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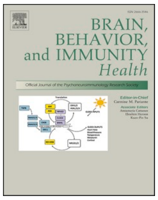

An inflammatory fingerprint in mild cognitively impaired patients is reversed by physical and cognitive training

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ABSTRACT

Background: Alzheimer's disease (AD) is a major global health concern, with number of affected individuals expected to rise to 139 million by 2050. Lifestyle factors play a significant role in modulating cognitive decline, and multidomain interventions have demonstrated effectiveness in improving outcomes for populations at risk. The Train the Brain (TTB) program—a combined physical and cognitive training delivered in a social setting—has previously demonstrated cognitive benefits within 7 months. However, the underlying biological mechanisms remain unclear. Given the role of inflammation in aging and neurodegeneration, we investigated whether specific immune biomarkers reflect the efficacy of this intervention.

Methods: We enrolled 76 individuals with Mild Cognitive Impairment (MCI) aged 65–80, into the TTB program. Participants underwent neurological assessment, MRI and blood sampling at baseline and after the intervention. Plasma levels of a comprehensive panel of immune – related biomarkers were measured through Proquantum and ELLA platforms.

Results: At baseline, MCI participants displayed elevated levels of IL-17A, CX3CL1, CCL11, with a borderline increase of IL-6 and TNFα. Following the TTB intervention, we observed reductions in IL-6, IL-17A, TNFα, and CCL11 levels. In contrast, anti–inflammatory cytokines (IL-10, TGFβ, IL-4) and BDNF declined in control group but were maintained or increased in the intervention group.

Conclusion: The TTB intervention not only improved cognitive and physical outcomes but also modulated key immune markers associated with neuroinflammation and aging. IL-10, in particular, emerged as potential peripheral biomarker of training efficacy. These findings support the utility of immune profiling in monitoring response to multidomain interventions and guiding personalized strategies for cognitive risk reduction.

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1. Background

Alzheimer's disease (AD) represents one of the most relevant health and social problems of the last century, with an estimated 315 million people globally at risk (Gustavsson et al., 2023). While genetic predisposition plays a role (Livingston et al., 2020), there is growing recognition of the importance of modifiable environmental and lifestyle factors in influencing cognitive trajectories and dementia risk (Salzman et al., 2022; Frisoni et al., 2023).

Over the past two decades, multidomain lifestyle interventions have emerged as promising strategies to enhance cognitive function and delay cognitive decline in older adults. Interventions targeting cognitive training have demonstrated improvements in executive function, memory, and processing speed in both healthy aging and at-risk populations, as shown in landmark trials such as ACTIVE (Smith et al., 2009) and IMPACT (Smith et al., 2009). Similarly, physical activity interventions, particularly aerobic and resistance training, have been consistently associated with improved cognitive outcomes and increased hippocampal volume, as shown in randomized controlled trials and meta-analyses (Northey et al., 2018; Kang et al., 2025; Lee et al., 2023; Fernandez-Gamez et al., 2025). The evidence for combined interventions, integrating cognitive and physical activity—often delivered in social or group formats—is more recent but it is constantly growing, with several studies indicating synergistic effects that surpass the benefits of single-modality interventions (Ngandu et al., 2015a; Gavelin et al., 2021; Rieker et al., 2022; Vásquez-Carrasco et al., 2025).

The Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) remains the most influential example of a large scale multidomain trial. Involving more than 1200 older adults at risk of dementia, FINGER demonstrated that a two-year intervention combining nutritional guidance, physical activity, cognitive training and social engagement, led to a 25 % improvement in cognitive performance with respect to the control group (Ngandu et al., 2015b; Wimo et al., 2023).

More recently, other multidomain trials, including MAPT, PreDIVA and GREAT, have shown mixed but generally supportive findings, contributing to an evolving consensus around the value of early, multifactorial intervention for brain health (Andrieu et al., 2017; van Charante et al., 2016; Clare et al., 2019; Vellas et al., 2014).

Building on this foundation, the Train the Brain (TTB) study, study evaluated a seven-month, socially enriched multidomain program combining physical and cognitive training in individuals aged 65–89 years. The intervention showed promising effects not only on cognition but also on neuroimaging biomarkers, including grey matter volume, cerebral blood flow, and BOLD signals in memory-related regions, suggesting that shorter, structured interventions may yield measurable neurobiological benefits even in individuals with mild cognitive impairment (MCI) (Train the Brain Consortium, 2017; Sale et al., 2023).

Despite this progress, important questions remain about the biological mechanisms underlying these interventions, particularly their impact on the immune system. Chronic low-grade inflammation, commonly referred to as neuro-inflammaging, has been implicated in the pathogenesis of AD and is characterized by dysregulated cytokine profiles detectable in both central and peripheral compartments (Franceschi et al., 2018; Soraci et al., 2024; Castro-Gomez and Heneka, 2024; Shen et al., 2019). Although previous studies have shown that physical exercise can reduce levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Nascimento et al., 2014; Souza et al., 2013; Collao et al., 2020; Gleeson et al., 2011; Cerqueira et al., 2020; Katsipis et al., 2024; Pesce et al., 2017), the immune effects of combined cognitive-physical training, especially in socially enriched contexts, remain poorly understood.

