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► **To cite this version:**

Francesco Mori, Silvia Rossi, Giulia Sancesario, Claudia Codecà, Giorgia Mataluni, et al.. Cognitive and cortical plasticity deficits correlate with altered amyloid- β CSF levels in Multiple Sclerosis. *Neuropsychopharmacology*, 2010, 10.1038/npp.2010.187 . hal-00585468

HAL Id: hal-00585468

<https://hal.science/hal-00585468>

Submitted on 13 Apr 2011

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Cognitive and cortical plasticity deficits correlate with altered amyloid- β CSF levels in Multiple Sclerosis

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Abbreviated title: *Amyloid- β and cognition in MS*

Number of figures: 5

Number of tables: 0

Number of pages: 32

Number of words for Abstract: 241 (max 250)

Number of words for Introduction: 597 (max 750)

Number of words for Materials and Methods: 1476 (max 1500)

Number of words for Discussion: 1444 (max 1500)

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ABSTRACT

Cognitive dysfunction is of frequent observation in Multiple Sclerosis (MS). It is associated with grey matter pathology, brain atrophy and altered connectivity, and recent evidence showed that acute inflammation can exacerbate mental deficits independently of the primary functional system involved.

In the present study, we measured cerebrospinal fluid (CSF) levels of amyloid- β_{1-42} and tau protein in MS and in Clinical Isolated Syndrome (CIS) patients, since both proteins have been associated with cognitive decline in Alzheimer's disease (AD). In AD, amyloid- β_{1-42} accumulates in the brain as insoluble extracellular plaques, possibly explaining why soluble amyloid- β_{1-42} is reduced in the CSF of these patients. In our sample of MS patients, amyloid- β_{1-42} levels were significantly lower in patients cognitively impaired and were inversely correlated with the number of Gadolinium-enhancing (Gd⁺) lesions at the magnetic resonance imaging (MRI). Positive correlations between amyloid- β_{1-42} levels and measures of attention and concentration were also found. Furthermore, abnormal neuroplasticity of the cerebral cortex, explored with theta burst magnetic stimulation (TBS), was observed in cognitively impaired patients, and a positive correlation was found between amyloid- β_{1-42} CSF contents and the magnitude of long-term potentiation (LTP)-like effects induced by TBS. No correlation was conversely found between tau protein concentrations and MRI findings, cognitive parameters, and TBS effects in these patients.

Together, our results indicate that in MS central inflammation is able to alter amyloid- β metabolism, by reducing its concentration in the CSF, and leading to impairment of synaptic plasticity and cognitive function.

Key words: cognition, inflammation, LTP, tau protein, transcranial magnetic stimulation

INTRODUCTION

Multiple Sclerosis (MS) causes cognitive deficits since the early stages of the disease, by altering memory, attention and executive functions (Amato *et al*, 2004, 2006a, 2008a,b; Chiaravalloti and DeLuca, 2008; Genova *et al*, 2009).

The synaptic and molecular mechanisms at the basis of cognitive impairment in MS are still poorly understood. A large body of evidence converges in indicating that learning and memory processes are encoded in brain circuits as experience- and activity-dependent long-term synaptic changes. In particular, long-term potentiation (LTP) is a form of synaptic plasticity in which repeated stimulation connects neurons by making synapses more responsive to future activation, and is commonly considered a major candidate for representing the substrate of learning and memory processes (Bliss and Collingridge, 1993; Malenka, 2003).

LTP induction is regulated by a variety of factors, able to interfere with critical receptor and post-receptor events at excitatory synapses (Bliss and Collingridge, 1993; Malenka, 2003; Feldman, 2009; Kessels and Malinow, 2009; Minichiello, 2009). Among these factors, amyloid- β , particularly the isoform amyloid- β_{1-42} , that was consistently found to be reduced in the cerebrospinal fluid (CSF) of Alzheimer's disease (AD) patients, plays a major role in LTP deficits and cognitive impairment in AD. More specifically, amyloid- β_{1-42} , aggregates in oligomers that impair synaptic plasticity mechanisms (Klyubin *et al*, 2005; Shankar *et al*, 2008; Walther *et al*, 2009; Townsend *et al*, 2010). Its role in other disorders associated with mental deterioration is however still unclear.

Amyloid- β peptides are formed by proteolytic cleavage of a transmembrane protein, the amyloid precursor protein (APP) by β -site APP-cleaving enzyme 1 (BACE). BACE cleaves APP, causing the secretion of an extracellular soluble fragment of APP, termed sAPP- β , and the retention of a 99 residue C-terminal fragment. This fragment can undergo further cleavage by γ -secretase to release 40 or 42 amino acid long amyloid- β fragments into the cytoplasm. However, APP may undergo at least two and most likely several different processing pathways. In the non-amyloidogenic pathway,

APP is cleaved at the α -site in the middle of the amyloid- β domain. This cleavage obliterates amyloid- β formation, instead yielding an N-terminal sAPP- α fragment (Andreasson *et al*, 2007).

We postulated that, in MS, acute inflammation is able to interfere with amyloid- β_{1-42} or tau metabolism, and that, like in AD, this abnormal metabolism could be associated with cognitive dysfunction and synaptic plasticity. Notably, although both amyloid- β_{1-42} and tau protein alter LTP induction and cognitive performances in animal models (Oddo *et al*, 2003; Klyubin *et al*, 2005; Rosenmann *et al*, 2008; Shankar *et al*, 2008; Polydoro *et al*, 2009; Walther *et al*, 2009; Townsend *et al*, 2010), their role in human synaptic plasticity has never been explored.

In humans, LTP can be explored in a safe and non-invasive manner by using paradigms of repetitive transcranial magnetic stimulation (rTMS) (Ziemann, 2004; Cooke and Bliss, 2006; Di Lazzaro *et al*, 2008). A novel LTP-inducing protocol has been recently developed, in the attempt to mimic the physiological activity of hippocampal neurons during learning episodes (Huang *et al*, 2005; Di Lazzaro *et al*, 2008). This new protocol has been termed theta burst stimulation (TBS) and, according to *in vitro* experiments in hippocampal preparations, it produces a NMDA receptor-dependent LTP in the human cortex, by enhancing corticospinal excitability for several minutes after the end of the stimulation (Huang *et al*, 2005).

Thus, in the current study we investigated whether altered CSF concentrations of amyloid- β_{1-42} metabolism are associated with abnormal patterns of cognitive performances and of cortical plasticity in a sample of MS patients that were submitted to an extensive neuropsychological examination and to neurophysiological investigations of cortical plasticity by means of TBS.

SUBJECTS AND METHODS

The study was approved by the Ethics Committee of the University Hospital Tor Vergata, Rome.

Abbreviations used throughout the text are provided in the Supplementary Text.

MS patients

We collected CSF from 42 patients (30 females and 12 males, aged 23-54 years), admitted to the neurological clinic of the University Hospital Tor Vergata of Rome, and later diagnosed as suffering from clinically isolated syndrome (CIS) (n=7), relapsing-remitting (RR) MS (n=30), or primary progressive (PP) MS (n=5). After their admittance, all patients underwent, in sequence, brain (and in selected cases also spinal) magnetic resonance imaging (MRI) scan, neuropsychological assessment, TMS and CSF withdrawal within 24 hours. Corticosteroids or other MS-specific immunosuppressive therapies were initiated later when appropriate.

The diagnosis of CIS or MS was established by clinical, laboratory and MRI parameters, and matched published criteria (McDonald *et al*, 2001; Polman *et al*, 2005). In all instances, patients underwent detection of oligoclonal banding in the CSF (positive in 92% of cases). Expanded Disability Status Scale (EDSS) scores were between 0 and 5.

As controls, we used CSF from 12 age- and gender-matched individuals (8 females and 4 males, aged 25-52 years) without inflammatory or degenerative diseases of the central or peripheral nervous system. These subjects underwent lumbar puncture because of a clinical suspect of acute peripheral neuropathy, meningitis, or subarachnoidal hemorrhage, which were not confirmed. All the subjects gave their written informed consent to the study.

MRI acquisition and analysis

Three Tesla MRI scan consisted of dual-echo proton density, FLAIR, T2-weighted spin-echo images and pre-contrast and post-contrast T1-weighted spin-echo images. All images were acquired in the axial orientation with 3 mm-thick contiguous slices. T2 lesion volume was determined by manual tracing and the number of gadolinium-enhancing (Gd+) (0.2 ml/Kg e.v.) lesions was counted by a neuroradiologist who was unaware of the patient's clinical details (Centonze *et al*, 2007).

CSF determination of amyloid- β_{1-42} and total tau

Immediately after its collection, CSF was centrifuged to eliminate cells and cellular debris and stored at -80°C until analyzed (Centonze *et al*, 2007). Levels of both amyloid- β_{1-42} and total tau were determined according to standard procedures, using commercially available sandwich enzyme-linked immunosorbent assays (Innotest β -Amyloid 1-42, Innotest h-tau Ag, Innogenetics, Ghent, Belgium) (Sancesario *et al*, 2010). Briefly, 25 μl of the CSF from each patient were dispensed into corresponding 96-well ELISA plates, pre-coated either with the monoclonal antibody 21F12 for amyloid- β_{1-42} , or AT120 for total tau, and incubated respectively with the biotinylated antibody 3D6 or HT7. Bound antibodies were then detected by a peroxidase-labeled streptavidin, after addition of a substrate solution. The reaction was stopped by sulphuric acid. The absorbance of the reaction product was read at 450 nm. The biomarker concentrations in the samples were calculated based on the amyloid- β_{1-42} and tau standard sigmoid curve equation.

Neuropsychological assessment

Cognitive functions were assessed through the Brief Repeatable Neuropsychological Battery (BRB) (Rao, 1990) by an expert trained clinician in a subgroup of 21 MS patients (4 CIS and 17 RR MS) who gave consent to the examination. The BRB assesses the cognitive domains most frequently impaired in MS (Amato *et al*, 2004), and incorporates tests of verbal memory (Selective Reminding Test [SRT]); visual memory (10/36 Spatial Recall Test); attention, concentration, and speed of information processing (Paced Auditory Serial Addition Test [PASAT], Symbol Digit Modalities Test [SDMT]); and verbal fluency (Word List Generation). Performance on each test of the BRB was assessed by applying the available Italian normative values (Amato *et al*, 2006b). Failure of a test was defined when the score was at least 2 standard deviations below the mean normative values.

Consistently with previous works (Amato *et al*, 2006a, 2008b), those patients who failed at least three tests were considered cognitively impaired (CI), and those who failed less than three tests

were considered cognitively preserved (CP). No subjects were taking psychoactive drugs or substances that might interfere with neuropsychological performance.

