposing  
25 beta activity as an efficient cortical frequency through which the brain networks communicate  
26 relevant information, playing a pivotal role on different memory processes (Feurra et al. 2016).

1       The main limitation of this study is the relatively small sample size. However, in order to  
2       select a homogeneous cohort, the clinical diagnosis of AD was supported by the use of CSF  
3       biomarkers in all participants, according to the current diagnostic criteria (Dubois et al. 2016). In  
4       addition, the low number of electrodes used for EEG recordings limits the spatial resolution of our  
5       conclusions, especially for the source analysis. In spite of these limitations, to our knowledge, this  
6       is the first study that investigated both the behavioral and neurophysiological effects of an rTMS  
7       protocol in AD patients. From a methodological point of view, we demonstrated the reliability of  
8       the TMS-EEG approach in revealing specific cortical changes that were not detectable by the  
9       resting EEG analysis.

10       In conclusion, our results show novel evidence that rTMS may be a potential effective  
11       strategy for treating patients with early AD for whom, currently, there is no available therapy. Our  
12       work is in line with an emerging framework considering circuit-based dysfunctions as a model for  
13       cognitive impairment (Canter et al. 2016), and identifies the PC as a novel interventional target to  
14       successfully improve memory in AD.

15

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1 **Table 1. Neuropsychological assessment at baseline of AD patients.**

<b>Cognitive Domain</b>	<b>Neuropsychological test</b>	<b>score</b>
LONG TERM MEMORY		
Verbal memory	<b>Free and cued selective reminding test</b>	
	Immediate Free Recall (cut-off $\geq 19.59$ )	17.0 (1.3)
	Immediate Total Recall (cut-off $\geq 35$ )	26 (3)
	Delayed Free Recall (cut-off $\geq 6.31$ )	4.0 (1.2)
	Delayed Total recall (cut-off $\geq 11$ )	8 (1.7)
	<b>Short story test</b>	
	Immediate Recall (cut-off $\geq 3.1$ )	3.9 (2.7)
	Delayed recall (cut-off $\geq 2.6$ )	3.6 (2.3)
Visuo spatial memory	<b>Rey's Complex figure</b>	
	Immediate recall (cut-off $\geq 6,4$ )	11.2 (8.1)
	Delayed recall (cut-off $\geq 6.3$ )	7.9 (4.7)
SHORT TERM MEMORY		
Verbal	Digit span ( $\geq 3.7$ )	4.8 (1.3)
Spatial	Corsi block tapping task (3.5)	4.1(0.6)
LANGUAGE	Naming of object from BADA ( $\geq 22$ )	28.0(3.5)
REASONING	Raven's Progressive Matrices ( $\geq 18.9$ )	26.2 (6.3)
EXECUTIVE	Modified Card Sorting test	4.4 (0.7)

FUNCTIONS	criteria achieved (cut-off $\geq$ 4.2)	
	Modified Card Sorting test perseverative errors (cut-off $\geq$ 7.6)	6.0 (2.0)
	Phonological verbal fluency( $\geq$ 17.3)	33.8 (7.3)
PRAXIS ABILITIES	Copy of drawing (cut-off $\geq$ 7.1)	9.5 (1.3)
	Copy of drawing with landmarks cut-off $\geq$ 61.8	67.0 (1.8)
ATTENTION	Trail Making Test A ( $\leq$ 94 sec)	76 (13)
	Trail Making Test B ( $\leq$ 283 sec)	136 (21)
	Trail Making Test A-B ( $\leq$ 187 sec)	85 (17)

1

2 The table shows the mean (SD) performance scores obtained on neuropsychological testing. For  
3 each administered test appropriate adjustments for gender, age and education were applied  
4 according to the Italian normative data. Available cut-off scores of normality ( $\geq$  95% of the lower  
5 tolerance limit of the normal population distribution) are also reported for each test.