To address these gaps, we conducted a primary study using a novel cohort to replicate the TTB intervention and examine its effects on systemic inflammatory biomarkers. We analyzed a comprehensive panel of cytokines, chemokines, and related analytes associated with immune polarization and neurodegeneration, aiming to explore their potential as biomarkers of cognitive intervention efficacy.

The objectives of this study were twofold: (1) to determine whether blood-based inflammatory biomarkers can differentiate individuals with MCI from cognitively healthy controls, and (2) to assess whether these markers are modulated by a combined physical and cognitive training program. We hypothesized that the intervention would modulate pro-inflammatory and anti-inflammatory signals and that changes in specific biomarker patterns would reflect training responsiveness, providing insight into the biological underpinnings of cognitive improvement.

2. Methods

2.1. Subjects

Subjects have been recruited from the general population through several channels: network of medical centers, self-referral of patients and their relatives who received information on the study from informative printed material (poster and flyers) set in pharmacies, associations, National Research Council of Pisa, Center for Cognitive Disorders and Dementia (CDCD) at the Neurology Unit (Azienda Ospedaliera Universitaria Pisa, AOUP) of Santa Chiara Hospital, Pisa (Italy). Participants who met the inclusion and exclusion criteria were enrolled in the study at CDCD. The eligible population included elderly subjects aged between 65 and 80 years, with at least five years of education, and diagnosed with MCI, single-domain and multi-domain, confirmed at the neurological examination according to the current guidelines (Croisile et al., 2012; Albert et al., 2011) or "Healthy" subjects (normal cognitive functions). Exclusion criteria included neurological and cognitive disorders (e.g., moderate or severe dementia, epilepsy, sensory-motor deficits), psychiatric conditions (e.g., depression, drug addiction), severe cardiovascular conditions (e.g., aortic stenosis, hypertrophic cardiomyopathy, Leriche-Fontaine arteriopathy), respiratory and renal diseases (e.g., COPD, severe chronic renal insufficiency), uncontrolled type II diabetes, advanced cancer, recent cranial trauma, and orthopedic or musculoskeletal issues preventing participation in cognitive or physical training programs. All participants gave written informed consent. Study design and protocol, including subject privacy and sensitive data treatment, have been approved by the Regional Ethical Committee for Clinical Experimentation and all methods were carried out in accordance with the guidelines laid down in the 1975 Declaration of Helsinki. Recruitment has been performed between August 2018 and May 2022. The protocol was approved by the Local Ethics Committee, letter n. 40130, 2018/08/01. Subjects diagnosed with MCI and matching inclusion criteria underwent baseline evaluation (MRI and blood/-plasma evaluation).

2.2. Randomization and masking

MCI subjects were assigned to the training group (MCI-training) or to the no-training group (MCI-no training) in a ratio of 1:1, through a randomization procedure using a computer-generated randomization sequence. Randomization was carried out after the evaluation at T0 by a statistician not involved in other project procedures, not having contact with the study participants. Project outcome evaluators were blinded to the allocation of participants, who were instructed not to disclose which group they belong to.

2.3. Cognitive assessment

For the MCI diagnosis, the diagnostic criteria proposed by the National Institute on Aging and the Alzheimer's Association were applied (Albert et al., 2011; Petersen et al., 1999). In particular, the initial cognitive screening was conducted using the Mini Mental State Examination (MMSE) (Magni et al., 1996), a tool widely used to screen for cognitive impairment and estimate the degree of severity. The test globally assesses various cognitive areas, including orientation, memory, attention, calculation, and language. Following this, a comprehensive battery of neuropsychological tasks was administered to assess performance across several cognitive domains, such as episodic memory (both verbal and visuo-spatial), working memory, verbal fluency, language, executive functions, and attention, as well as visuo-spatial abilities and constructional praxis. The following tests were selected to ensure a comprehensive neuropsychological evaluation, using validated tools for a normative sample of the same nationality as the participants (Italian), with cut-off scores useful for differentiating between “normal” and “impaired” cognitive function, and norms corrected for age, education, and gender. This assessment is detailed in the **eMethods** section of **Supplement 1.**

2.4. Physical assessment

Both Healthy and MCI subjects have been administered with a battery of physical tests at the baseline and after seven months with the purpose to evaluate the eligibility of receiving the interventional approach and the efficacy of the training, respectively. All participants were evaluated for physical performance, speed, balance, and joint motility (Jones and Rikli, 2002; Gian Nicola Bisciotti, 2012). The following physical assessments have been delivered: the Six Minute Walk Test (6MWT), Chair Sit-Up Test (CSST) (Jones et al., 1998; Whitney et al., 2005) and Arm Curl Test (ACT) (Jones and Rikli, 2002); the Feet Up and Go Test (Podsiadlo and Richardson, 1991) and Romberg Test (RT) (Khasnis and Gokula, 2003); the Sit and Reach Test (SRT) (Jones and Rikli, 2002) and the Back Scratch Test (BST) (Gian Nicola Bisciotti, 2012). Detailed description of the physical tests is provided in the **eMethods** section of **Supplement 1**.