TMS

TMS protocols were performed in a subgroup of 30 MS patients (5 CIS, 23 RR MS and 2 PP MS) who gave consent to the examination and were asymptomatic in the upper right limb. Electromyographic (EMG) traces were recorded from the right first dorsal interosseus muscle (FDI) with surface cup electrodes. The active electrode was placed over the muscle belly and the reference electrode over the metacarpophalangeal joint of the index finger. Responses were amplified with a Digitimer D360 amplifier (Digitimer, Welwyn Garden City, Hertfordshire, United Kingdom) through filters set at 20 Hz and 2 kHz with a sampling rate of 5 kHz, then recorded by a computer with SIGNAL software (Cambridge Electronic Devices, Cambridge, United Kingdom).

MEPs were evoked through a figure-of-eight coil with external loop diameter of 70 mm connected to a Magstim 200² magnetic stimulator (Magstim Company, Whitland, Wales, UK). Coil position was adjusted to find the optimal scalp site to evoke motor responses in the contralateral FDI, the motor “hot spot”, at the beginning of each experimental session and marked over the patients scalp with a pencil. The coil was held tangentially to the scalp surface with the handle pointing posteriorly and laterally at about 45° with respect to the mid-sagittal axis of the head.

iTBS

Intermittent TBS (iTBS) was delivered over the motor “hot spot” of the right FDI through a Magstim Rapid² stimulator. The active motor threshold (AMT) was defined as the minimum stimulation intensity required to evoke a liminal motor potential from the FDI during voluntary contraction (about 200 μ V in 50% of 10 trials). Stimulation intensity was 80% of AMT. The iTBS protocol consisted of 10 bursts, each burst composed of three stimuli at 50 Hz, repeated at a theta frequency of 5 Hz every 10 s for a total of 600 stimuli (200 s). Sixty MEPs were collected before

iTBS (baseline) and at two different time points (0 and 15 minutes) after the end of iTBS. Stimulation intensity was set to induce a stable MEP of approximately 1 mV peak to peak amplitude in the relaxed right FDI at baseline and remained unchanged until end of recordings. MEP's amplitudes were then averaged at each time point and normalized to the mean baseline amplitude.

Intracortical circuits in right M1

We also tested, through paired-pulse (pp) TMS, short interval intracortical inhibition (SICI, mediated by intrinsic GABAergic circuits) (Kujirai *et al*, 1993), intracortical facilitation (ICF, believed to follow the preferential recruitment of intrinsic excitatory fibers) (Kujirai *et al*, 1993), short intracortical facilitation (SICF, likely mediated by excitatory cortical interneurons) (Ziemann *et al*, 1998), and long interval intracortical inhibition (LICI, mediated by local GABAB pathways) (Valls-Solé *et al*, 1992) of the left M1. One figure of eight coil, external diameter 70 mm was held tangentially to the scalp over the motor "hot spot" for right FDI muscle. Stimulation intensity for TS was adjusted in each experiment to evoke a MEP of approximately 1 mV peak to peak amplitude in the relaxed right FDI.

SICI and ICF were tested using ppTMS with a subthreshold CS preceding a suprathreshold TS (Kujirai *et al*, 1993; Rothwell, 1997). CS stimulus was set at 80% AMT. Three conditions were presented in a random order: control (TS given alone) and two pp conditions (TS preceded by CS) at one of two different ISI (2 and 10 ms).

For SICF, the intensity of CS was set to 90% RMT. Six randomly intermixed conditions were presented in a random order: TS given alone and five conditions with the TS followed by CS at one of five different ISI (1.5, 2.1, 2.7, 3.7, 4.5 ms) (Hanajima *et al*, 2002).

For LICI the intensity of CS was set at 120% RMT. Two conditions were presented in a random order: control (TS given alone) and one paired-pulse condition (TS preceded by CS) at 100 ms ISI (Valls-Solé *et al*, 1992). For each experiment ten responses were collected for the test stimulus

alone and for conditioned MEPs at each ISI. Changes in MEP amplitude at each ISI were expressed as percentage of the mean unconditioned MEP amplitude.

Data analysis

Differences in tau and amyloid- β_{1-42} CSF levels were assessed using analysis of variance, followed by post-hoc comparison using the Tukey highest significant difference procedure, to account for multiple comparisons and by T Test or Mann–Whitney test for comparisons between two groups. Relationships between tau and amyloid- β_{1-42} CSF levels and MRI findings were assessed using Spearman correlation test for nonparametric values and Pearson's correlation test for parametric values. Correlation analysis between amyloid- β_{1-42} CSF levels and cognitive variables was performed by calculating Spearman correlation coefficients.

Correlations between tau and amyloid- β_{1-42} CSF levels with iTBS at each timepoint, SICI, ICF, LICI and SICF at each ISI were calculated using Pearson's correlation coefficient. Differences between groups were assessed using repeated measures analysis of variance for iTBS, SICF, SICI, ICF and LICI. Bonferroni correction was used to correct for multiple comparisons. Data, expressed as mean \pm SEM, were considered significant at the 0.05 level.

RESULTS

CSF contents of amyloid- β_{1-42} and total tau protein in MS patients and their correlation with neuropsychological parameters

We first measured amyloid- β_{1-42} and tau protein levels in the CSF of MS patients. Amyloid- β_{1-42} levels were significantly reduced in the population of MS subjects, compared to control individuals (MS group: 267 \pm 22 pg/ml, n = 42; control group: 390 \pm 45 pg/ml, n = 12, p < 0.05) (Fig. 1A). Conversely, tau protein levels were not altered in the CSF of MS patients (MS group: 128 \pm 11 pg/ml, n = 42; control group: 143 \pm 14 pg/ml, n = 12, NS) (Fig. 1B).

MS patients were, then, classified as cognitively impaired (CI, n = 11) or cognitively preserved (CP, n = 10) on the basis of their neuropsychological performance, without differences in terms of demographic characteristics (CI: 7 females and 4 males, aged 25-40 years with 11±7 years of education; CP: 6 females and 4 males, aged 23-37 years with 11 ± 3 years of education).

The reduction of amyloid- β_{1-42} levels was related to cognitive dysfunction (CI MS group: 145±25 pg/ml, n=11; CP MS group: 297±46 pg/ml, n=10; control group: 390±45 pg/ml, n = 12; F = 9.81, p = 0.0005), since in CP MS amyloid- β_{1-42} levels were higher than in CI MS patients (p < 0.05), and indistinguishable from controls (NS) (Fig. 1C). In CI MS patients, tau protein CSF levels were conversely still indistinguishable from those measured in controls and in CP patients (CI MS group: 110±18 pg/ml, CP MS group: 176±37 pg/ml, control group: 143±14 pg/ml, F = 2.22, p = 0.12) (Fig. 1D).

The most frequently failed tests by examined MS patients were those assessing sustained attention and concentration (SDMT, PASAT) and verbal memory (SRT-LTS, SRT-CLTR). Interestingly, a moderate positive correlation was found between amyloid- β_{1-42} CSF levels and scores on measures of attention/concentration (SDMT: n = 21, r = 0.51, p = 0.02; PASAT: n = 21, r = 0.54, p = 0.01) (Fig. 1E, F). The scores on verbal memory tests were, conversely, not significantly related to amyloid- β contents (SRT-LTS: n = 21, r = - 0.08, p = 0.61; SRT-CLTR: n = 21, r = - 0.11, p = 0.58).

None of the neuropsychological measurements correlated to tau CSF levels in MS patients (n = 21, p = NS for all parameters, not shown).

Since pathology of corpus callosum (CC) may play a major role in cognitive impairment of MS patients (Ozturk et al, 2010), we further compared scores in all neuropsychological tests between patients showing lesions within the CC (n=6) and patients without CC lesions (n=15) at conventional MRI. Comparison of neuropsychological scores between CIS and RRMS was also performed. No significant differences emerged between groups (n = 21, all p = NS) (not shown).

Correlation between amyloid- β_{1-42} and total tau protein with Gd+ lesions in MS patients

Upon grouping of MS patients according to the presence of Gd+ lesions at the MRI (Gd+ MS group: n = 22 (14 patients complaining new symptoms since 10 days or less), Gd- MS group: n = 20 (4 patients with new symptoms since 10 days or less), control group: n = 12, F = 7.98, p = 0.0009), amyloid- β_{1-42} levels were lower in Gd+ MS patients (209±26 pg/ml) than in both Gd- MS patients (331±25 pg/ml, p < 0.05) and in controls (390±45 pg/ml, p < 0.01) (Fig. 2A). No significant alteration of tau protein CSF content was revealed by grouping Gd- and Gd+ patients (Gd+ MS group: 129±11 pg/ml, Gd- MS group: 124±20 pg/ml, control group: 143±14 pg/ml F = 0.3, p = 0.74) (Fig. 2B).

These results might indicate that acute brain inflammation is associated with alterations of amyloid- β metabolism, leading to decreased CSF detection of this biologically active peptide. We further explored this possibility by correlating both amyloid- β_{1-42} and tau protein levels with the number of Gd+ lesions seen at the MRI. These analyses uncovered a significant negative correlation between amyloid- β_{1-42} content and Gd+ lesions (n = 22, r = - 0.45, p = 0.03) (Fig. 2C), while no correlation was seen between amyloid- β_{1-42} and total lesion load in T2 MRI sequences (n = 42, r = 0.06, p = 0.69) (Fig. 2D). No correlation was also found between tau levels in the CSF of MS patients and both Gd+ lesions (n = 22, r = - 0.21, p = 0.34) and T2 lesion load (n = 42, r = - 0.08, p = 0.57) (Fig. 2E,F).

Correlation between TBS-induced LTP with amyloid- β_{1-42} and total tau levels in MS patients

Amyloid- β dimers isolated from AD brains completely block LTP induction in the rodent hippocampus, and consequently cause cognitive defects (Shankar *et al*, 2008). In an attempt to see if also in MS brains amyloid- β_{1-42} is able to alter LTP induction, we explored whether any correlation could be uncovered between CSF content of amyloid- β_{1-42} and the magnitude of LTP induced in MS patients with TBS. A significant positive correlation was found between the

magnitude of LTP measured 15 min after the TBS protocol and the levels of amyloid- β_{1-42} in the CSF ($r = 0.48$, $p = 0.007$), a finding which may suggest that also in MS, as in AD, reduced CSF contents of amyloid- β_{1-42} may be associated with defective memory-related synaptic plasticity (Fig. 3A,B).