6

1    **Table 2. Principal Demographic and Clinical Characteristics of AD patients.**

AGE, years (mean $\pm$ SD)	70.0 $\pm$ 5.1
SEX ,% female	50
EDUCATION, years (mean $\pm$ SD)	7.2 $\pm$ 3.0
MMSE (mean $\pm$ SD)	26.1 $\pm$ 1.8
ADL (mean $\pm$ SD)	5.6 $\pm$ 0.5
IADL (mean $\pm$ SD)	7.3 $\pm$ 0.6
CDR (mean $\pm$ SD)	0.3 $\pm$ 0.3
Disease duration (months)	13.8 $\pm$ 5.1
CSF beta 1-42 pg/mL (mean $\pm$ SD)	383.1 $\pm$ 16.2
CSF total tau pg/mL(mean $\pm$ SD)	558.3 $\pm$ 190.7
CSF p-tau pg/mL(mean $\pm$ SD)	72.8 $\pm$ 16.2
APOE $\epsilon$ 4+ %	65

2

3    Abbreviations: AD=AD; MMSE: Mini-Mental State Examination; ADL: Activities of daily Living;

4    IADL: Instrumental activities of Daily living; CSF: cerebrospinal fluid; SD: Standard deviation.

5

6



Cognitive test	pre rTMS	post rTMS	pre sham	post sham	ANOVA Group x time
MMSE	26.9± 1.9	27.3± 1.6	25.8± 2.1	26.7± 2.6	<i>p</i> =n.s.
RAVLT (IR)	25.4 ±6.5	26.4 ±7.7	25±8.0	25.9 ±8.2	<i>p</i> =n.s.
RAVLT (DR)	2.2 ± 2.7	3.0 ± 2.6	2.4 ±2.7	2.4 ±2.9	<i>p</i> =0.02*
FAB	14.1 ± 2.2	13.9±1.9	14.0±2.2	14.3±2.5	<i>p</i> =n.s.
DSST	89.4± 2.7	90.3± 1.7	89.3± 2.5	89.2± 2.7	<i>p</i> =n.s.

1 **Table 3. Cognitive performances of AD patients in different experimental conditions.**

2

3 Abbreviations: rTMS: repetitive transcranial magnetic stimulation; MMSE: Mini-Mental State

4 Examination; RAVLT: Rey Auditory Verbal learning Test (IR: Immediate Recall; DR: Delayed

5 Recall); DSST: Digit Symbol Substitution Test.

1 **Figure legends**

2 **Figure 1. Experimental design and source analysis reconstruction.**

3 (A) AD patients (n=14) were randomly assigned to either a first neuronavigated rTMS or control  
4 stimulation (sham) arm. rTMS/sham was daily applied in a 10-session course, Monday to Friday,  
5 for a total duration of two weeks. A two-week washout interval was then applied, following which  
6 patients were crossed over to the alternative study arm for two additional weeks. The order of  
7 assignment to either study arm was counterbalanced across patients. (B) rTMS was applied over the  
8 PC at 20 Hz (1600 stimuli per day), using a neuronavigation system to ensure that the same spot  
9 was constantly stimulated across sessions. (C) Source analysis of TMS-evoked EEG activity  
10 showed a bilateral activation of the PC, as reconstructed at the peak fit of cortical response between  
11 60 and 90 ms from PC stimulation with single-pulse TMS.

12

13 **Figure 2. Behavioral results.**

14 (A) Learning curves for the 15 word-list of the Rey Auditory Verbal Learning Test before and after  
15 two weeks of treatment with rTMS (left panel) or sham (right panel). (B) Immediate (IR) and (C)  
16 Delayed Recall (DR) of the Rey Auditory Verbal Learning Test before and after two weeks of  
17 treatment with rTMS or sham. \*  $p < 0.05$ . Error bars indicate SEM.

18

19 **Figure 3. TMS/EEG evoked responses in the spatio/temporal domain.**

20 (A) rTMS, but not sham (B) increases cortical activity from 60 to 90 ms (red thick line) following a  
21 single-pulse TMS applied over the PC. Such increase is spatially distributed over the electrodes  
22 located on the medial fronto-parietal areas, as shown by the scalp maps of surface voltage  
23 distribution. (C, D) No significant effects are detectable when conducting the same analysis on the  
24 cortical response after single-pulse TMS of the l-PPC. \* $P < 0.05$ . Line shading indicated SEM

25

26 **Figure 4. TMS/EEG evoked responses in the time/frequency domain: ERSF**

1 (A) rTMS, but not sham (B), enhances PC beta activity in terms of spectral power, as revealed by  
2 event-related spectral perturbation (ERSP). No significant effects are detectable when the same  
3 analysis is conducted on the l-PPC (C,D). \* $P < 0.05$ . Error bars indicated SEM.

4

5 **Figure 5. TMS/EEG evoked responses in the time/frequency domain: ITC**

6 (A) rTMS, but not sham (B), synchronized PC beta oscillatory activity, as revealed by inter-trial  
7 coherence (ITC). No significant effects are detectable when the same analysis is conducted on the l-  
8 PPC (C,D). \* $P < 0.05$ . Error bars indicated SEM.