2.5. Blood collection and analytes measurements

Subjects enrolled to different groups underwent blood withdrawal at baseline (cognitively Healthy and MCI-T0) and after seven months of recruitment, at the end of training protocol (T7) (Thavasu et al., 1992). Following an overnight fast, peripheral venous blood was collected from all participants between 07:00 and 09:00 a.m. by a trained phlebotomist using standard venipuncture techniques. A 21-gauge butterfly needle was used to draw blood into either K-EDTA or CAT (Serum Clot activator) vacuette tubes for plasma and serum separation, respectively. Blood samples have been processed within 30 min from withdrawal. Samples have been centrifuged at 1500×*g* speed for 20 min and supernatants (plasma and serum) have been aliquoted and rapidly stored at −80 °C for subsequent processing. A battery of cytokines, chemokines and other circulating analytes has been analyzed through Proquantum High Sensitivity Immunoassay (Thermo Fisher), via the Proximity Ligation Assay (PLA) technique, according to the manufacturer protocol. Briefly, 5ul of plasma or serum have been diluted and Standard vial has been reconstituted with Assay Dilution Buffer. After resuspension, Standards have been allowed to reconstitute 15 min prior use at room temperature. Standards have been generated by serial dilution from the stock. Antibody A and B have been conjugated in a ratio 1:1 and then 2 μl of antibody mix have been pipetted into a 384-well plate. 2 μl volume of standards or diluted samples have been then added to the antibody mix in the 384-well plate, mixed thoroughly, sealed and centrifuged for 1 min to remove bubbles and let them stand 1 h at room temperature for conjugation. After that period the Master Mix supplemented with appropriate volume of Ligase has been added with a final reaction volume of 10 μl, the plated has been sealed, then centrifuged and run into the ViiA™ 7 Real-Time PCR System. Exported data have been analyzed with the Proquantum Software according to the manufacturer’s protocol. The following Immunoassays have been performed: Human IL-6, IL-17, IL-12p70, TNFα, INFγ, IL-1β, IL-4, IL-10, TGFβ, CCL11, MCP-1/CCL2, CX3CL1, Leptin, VEGF. Ella platform was additionally used to assay three analytes, Human NFL, soluble TREM-2 (sTREM-2) and BDNF via automated immunoassay technology. Briefly, 30 μl of samples have been diluted 1:1 with dilution Buffer, then 50 μl of diluted samples have been added to the ELLA cartridge, and immediately run with the platform. Exported data have been analyzed with Simple Plex Runner according to the manufacturer’s protocol. Triplicates of each sample have been assayed with Proquantum platform; automatic triple measurements were performed with ELLA platform. Any of the biomarkers assayed resulted undetectable.

2.6. Magnetic resonance imaging

All subjects underwent a 1.5T MR exam of the brain at both T0 and T7 time points. In addition to a number of clinical-oriented sequences, the MRI acquisition protocol included a 3D T1-weighted sequence and a 3D pseudo-continuous Arterial Spin Labeling (pCASL) sequence, aimed to estimate respectively brain volumetry and cerebral perfusion. MRI data were processed and analyzed using two approaches: a voxel-wise analysis (Voxel-based Morphometry, VBM) and an ROI-based analysis. All details about data preprocessing and analyses are described in the **eMethods** section of **Supplement 1**.

2.7. The train the brain intervention

MCI subjects enrolled in the study underwent a program of cognitive and physical training, in a socially-enriched environment, lasting 7 months. The training has been administered according to the protocol extensively described in the original study by Train the Brain consortium (Train the Brain Consortium, 2017), in a structure built on purpose within the National Research Council Area of Research in Pisa. The training program was based on 8 cycles. Each cycle was composed of 18 sessions of cognitive stimulation, with exercises and activities aimed at stimulating multiple cognitive functions. Each cycle lasted 3 weeks with exercises and activities of increased complexity compared to the previous cycle. In addition to the gradual progression of activities, which are distinctive features of cognitive training, intermediate monitoring provided the opportunity to adjust the activities based on individual and group needs, allowing for a more targeted and effective intervention. Subjects were included into mixed-sex classes of 7–10 subjects each and given two sessions/day of supervised cognitive training lasting 60 min each, 3 times a week, in the morning, every other day from Monday to Friday (for a total of 6 h/week). The cognitive training sessions featured a variety of exercises aimed at stimulating a broad range of cognitive abilities, with the complexity gradually increasing from one cycle to the next. The targeted functions included auditory and visual attention, spatial and visual memory, imagination, orientation in space and time, verbal memory, vocabulary, semantic memory, emotional memory, reading comprehension, recognition of faces and names, and logical reasoning. To diversify the cognitive input and maintain engagement, the program combined paper-and-pencil exercises, group-based social games, and multimedia computer tasks. These were carefully designed to avoid overlap with the tools used for neuropsychological assessment. The training alternated between single-modality and multimodal sessions. Single-modality activities focused on specific domains like memory and executive functioning, while multimodal tasks encouraged interaction among participants, aiming to promote socialization and enhance well-being in daily life. Additionally, a monthly “Cineforum” session brought all participants together to watch and discuss a film. Weekly music therapy sessions (1 h each) included both passive listening and active participation, such as singing, playing instruments, and rhythm-based movement (see also **eMethods, Supplement 1**). Alongside the cognitive training, participants also engaged in a supervised aerobic exercise program lasting 7 months (1-h lesson/day, three times/week). Each training session was studied and delivered according to the American College of Sports Medicine guidelines (Thompson et al., 2010). Each session began with aerobic activity on a cycle ergometer, with the duration gradually increasing from 10 to 20 min over time. This was followed by exercises designed to enhance muscle strength, physical performance (both static and dynamic), neuromuscular coordination, and flexibility (see also **eMethods**, **Supplement 1**).