Correlation between neuropsychological assessments and TBS- induced LTP

To investigate whether cognitive impairment was associated with reduced cortical LTP in MS subjects, we compared the mean percentage values of peak to peak MEP amplitudes before and after TBS in CI and CP MS patients. A repeated measures ANOVA with TIME (3 levels: baseline, 0 and 15 min post TBS) as within subjects and GROUP (2 levels: CI and CP) as between subjects main factors showed a significant effect of time ($F = 8.04$, $p = 0.002$), a significant effect of GROUP ($F = 6.82$, $p = 0.023$) and a significant TIME x GROUP interaction ($F = 5.96$, $p = 0.031$). At post-hoc contrasts CI patients showed a lower effect of TBS at both timepoints (0 minutes post TBS: 1.09 ± 0.14 ; 15 minutes post: 1.18 ± 0.12 ; all $p < 0.05$) compared to CP patients (0 minutes post TBS: 1.67 ± 0.16 ; 15 minutes post: 1.71 ± 0.16) (Fig. 4). We also performed a T-Test for independent data on the peak effect of TBS represented by the post TBS time-point with the highest mean MEP amplitude. A significantly lower peak effect of TBS in CI ($n = 10$, mean \pm SE = 1.39 ± 0.15) compared to CP ($n = 10$, mean \pm SE = 2.32 ± 0.23) MS patients ($p = 0.008$) was observed (not shown). These findings, showing a lower effect of TBS in CI patients, are in line with previous observations that rTMS has an altered effect in AD patients (Inghilleri *et al*, 2006; Battaglia *et al*, 2007) and show for the first time that cognitive impairment in MS patients is paralleled by an alteration of NMDAR dependent synaptic plasticity.

Comparison of TBS effect between CIS, RRMS and PPMS did not show any significant difference.

Role of amyloid- β_{1-42} in the modulation of excitatory and inhibitory synaptic transmission in MS

LTP is a complex phenomenon, and changes in glutamate-mediated synaptic drive or GABA-mediated inhibition alter its induction. Accordingly, blockade of NMDA glutamate receptors prevents LTP (Bliss and Collingridge, 1993; Cooke and Bliss, 2006), as also does the enhancement of GABA-mediated inhibition (Matsuyama *et al*, 2008; Gong *et al*, 2009; Pan *et al*, 2009). Thus, we tried to investigate whether amyloid- β_{1-42} -mediated inhibition of LTP could be secondary to impaired basal synaptic transmission in cortical neurons of MS patients. In ppTMS experiments, however, we found that the degree of SICI, ICF, SICF, and LICI was not related to the CSF levels of amyloid- β_{1-42} in MS patients, suggesting that this protein affects synaptic plasticity but not basal excitatory or inhibitory transmission (Fig. 5). Comparisons between CI and CP MS patients also did not reveal any significant difference (not shown).

DISCUSSION

Grey matter pathology, brain atrophy, and loss of anatomical connectivity are the most accredited determinants of progressive cognitive decline in MS (Calabrese *et al*, 2009, Calabrese *et al*, 2010; Staff *et al*, 2009; Tiemann *et al*, 2009). Acute and reversible mental deterioration also occurs during clinical relapses, independently of the primary functional system involved (Foong *et al*, 1998). The presence of Gd⁺ lesions at the brain MRI also correlates with poor cognitive performance in asymptomatic and symptomatic MS patients (Foong *et al*, 1998; Bellmann-Strobl *et al*, 2009), suggesting that focal inflammation is able to diffusely disrupt the neuronal substrate of information processing, leading to acute cognitive impairment. Given that amyloid- β_{1-42} metabolism is regulated by inflammation (Griffin *et al*, 2006; Hickman *et al*, 2008; Schmidt *et al*, 2008) a recent study performed in a large sample of subjects, reported that in MS patients there is an overall decrease of CSF amyloid- β_{1-42} in comparison with healthy controls (Mattsson *et al*, 2009). These results seem to indicate that in MS there could be an altered expression of amyloid- β_{1-42} , possibly related to the underlying inflammatory processes.

The present study shows that during the inflammatory phase of MS, amyloid- β_{1-42} metabolism is altered leading to its reduced detection in the CSF. We found, in fact, that amyloid- β_{1-42} levels were significantly lower in subjects with Gd⁺ lesions at the MRI. Interestingly, these findings are in good agreement with previous studies showing that IL-1 β , an important pro-inflammatory cytokine, alters amyloid- β metabolism in the cerebral cortex, increasing APP levels in rat brain homogenate (Fan *et al*, 2009). There is no information, conversely, about IL-1 β effect on CSF amyloid β_{1-42} levels. However, inflammation increases BACE activity (Hong *et al*, 2003), and BACE activity levels were found to be higher in patients with low amyloid β_{1-42} levels in the CSF (Mulder *et al*, 2010). Anti-inflammatory cytokines, on the other hand, enhance the activity of amyloid- β degrading enzymes, thus favouring amyloid- β tissue deposit clearance (Shimizu *et al*, 2008).

The present study shows, for the first time, a difference in cognitive profile of MS patients related to CSF levels of amyloid- β_{1-42} . Interestingly, the cognitive domains that seemed to be affected in association with low amyloid- β CSF levels were those regarding attention, concentration and information-processing speed, the same influenced by the presence of radiological disease activity (Bellmann-Strobl *et al*, 2009). Gd enhancement has been associated with poor PASAT performance in otherwise physically stable MS patients (Bellmann-Strobl *et al*, 2009) and to CSF amyloid- β_{1-42} levels (present work), in line with the idea that CSF amyloid- β_{1-42} expression, altered by brain inflammation, may cause a diffuse impairment of cerebral connectivity with a negative impact on cognitive functioning. Conversely, neuropsychological test scores examining memory did not correlate with amyloid- β_{1-42} levels.

Cortical atrophy was not measured in the present study since imaging was performed for clinical purposes, thus only conventional MRI scans were collected. However CSF tau levels, already reported to be associated with neurodegenerative damage (Vemuri *et al*, 2009; Ghoshal *et al*, 2002) were found to be normal in our patients. A further limitation of the present study may be represented by anxiety, due to diagnosis communication or expectancy for the disease. Anxiety is

known to influence the performance in the neuropsychological tests (Peretti, 1998; Tombaugh, 2006), especially those addressing concentration and attention. However, all participants were diagnosed as suffering from CIS or MS during hospitalization, and diagnosis was communicated after all data collection.

The synaptotoxic properties of amyloid- β_{1-42} have long been recognized, as well as its ability to interfere with LTP and with cognitive abilities (Klyubin *et al*, 2005; Battaglia *et al*, 2007; Shankar *et al*, 2008; Nygaard *et al*, 2009; Walther *et al*, 2009; Townsend *et al*, 2010). A recent study, reported that amyloid- β dimers isolated from AD patients can impair hippocampal LTP and memory in mice, and induce dendritic spine retraction in neurons (Shankar *et al*, 2008). Of note, while the role of amyloid- β in LTP disruption has been demonstrated in a variety of experimental models of AD (Klyubin *et al*, 2005; Battaglia *et al*, 2007; Shankar *et al*, 2008; Walther *et al*, 2009; Townsend *et al*, 2010), its correlates with LTP measured in humans have never been demonstrated before. Thus, the correlation between amyloid- β_{1-42} CSF levels and LTP amplitude reported in the present study is a first indication that amyloid- β is a potent regulator of synaptic plasticity also in the human brain, with potentially relevant consequences for understanding the mechanisms of cognitive deficits not only in MS subjects but also in other neurological disorders, obviously including AD. Importantly, although both altered cognitive performances and LTP induction was found in mice overexpressing tau protein (Polydoro *et al*, 2009), tau levels did not correlate with TBS-induced LTP amplitude in MS patients (Fig. 3C,D). In this respect, it is noteworthy that only LTP induced by high-frequency stimulation but not that induced by TBS was altered in mice overexpressing tau (Polydoro *et al*, 2009).

Previous investigations found that in MS patients SICI was lower in secondary progressive MS (SPMS) in comparison with RRMS patients in the remitting phase, and that SICI correlated significantly with EDSS scores (Conte *et al*, 2009). These results are in apparent contrast with our findings. However our study group did not include patients with SPMS and the mean age and disease duration of these patients (age: 51 ± 0 , disease duration: 21.0 ± 2) are also very different in

comparison with our study group (age: 34.3 ± 1.4 years, disease duration: 2.7 ± 0.8 years). Other investigators found that fatigue in MS is associated with lower SICI compared to MS patients without fatigue and healthy subjects (Liepert *et al*, 2005). In our study group only very few patients complained fatigue and also in this case our group differed in terms of mean age (41.4 ± 4.75 years). Moreover one of the inclusion criteria in the study from Liepert was that patients had to be in the remitting phase of the disease, thus presumably also had longer disease durations. Finally, a similar reduction of SICI was found in the relapsing phase of the disease in comparison with remitting patients in a study by Caramia *et al* (2004). Also in this case the study group had longer disease duration (mean 4.29 ± 3.26 years), included only patients with a definite diagnosis of RRMS. Thus differences in the results may be explained by the different clinical characteristics of the samples studied.

A recent investigation based on a different TMS protocol to induce neuroplastic changes, suggested that motor plasticity in MS patients is comparable to that found in healthy subjects (Zeller *et al*, 2010). Neuroplasticity in this study was however assessed only in patients in the remitting phase of the disease, with a stable clinical condition, while the alterations observed in our study were evident in patients with cognitive impairment and presence of Gd⁺ lesions.

The reduction in CSF amyloid- β_{1-42} in AD has been postulated to reflect deposition of the aggregated insoluble fibrillar amyloid- β peptides in senile plaque, with lower levels of diffusion into the CSF. A limitation of the present study is that we do not provide any evidence of amyloid- β tissue deposition in our patients. We cannot argue, therefore, that the decrease in amyloid- β_{1-42} CSF levels observed is consequent to tissue deposition. However, AD neuropathology apart from amyloid- β deposition includes variable amounts of soluble species that show much stronger correlations with severity of dementia than fibrillar amyloid- β (Terry 1996; Lue *et al*, 1999; McLean *et al*, 1999; Gandy *et al*, 2010). Synthetic amyloid- β dimers are extremely potent inhibitors of synaptic plasticity both in vivo and in vitro whereas even relatively high concentrations of amyloid- β monomers are inactive (Selkoe, 2008; Hu *et al*, 2008; Shankar *et al*, 2008). Moreover,

human ex vivo samples of CSF that contain amyloid- β oligomers but not monomers, potentially inhibit LTP (Klyubin *et al*, 2005). The lowering of natively measured amyloid- β_{1-42} in the CSF of our patients may be consequent to oligomerization as observed in AD (Englund *et al*, 2009). Finally a transgenic mice, overexpressing APP^{E693Q} develops learning and memory deficits in association with formation of amyloid beta oligomers without generating amyloid plaques in their brains at any age (Gandy *et al*, 2010). These observations, even though only at a speculative stage, could help explaining the findings of our iTBS experiments. Another possibility is that the observed findings could be related to inflammation-induced downregulation of β -site APP cleavage. Accordingly, BACE activity is modulated by inflammation (Mattsson *et al*, 2009), and is also involved in the promotion of myelination by oligodendrocytes (Hu *et al*, 2006).