2.8. Sample size

The results of the previous Train the Brain study were considered (Train the Brain Consortium, 2017). In this study a difference of 3.5 points in ADAS-Cog in cognitive decline between MCI-training and MCI-no training subjects was found; a sample size of 35 subjects per group would be sufficient to evaluate a significant difference between groups in ADAS-Cog, considering a drop-out of 14 % (as that found in (Train the Brain Consortium, 2017), setting significance level to 5 % and power of 80 %).

In relation to inflammation markers derived from blood samples, considering an a-priori power analysis based on previous studies evaluating strictly associated parameters (Gorska-Ciebiada et al., 2015), it was estimated that a sample size of 70 subjects (35 Healthy and 35 MCI-training; or 35 MCI-training and 35 MCI-no training) would allow to evaluate an absolute effect size of 0.35, with a power 80 %, a 5 % significance level, and considering a 14 % drop-out.

2.9. Statistical analysis

Two patterns of analyses were developed: the first aimed to compare Healthy and MCI subjects, the second focused only on subjects with MCI, to assess the effect of Train the Brain treatment (MCI-training vs. MCI-no training).

Characteristics at the baseline of the study (T0) of MCI and Healthy individuals were compared considering the $\chi 2$ test or Fisher's exact method for categorical variables, Generalized Linear Models after testing for homoschedasticity or the nonparametric Wilcoxon rank sum test for quantitative ones. Neuropsychological tests, physical performance tests, MRI measures and neuroinflammatory markers at T0 were compared also adjusting for sex, age, and education (Koch et al., 1998). The partial Spearman's rank correlation, implemented in the PResiduals package for R, was computed for every pair of continuous variables adjusting for age, sex and scholarity (Liu et al., 2020; R Core Team, 2023). The data distribution at T0 was visualized with non-metric multidimensional scaling (NMDS), implemented in the vegan package for R (Oksanen et al., 2022).

Changes in cognitive scores, in physical performance tests, in MRI measures and in neuroinflammatory markers in MCI participants were studied through mixed-effects models considering group (MCI-training; MCI-no training), time (T0, T7), and group*time interaction effects, adjusting for baseline score, sex, age and education. Intent-to-treat analyses were applied using multiple imputation of missing values (Markov chain Monte Carlo multiple imputation, and fully conditional specifications (FCS) method for categorical variables (Berglund, 2015), 20 imputed datasets were combined considering Proc MI Analyze (Allison, 2009).

Statistical significance was assumed for a p-value <0.05. The analyses were performed using SAS statistical package, release 9.4 (SAS Institute Inc., Cary, NC). A full description of statistical analysis is described in **eMethods** section of **Supplement 1**.