Nevertheless, the mechanism by which amyloid- β_{1-42} is reduced during inflammation in MS remains unclear and further studies are needed to better clarify this issue.

DISCLOSURE/CONFLICT OF INTERESTS

The authors have no financial interests or conflicts of interest to disclose.

ACKNOWLEDGEMENTS

This investigation was supported by the Italian National Ministero dell'Università e della Ricerca to DC, by the Italian National Ministero della Salute, and by Fondazione TERCAS to DC.

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FIGURE LEGENDS

Figure 1. CSF contents of amyloid- β_{1-42} are reduced in MS patients and correlate with their cognitive functions. A. Amyloid- β_{1-42} levels were significantly reduced in the CSF of MS subjects, compared to control individuals. B. Tau protein levels were not altered in the CSF of MS patients. C. In a subgroup of 21 MS patients, who gave consent to the Neuropsychological assessment, that, amyloid- β_{1-42} levels were lower in cognitively impaired (CI) MS patients than in both cognitively preserved (CP) MS patients and controls. D. Tau protein CSF levels were normal also in CI MS patients. E. Correlation plot between amyloid- β_{1-42} CSF levels and PASAT score in MS subjects. F. Correlation plot between amyloid- β_{1-42} CSF levels and SDMT in MS subjects. In this figure and in the following ones, amyloid- β_{1-42} is abbreviated in “amyloid- β ”

* = $p < 0.05$ versus control ** = $p < 0.01$ versus control # = $p < 0.05$ versus CP MS.

PASAT = Paced Auditory Serial Addition Test; SDMT = Symbol Digit Modalities Test.

Figure 2. Disease activity at MRI correlates with reduction of amyloid- β_{1-42} levels in the CSF of MS subjects. A. The graph shows that, upon grouping of MS patients according to the presence of Gd+ lesions, amyloid- β_{1-42} levels were lower in Gd+ MS subjects than in both Gd- MS subjects and controls. B. Tau protein CSF levels were normal also in Gd+ MS patients. C. Correlation plot between amyloid- β_{1-42} CSF levels and the number of Gd+ lesions in Gd+ MS subjects. D. Correlation plot between amyloid- β_{1-42} CSF levels and T2 lesion volume in MS subjects. E,F. Correlation plots between tau CSF levels and the number of Gd+ lesions (E) or T2 lesion volume in MS subjects (F).

** = $p < 0.01$ versus control # = $p < 0.05$ versus Gd- MS

Gd+ = Gadolinium-enhancing; Gd- = non Gadolinium enhancing.

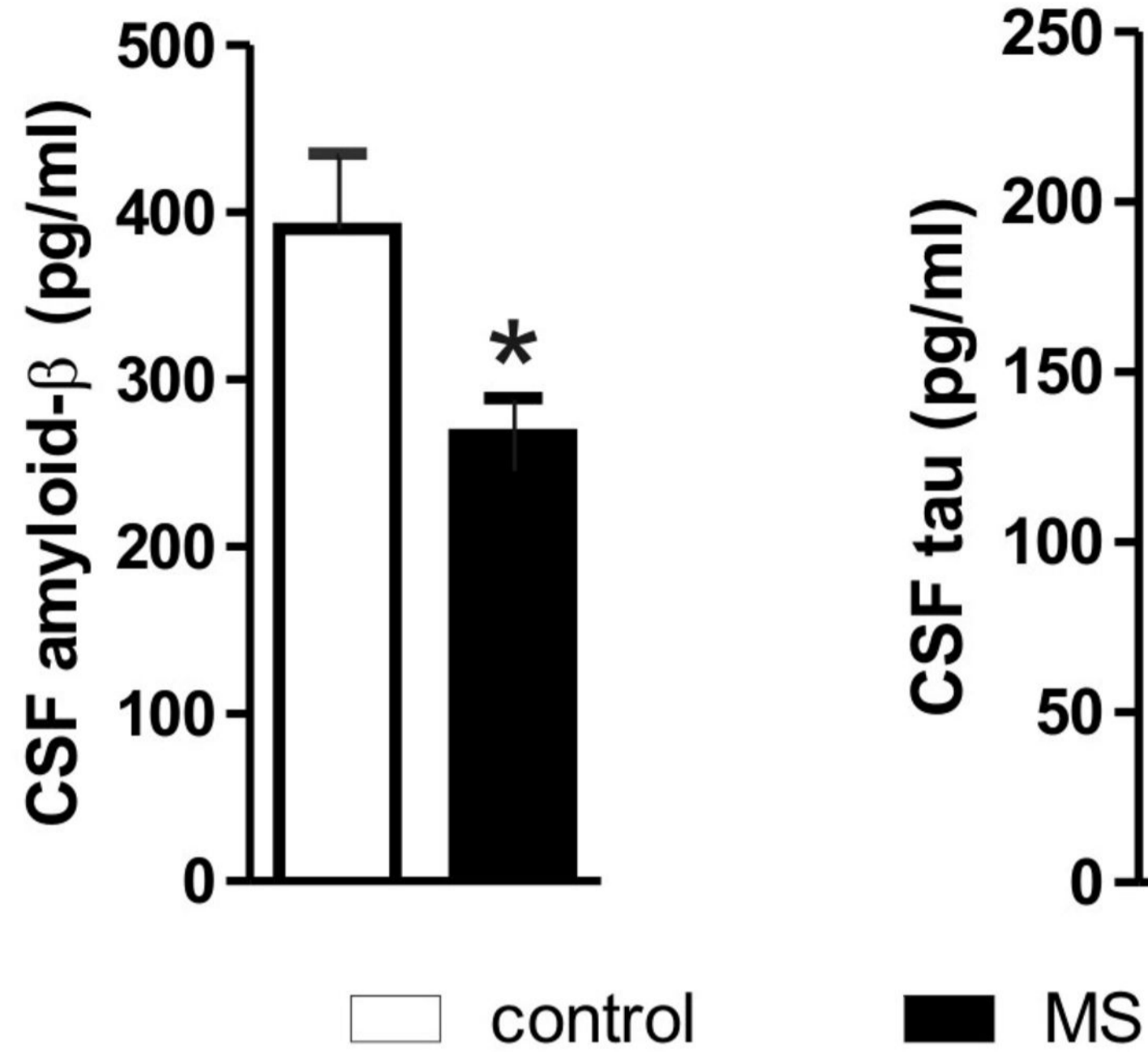
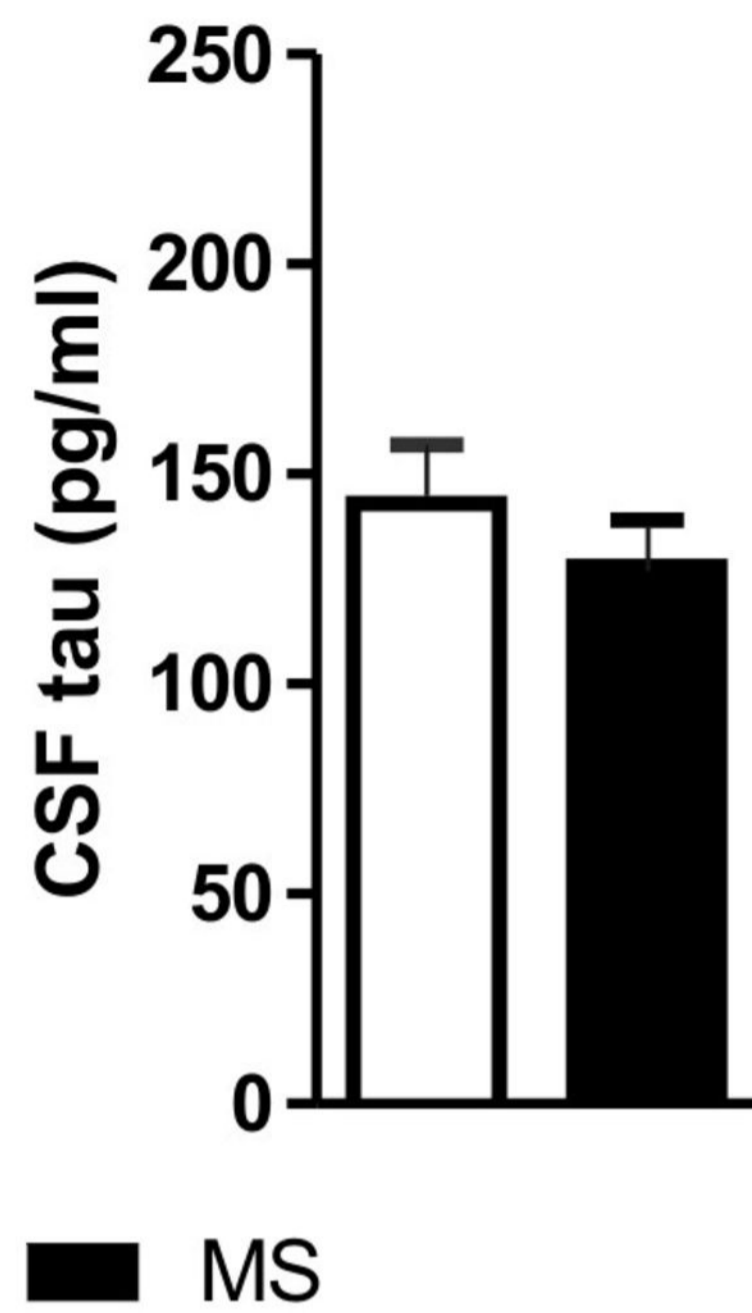
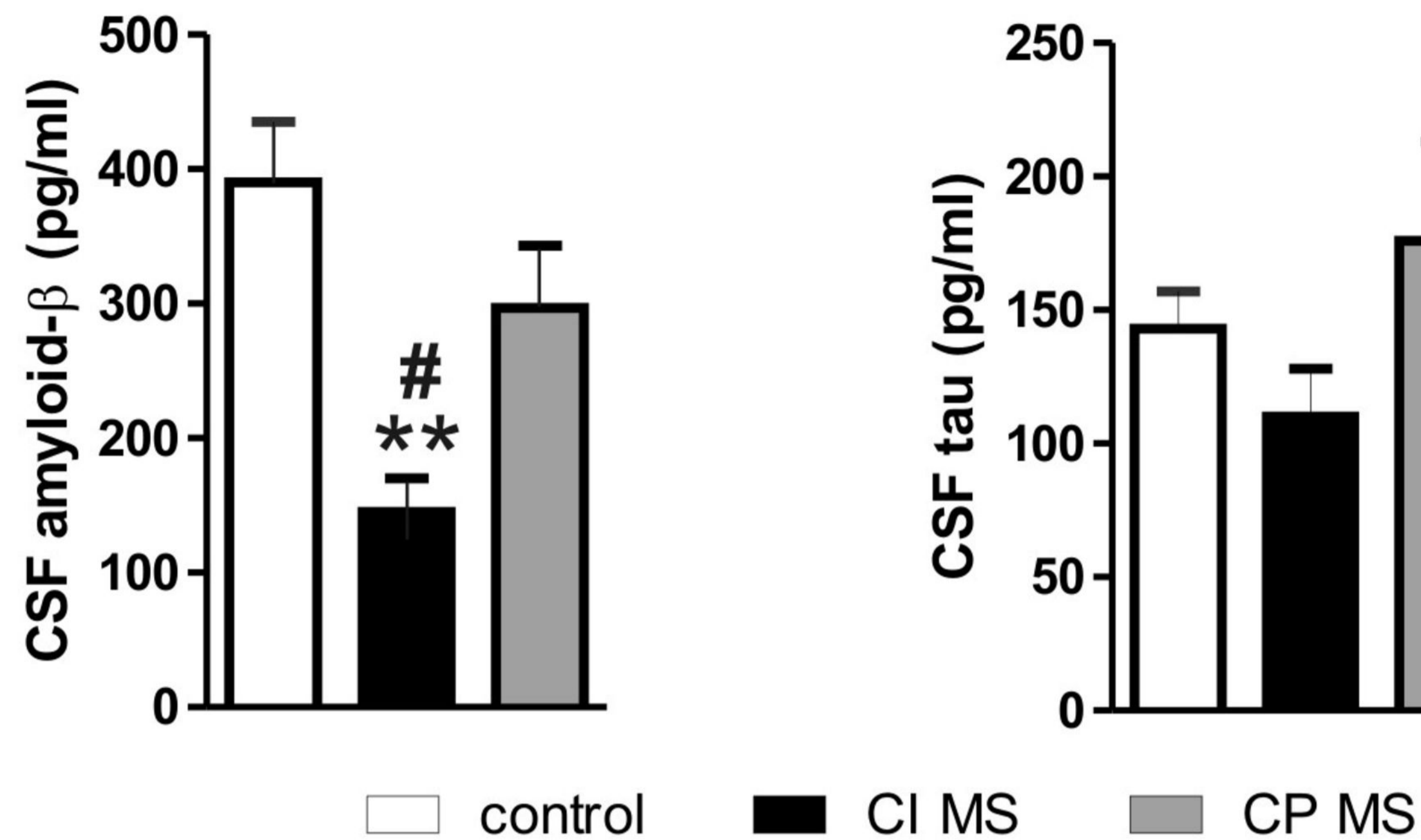
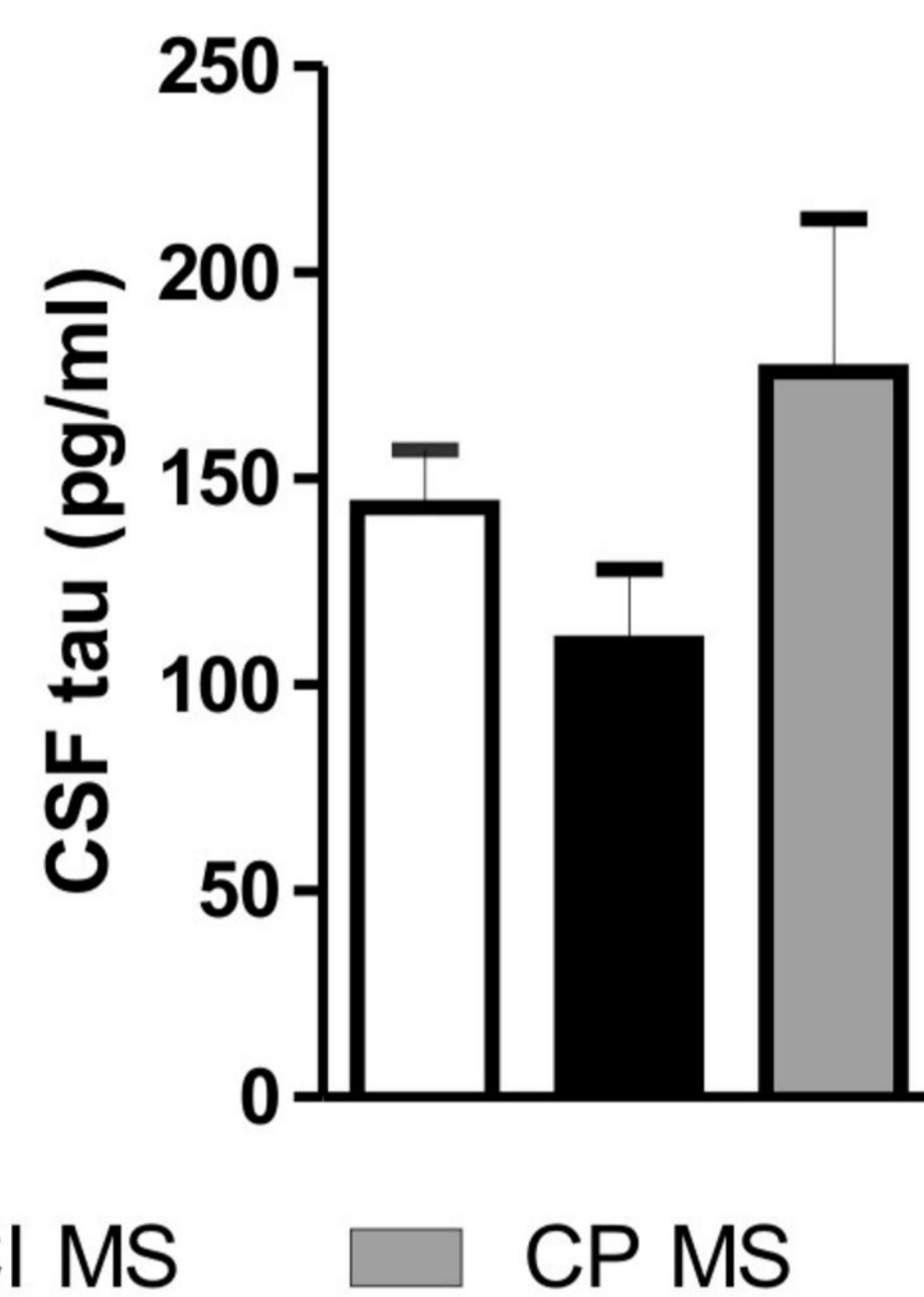
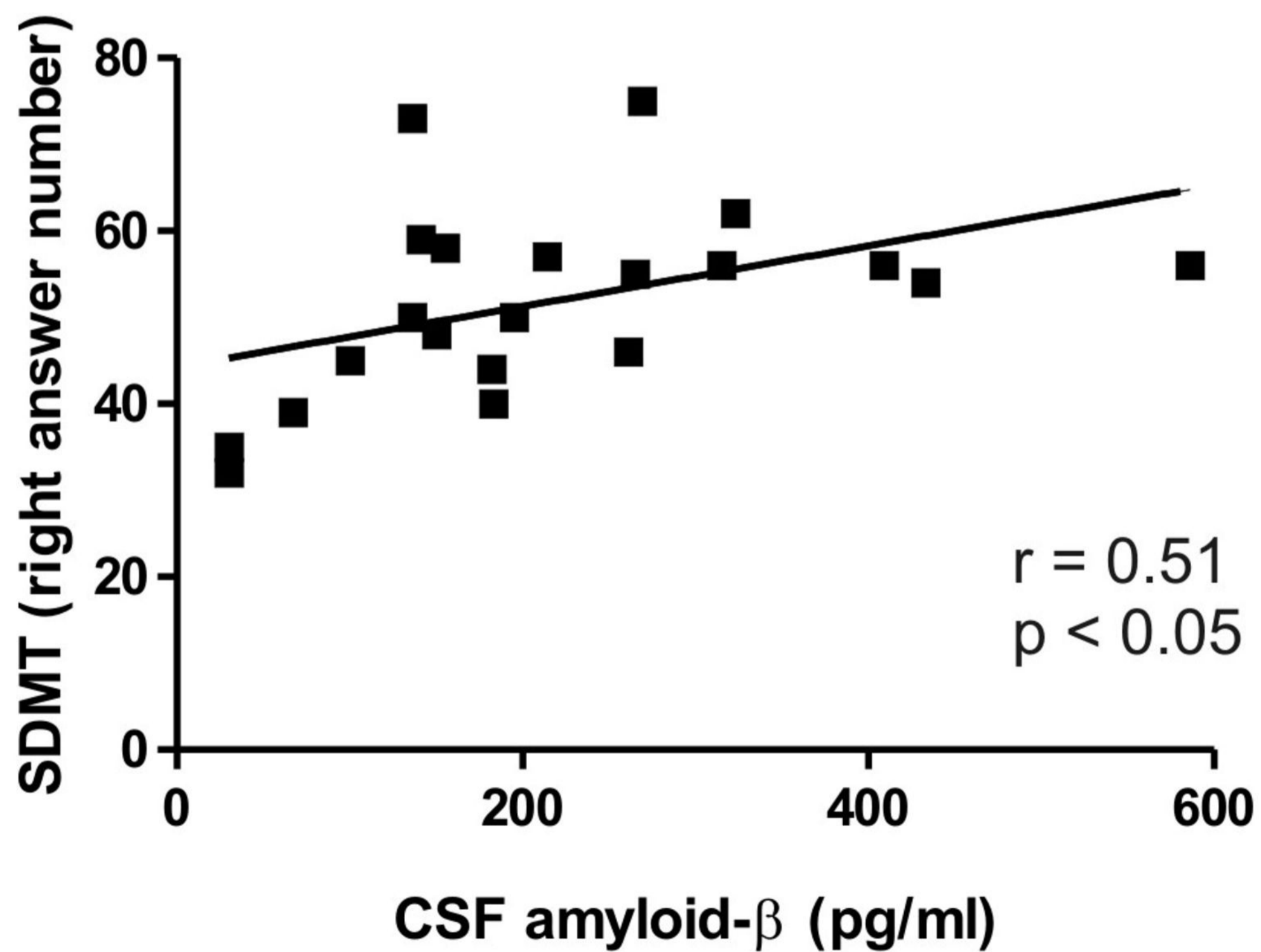
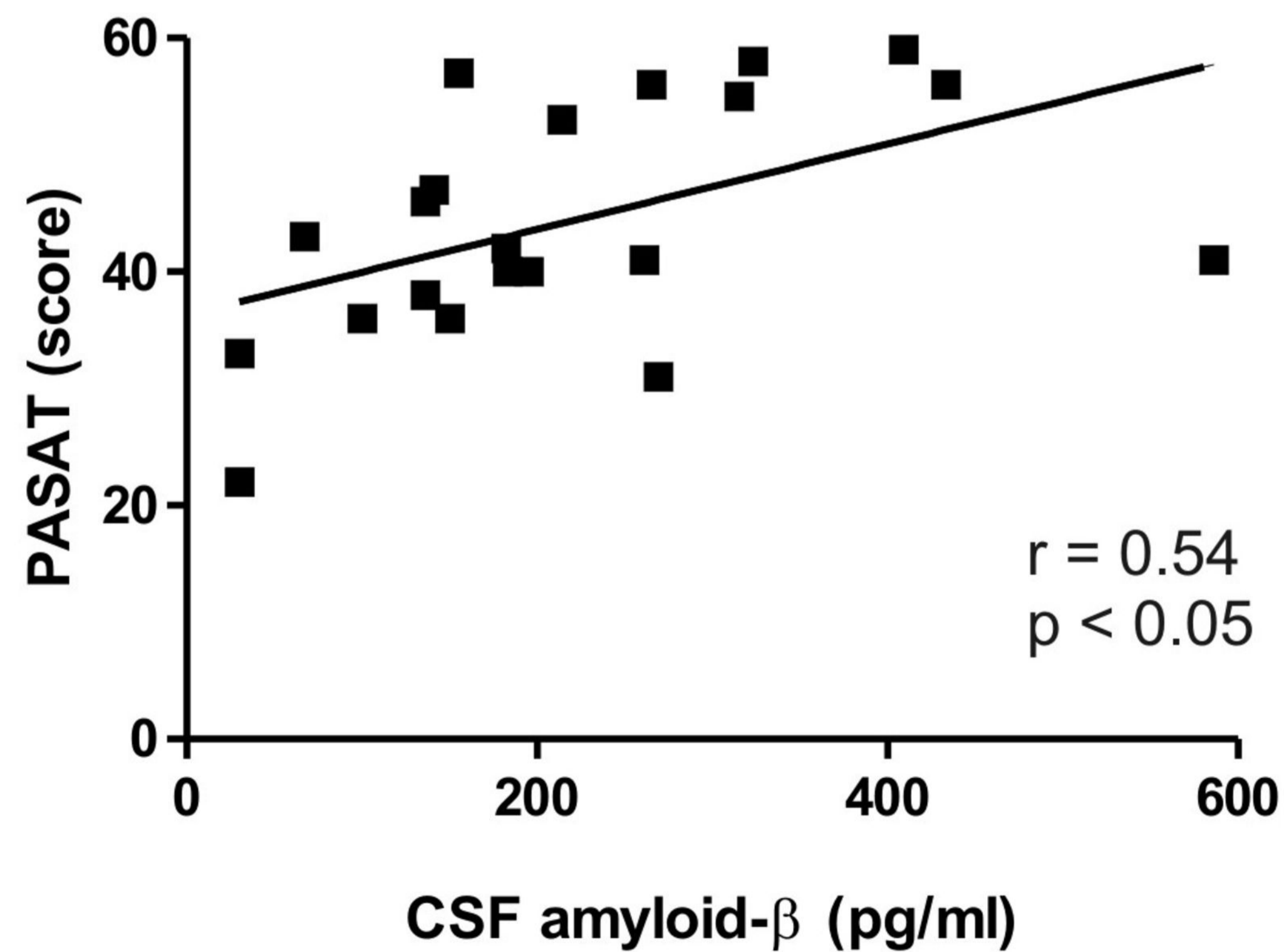
Figure 3. TBS induced LTP magnitude correlates with amyloid- β_{1-42} levels in the CSF of MS subjects. A. Correlation plot between amyloid- β_{1-42} CSF levels and MEP amplitude changes, expressed as percentage of baseline, immediately after TBS, and B) 15 minutes after TBS. C, D. Correlation plots between tau CSF levels and TBS-induced LTP.