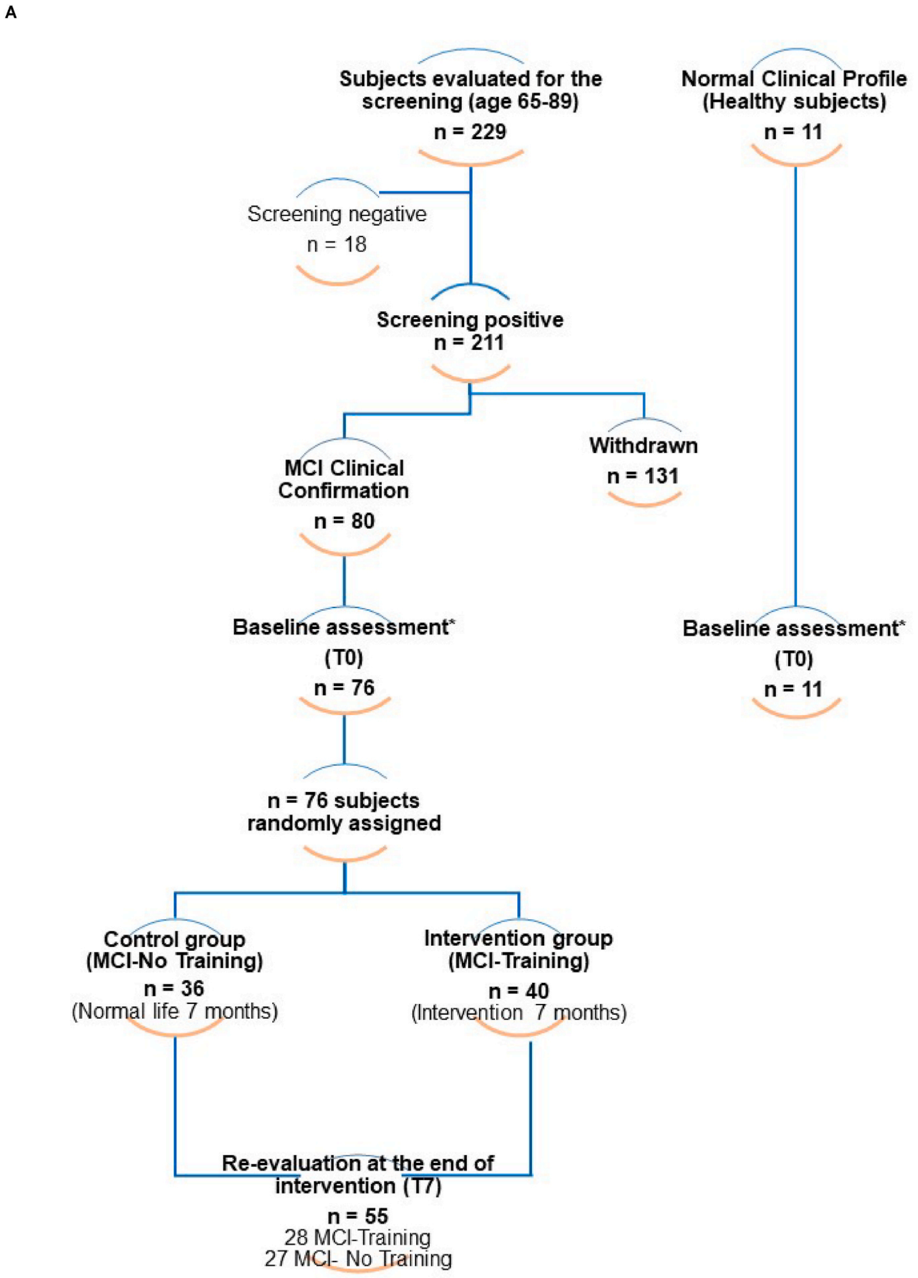
3. Results

3.1. Cohort description

A total of 229 subjects were evaluated for the screening phase. Of these 211 were submitted to the clinical confirmation phase (positive screening) resulting in 80 individuals receiving a diagnosis of mild cognitive impairment (MCI). An additional 11 aged-matched subjects with normal cognitive profiles ("Healthy" participants) were enrolled for comparison. Before randomization 4 MCI participants withdrew from the study, leaving 76 individuals who were randomized into either the intervention group (MCI-training, n = 40) or the control group (MCI-no training, n = 36). In the MCI-training group, 7 participants did not complete the training due to the health emergency related to the COVID-19 pandemic and 5 dropped out. As a result, 28 participants had both baseline (T0) and post-intervention (T7) assessments available. In the MCI-no training group, 9 participants dropped out, leaving 27 with complete T0 and T7 assessments (Fig. 1). No significant differences in dropout rates were found between groups (p = 0.4291), or based on sex (p = 0.1703), age (p = 0.4380) or education level (p = 0.5850). At baseline, MCI and Healthy groups were similar in age, but significant differences emerged in education and sex distribution. MCI participants had a lower median number of years of education with respect to the Healthy group (11 years for MCI vs 17 years for Healthy, p = 0.0013). There was also a significantly higher percentage of females in the Healthy group compared to the MCI group (50 % for MCI vs 81.8 % for Healthy, p = 0.0478) (eTable 1**, Supplement 2).** Additionally, the MCI cohort had lower fat mass (borderline significance) and a significantly higher heart rate compared to Healthy controls (eTable 1**, Supplement 2).** No significant group differences were observed in the systolic or diastolic blood pressure or oxygen saturation (eTable 1**, Supplement 2**). There were no major baseline differences between MCI cohort and cognitively Healthy group relatively to chronic conditions such as hypertension, diabetes mellitus, dyslipidemia, obesity, angina, myocardial infarction, pulmonary and renal diseases (eTable 1**, Supplement 2**). Overall, no significant differences at baseline were found between MCI-training and MCI-no training groups (eTable 1a**, Supplement 2**). Individuals with major chronic diseases known to have a pro-inflammatory profile were excluded from the study.

Neuropsychological and physical assessments, MRI scans and plasma biomarker analyses were conducted at the time of recruitment (Fig. 2A). This multi-level approach enabled a comprehensive characterization of each participant based on a wide range of measured variables. The Nonmetric MultiDimensional Scaling (NMDS) plot (Fig. 2B) indicated that MCI patients and Healthy controls formed two relatively homogenous, hough partially overlapping, populations. As expected and consistent with existing literature, MCI patients demonstrated significantly lower scores on the ADAS-Cog and MMSE compared to Healthy individuals when assessed using a standardized battery of neuropsychological tests (Fig. 2C; eTable 2, Supplement 2). Specifically, MCI participants showed significant impairments in: i) short term memory-related tasks, particularly in Rey auditory Verbal Learning and Rey-Osterrieth Complex Figure tests (immediate recall), ii) retrospective memory, assessed through delayed recall in the same tests; iii) attention -related functions, assessed by the Stroop test. These differences remained significant after adjusting for sex, age, and education (Fig. 2C; eTable 2, Supplement 2). In contrast, no significant impairments were found in physical performance measures, such as speed, balance and joint motility, in the MCI group compared to Healthy controls **(**eTable 3**, Supplement 2)**, supporting their eligibility to undergo the planned intervention.