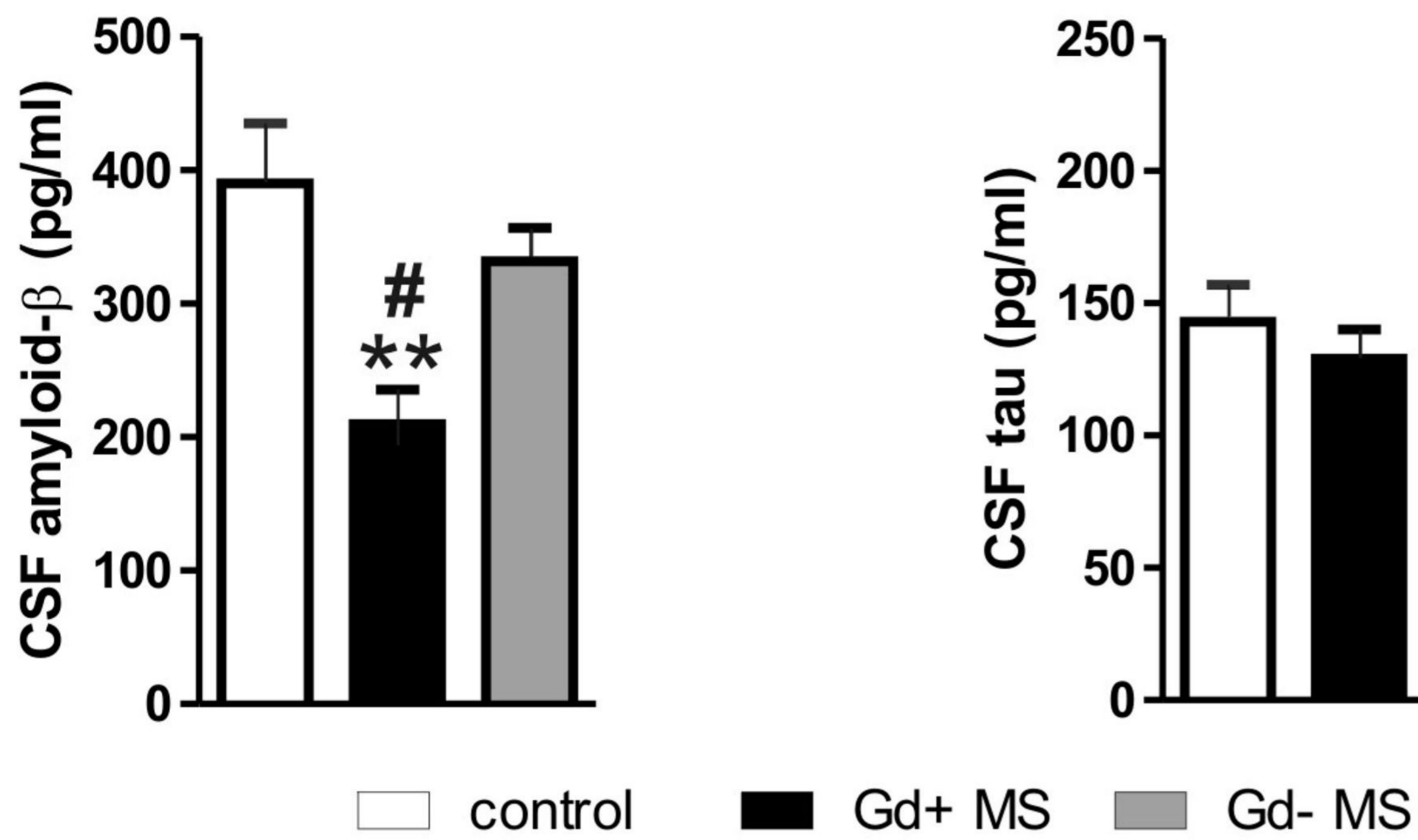
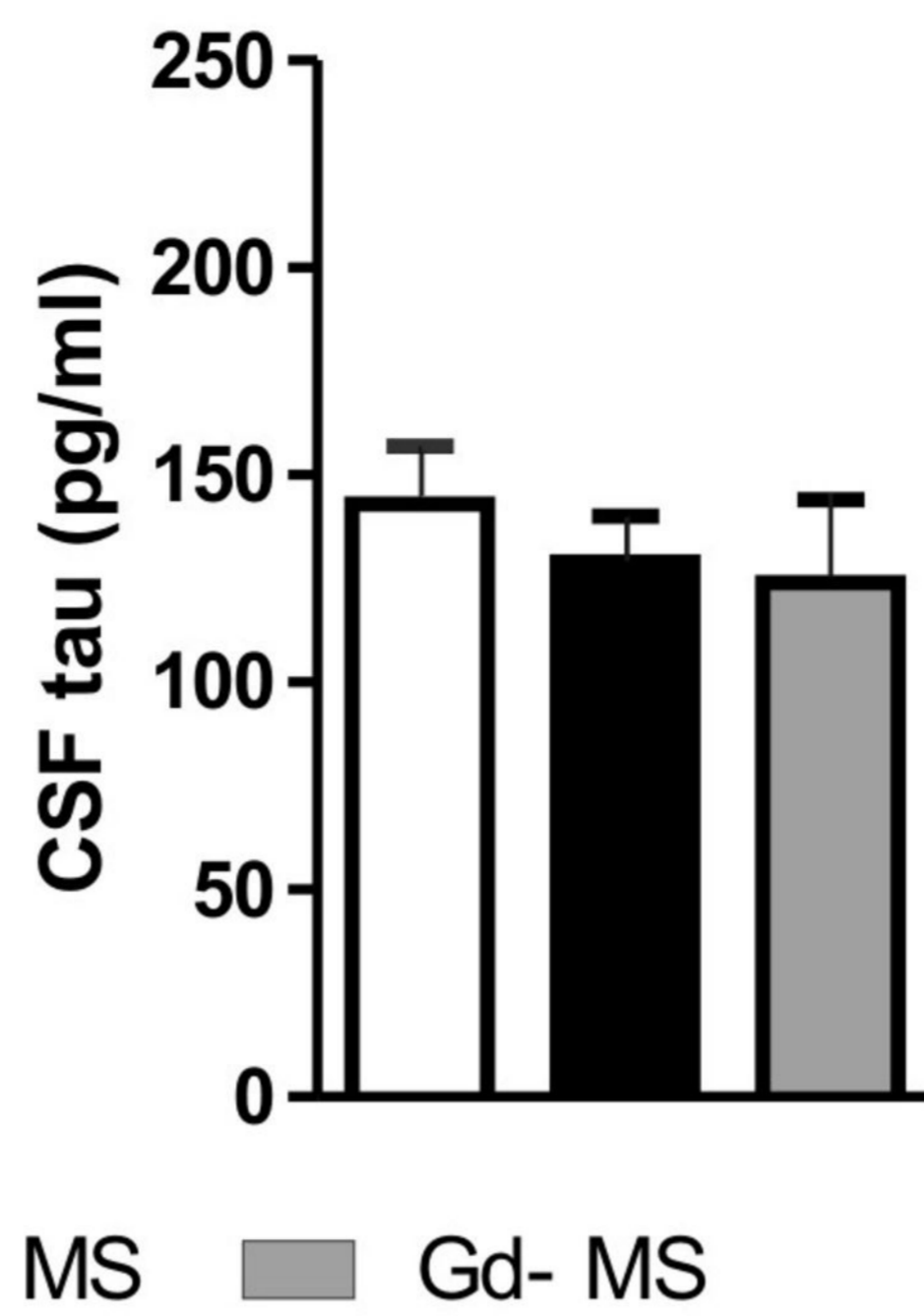
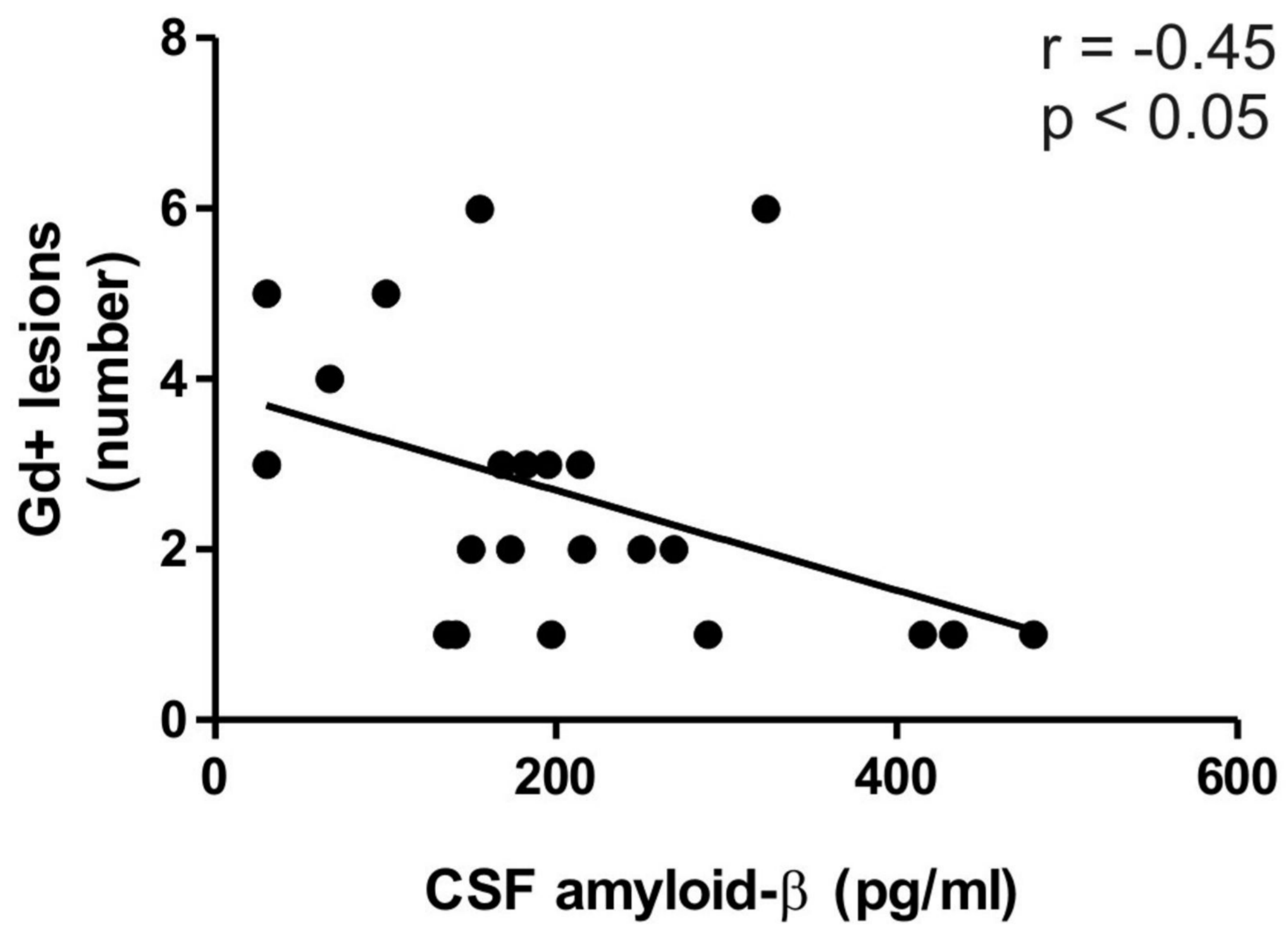
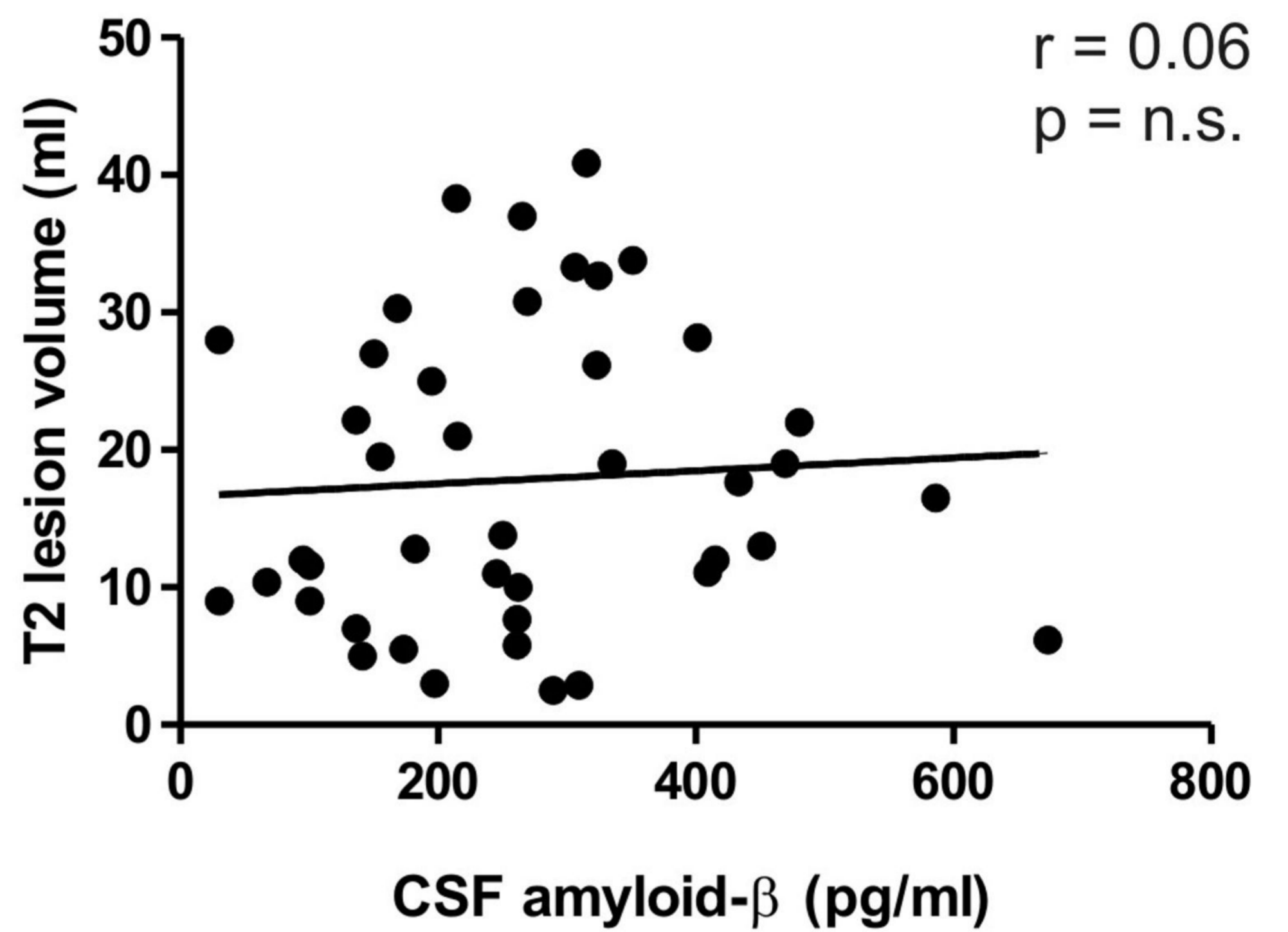