Baseline MRI analyses using Voxel Based Morphometry (VBM) revealed significant differences in brain volumetry - specifically, grey matter concentration - between MCI and Healthy controls (Fig. 2D, left; eTable 4, Supplement 2). Compared to Healthy individuals, the MCI group exhibited a significantly lower concentration of grey matter in several regions associated with a well-known circuit including the medial temporal lobe, i.e. parahippocampal gyrus and hippocampus, the cortex along a temporal–parietal–frontal trajectory, occipital visual areas, cingulate cortex as well as motor areas (Pini et al., 2016; Zacková et al., 2021). Areas with significant differences (p-uncorr<0.0001, cluster size>5 voxel) are reported in Fig. 2D, left; eTable 4, Supplement 2. As expected, no significant differences in grey matter concentration were observed at baseline between MCI training and MCI no-training at T0. The ROI-based analysis (eTable 4**, Supplement 2**) further confirmed



*including: neuropsychological evaluations, MRI, blood withdrawal, according to the study protocol

Fig. 1. Overall study design flowchart.

structural alterations in the MCI group, including reduced cortical thickness of the left entorhinal cortex and a reduced volume of the right hippocampus in the MCI with respect to Healthy controls. Similar trends were observed in the contralateral regions, though these reached only borderline significance after adjusting for covariates. No significant differences were found between MCI no-training and MCI training at T0, as expected (eTable 12, **Supplement 2**). In terms of cerebral perfusion, no significant differences were detected between MCI and Healthy control at T0, neither in ROI-based nor in voxel-wise analysis (Fig. 2D, right; p-uncorr <0.0001).

3.2. *A complex inflammatory cytokine network in MCI patients*

A panel of cytokines and chemokines was selected to capture key features of immune polarization, focusing on robust indicators of pro- and anti-inflammatory states. Specifically, we selected IL-6 (a common upstream initiator of inflammation); IL-12p70, IL-1β, IFN$_\gamma$, TNFα (the four most robust Th1-polarization-associated cytokines); IL-4 (the most abundant Th2-polarization cytokine); IL-17A (the most abundant Th1-polarization cytokine); IL-10, TGFβ (the only soluble cytokines produced by anti-inflammatory Treg (Sakaguchi et al., 2009). We also assessed CCL2 and CXC3CL1 (also known as fractalkine), chemokines known to amplify Th1 responses in many difference disease contexts (Garetto et al., 2015; Martini et al., 2019) and CLL11/eotaxin-1, which is reduced in mice exposed to environmental enrichment and is implicated in cognitive benefits (Scabia et al., 2021). We finally included soluble TREM2 (sTREM), which is involved in neurodegenerative processes (Suárez-Calvet et al., 2019); Neurofilament Light (NFL), a marker of

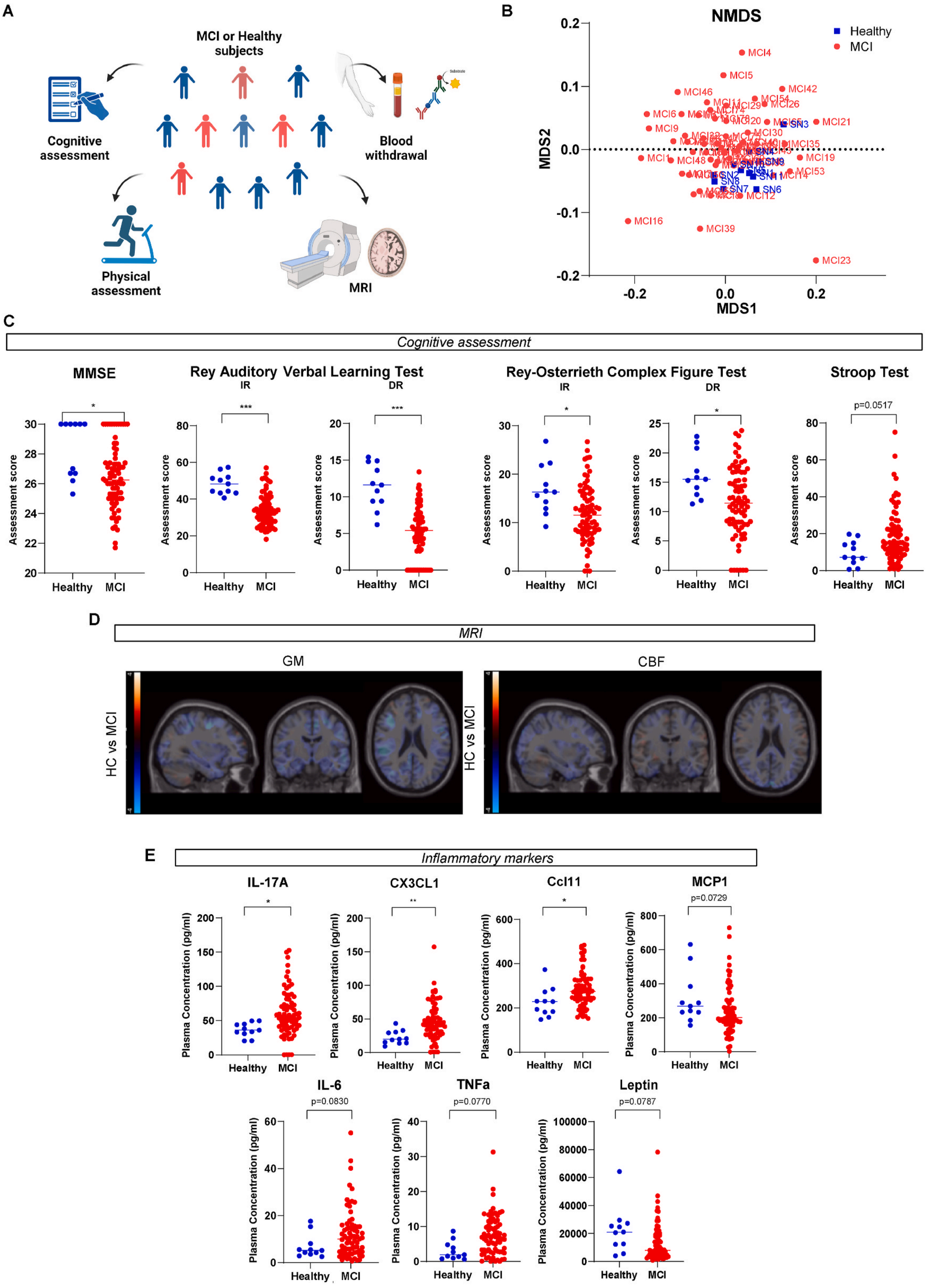


Fig. 2. MCI cohort is featured with a unique fingerprint. A. Representative cartoon showing the experimental procedure for the study. **B.** Non Multi-Dimensional Scaling (NMDS) plots describing homogenous, although partially overlapping, populations of MCI and Healthy subjects enrolled in this study. **C.** Panel of cognitive assessment evaluations in MCI vs Healthy subjects at baseline (T0), showing, from left to right: MMSE score, Rey Auditory Verbal Learning task assessing short (Immediate Recall – IR) and long (Delayed Recall – DR) term verbal memory, Stroop time assessing attention, Rey Osterrieth Complex Figure tests, assessing short (Immediate Recall – IR) and long (Delayed Recall – DR) visual memory and executive functions. p-value from ANCOVA analysis adjusted for age, sex and education; *, $p < 0.05$; **p, <0.01; ***p, <0.001. **D.** T-maps (t range = [-4, 4]), overlayed on T1w structural images in MNI space, obtained by *t*-test statistical analysis (ANCOVA analysis with sex and age as covariates), comparing HC and MCI subjects (HC > MCI), for grey matter concentration (GM, left panel) and cerebral perfusion (CBF, right panel). Images in radiological convention. **D.** Panel of blood measurements of inflammatory markers in MCI vs Healthy subjects, from up, left to down, right: Interleukin-17A (IL-17A), Fractalkine (CX3CL1), Eotaxin (CCL11), Monocyte Chemoattractant Protein-1 (MCP-1/CCL2), Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha (TNFα), Leptin. p-values from ANCOVA analysis adjusted for age, sex and education; *, $p < 0.05$; **p, <0.01; ***p, <0.001.

neuronal damage (Bavato et al., 2024); BDNF, a neuroprotective factor (Charlton et al., 2023); Leptin, a regulator of neuronal excitability and cognitive function (Signore et al., 2008), and VEGF, a marker associated with optimal brain aging and brain perfusion (Hohman et al., 2015). These markers were quantified at baseline (T0) in 11 Healthy participants (complete data) and 73 MCI participants (3 participants with missing data) (eTable 6, 6a**, Supplement 2**). Despite the smaller sample size, Healthy participants exhibited low variability in inflammatory marker profiles (eTable 6**, Supplement 2;** Fig. 2E). In contrast, MCI participants showed a markedly dysregulated inflammatory and neuroimmune profile. As shown in Fig. 2E and eTable 6 of Supplement 2, they exhibited significantly elevated plasma levels of IL-17A, -marker of Th17 cells-, CX3CL1 -marker of Th1 cells- and the eosinophil-specific chemokine CCL11 (Mantovani et al., 2010), compared to Healthy participants. Furthermore, MCI individuals exhibited, compared to cognitively Healthy individuals, lower levels of the Monocyte Chemoattractant Protein MCP/CCL2, higher levels of IL-6 and TNFα -though with borderline significance after adjustment for covariates ($p < 0.10$), and lower levels of Leptin, with borderline significance persisting also after adjustments. No significant differences were found between groups for other neuroinflammation markers, namely IL-1β, IL-4, IL-10, TGFβ, TREM2, VEGF ($p > 0.10$). However, trends toward increased levels of **IFNγ** and **IL-12p70**—both proinflammatory cytokines—were observed in the MCI group, although these did not reach statistical significance after adjustment. NFL and BDNF levels did not differ significantly between MCI patients and Healthy controls.

To better understand the interplay between immune markers, cognitive status, and brain structure, we performed correlation analyses across all measured parameters. This approach aimed to identify potential biological signatures of aging and MCI progression, as well as pathways potentially modifiable by intervention.

Multiple correlation analyses (see **eMethods, Supplement 1**) revealed distinct immuno-cognitive interaction patterns in Healthy versus MCI individuals. In Healthy controls, cytokine relationships appeared to reflect physiological aging. A filtered heatmap of significant correlations ($p < 0.05$; Fig. 3A; eFig. 1A Supplement 2) showed a scattered pattern dominated by inverse associations between plasma markers and cognitive or anatomical parameters. In particular, IL-6 and IL-17A were strongly inversely correlated with spatial memory performance (Rey–Osterrieth Complex Figure test); TNFα and NFL negatively correlated with verbal memory (Rey Auditory Verbal Learning task); IL-1β was inversely associated with MMSE scores; IL-6 also negatively correlated with hippocampal volume (left and right). Other key associations included CX3CL1 showing mild positive correlations with MMSE, IL-10, and IL-12p70, and BDNF, positively correlated with verbal fluency (FAS) and inversely with IFNγ. Also, a strong inverse link between IL-6 and IL-4, and between CCL11 and IL-10. These findings suggest that in normal aging, low-grade inflammation interacts with cognitive resilience and structural brain integrity through a complex but largely inverse pattern of associations.