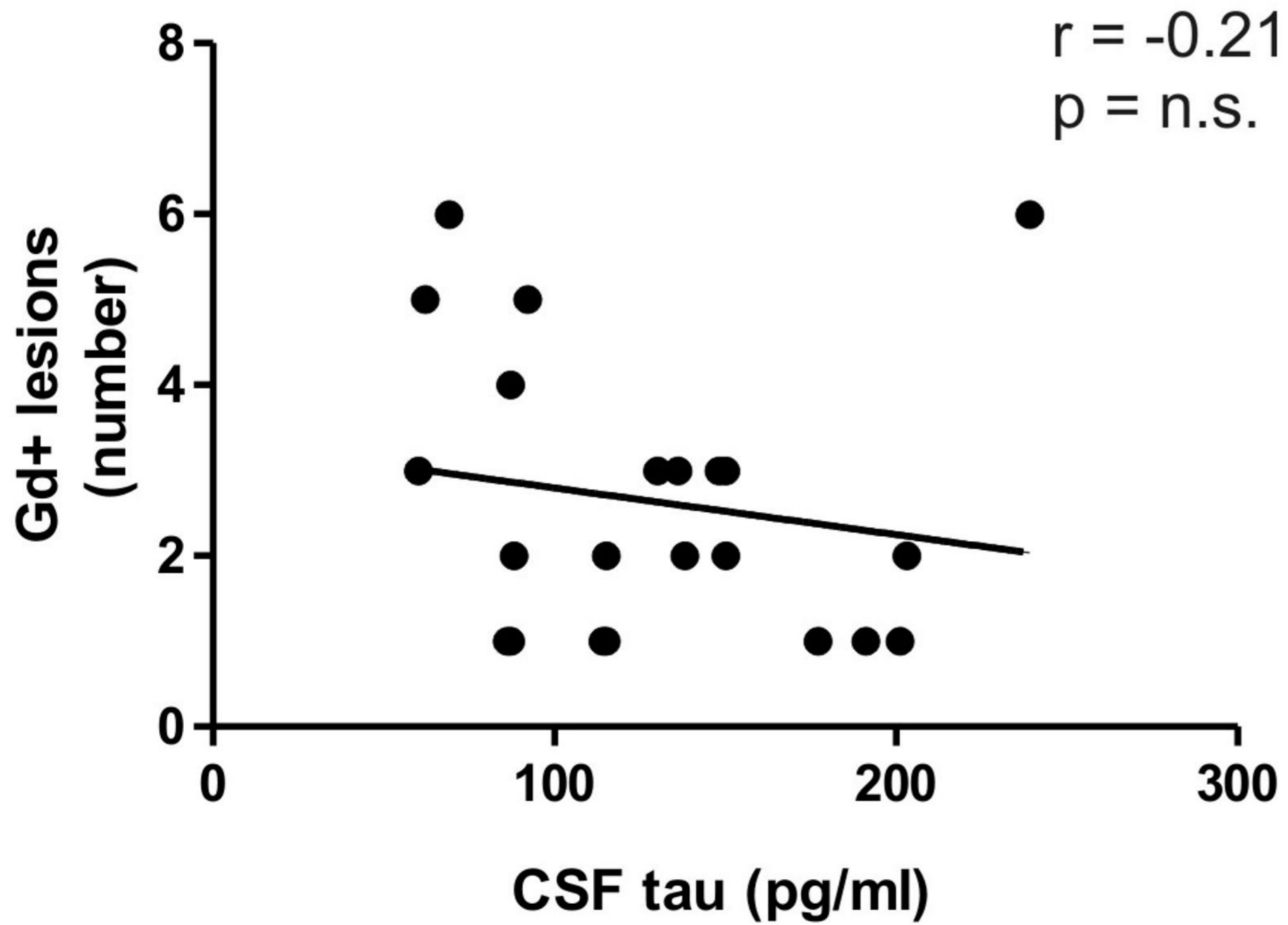
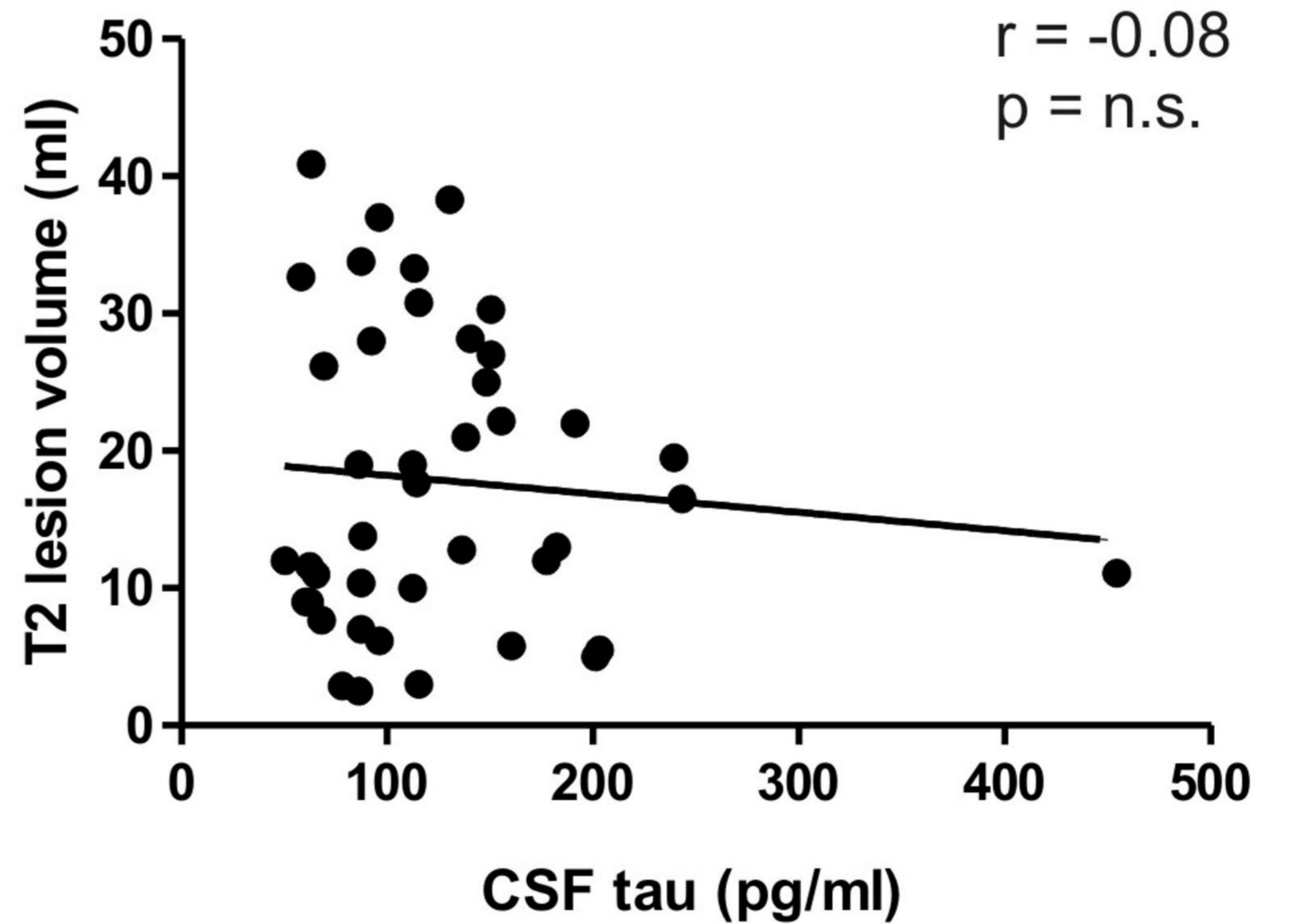
LTP = long term potentiation; TBS = theta burst stimulation.

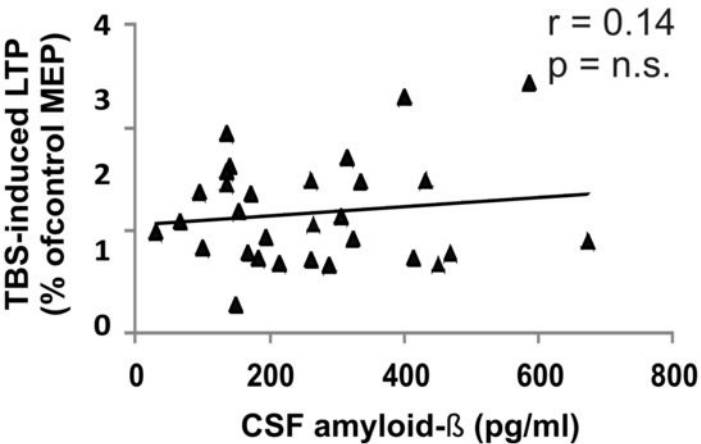
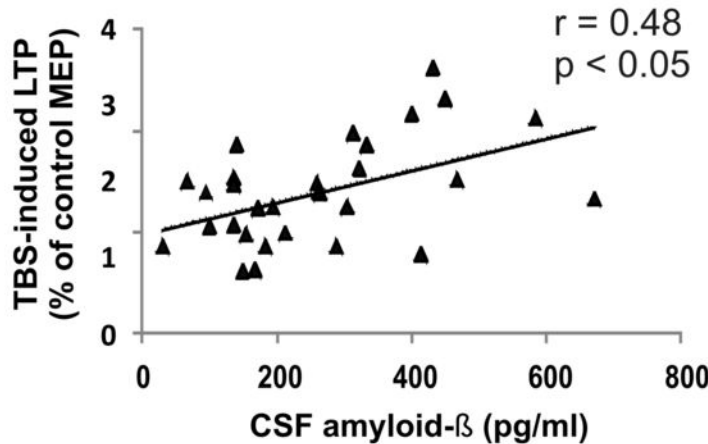
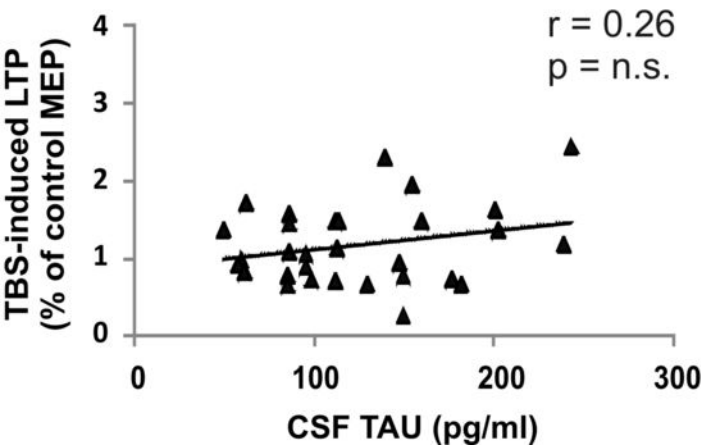
Figure 4. TBS induced LTP differs between cognitively impaired and cognitively preserved MS patients. Differences between the two groups were observed at both 0 and 15 minutes after TBS time-points. * = $p < 0.05$

Figure 5. Correlation plots between amyloid- β_{1-42} CSF levels and MEP amplitude changes, expressed as percentage of baseline, induced by A) SICI at 2 ms ISI, B) ICF at 10 ms ISI, C) LICI at 100 ms ISI, D) SICF at 1.5 ms ISI and E) at 2.7 ms ISI.

MEP = motor evoked potential; SICI = short interval intracortical inhibition; ISI = interstimulus interval; ICF = intracortical facilitation; LICI = long interval intracortical inhibition; SICF = short intracortical facilitation.

A**B****C****D****E****F**

A**B****C****D****E****F**

A**0 min after iTBS****B****15 min after iTBS****C****0 min after iTBS****D****15 min after iTBS**