In contrast, MCI patients displayed a markedly different, more structured correlation network (Fig. 3B; eFig. 1B, Supplement 2), including the three main clusters, pro-inflammatory cytokines, cognitive performance, and brain structure. Key findings include i) the presence of a tightly interconnected pro-inflammatory cytokine network (IL-6, IL-1β, IL-17A, IL-12p70, IFNγ, TNFα), also including IL-4, IL-10, and CX3CL1, suggesting broad immune activation, and ii) the inverse association of BDNF with IL-1β, IFNγ, and CX3CL1, but its positive correlation with TGFβ, supporting a potential neuroprotective response. Cognitive performance also correlated with specific immune markers: IL-10 showed a strong positive correlation with both immediate and delayed recall in the Rey–Osterrieth test, highlighting its potential as a biomarker of cognitive preservation. Immediate recall scores were also positively associated with IL-1β, IL-12p70, and soluble TREM2—a microglial activation marker implicated in Alzheimer's disease (Morenas-Rodríguez et al., 2022; Suárez-Calvet et al., 2016) —despite these markers not being elevated in MCI versus Healthy subjects (eTable 6**, Supplement 2**). Short-term visual memory also correlated strongly with CX3CL1 levels. These findings suggest that in MCI, immune markers—particularly those involved in inflammation and microglial activity—are closely linked to memory performance and may serve as early indicators of cognitive decline.

In summary, the MCI cohort exhibits a distinct, coordinated immune signature characterized by mild, chronic inflammation and a compensatory anti-inflammatory response. This evolving cytokine–cognition–structure network underscores the role of immune imbalance in early neurodegeneration and highlights candidate biomarkers for early detection and monitoring of MCI progression.

3.3. *The Train the Brain intervention shapes inflammatory responses while improving cognition and cerebral perfusion in MCI patients*

After seven months, MCI participants who completed the training (T7) showed clear, multidimensional improvements compared to those who did not (Fig. 4A). On the cognitive front, significant gains were observed: the ADAS-Cog scores improved in the training group (between-group mean difference at T7, $p = 0.0278$), with a trend toward significance in the group*time interaction ($p = 0.0770$) (Fig. 4B, left; eTable 7, Supplement 2). The Short-term memory, assessed by the Rey–Osterrieth Complex Figure immediate recall, also ameliorated (mean difference at T7 $p = 0.0521$; interaction $p = 0.0360$) (Fig. 4B, right). Improvements were also observed in verbal memory (Rey Auditory Verbal Learning, immediate and delayed recall), visuospatial skills, and constructional praxis (Clock Drawing Test), although these did not reach statistical significance when compared to the no-training group (eTable 7, Supplement 2). Physically, the benefits were even more striking. Participants in the training group outperformed controls in flexibility, balance, and motor speed, with significant group differences and group*time interactions in Sit and Reach, Back Scratch, Romberg, and Foot Up and Go tests (Fig. 4C; eTable 8, Supplement 2).

Neuroimaging analyses confirmed these functional changes. Voxel-based morphometry (VBM) revealed a progressive atrophy in the no-training group, but not in the training group (Fig. 4D, left; eTable 9-10, Supplement 2). Also, trained individuals exhibited significant increase in cerebral blood flow (CBF), particularly in the left hippocampus and parahippocampal gyrus (Fig. 4D, right; eTable 11, Supplement 2). Overall, MRI measurements obtained in the ROI-based analysis, revealed a significant decrease in the CBF values within the no-training group in the left parahippocampal region ($p = 0.013$) with significant group*time interaction ($p = 0.027$) (Fig. 4E, left; eTable 12, Supplement 2) and a borderline effect in the right side ($p = 0.081$) (Fig. 4E, right; eTable 12 Supplement 2), while CBF values remained stable in the training group.

Plasma biomarker profiles echoed these changes, indicating a systemic anti-inflammatory shift in response to training. Significant reductions in IL-6, IL-17A, TNFα, and CCL11 were observed at T7 in the training group (Fig. 4F, upper panel; eTable 13, Supplement 2). Simultaneously, anti-inflammatory and neuroprotective markers—IL-10, TGFβ, IL-4, and BDNF—were preserved or increased, while they declined in the no-training group (Fig. 4F, lower panel). Group*time interactions were statistically significant for TNFα (reduced in training), IL-10 and TGFβ (enhanced in training), and showed a borderline effect for BDNF ($p = 0.0571$) (eTable 13, Supplement 2).

Taken together, these results reveal a clear divergence between the groups by T7, with the training protocol driving cognitive and physical resilience, stabilizing BDNF, activating anti-inflammatory pathways, and reducing specific pro-inflammatory cytokines. This multi-domain benefit highlights TTB as a promising intervention to counteract early neurodegenerative decline.

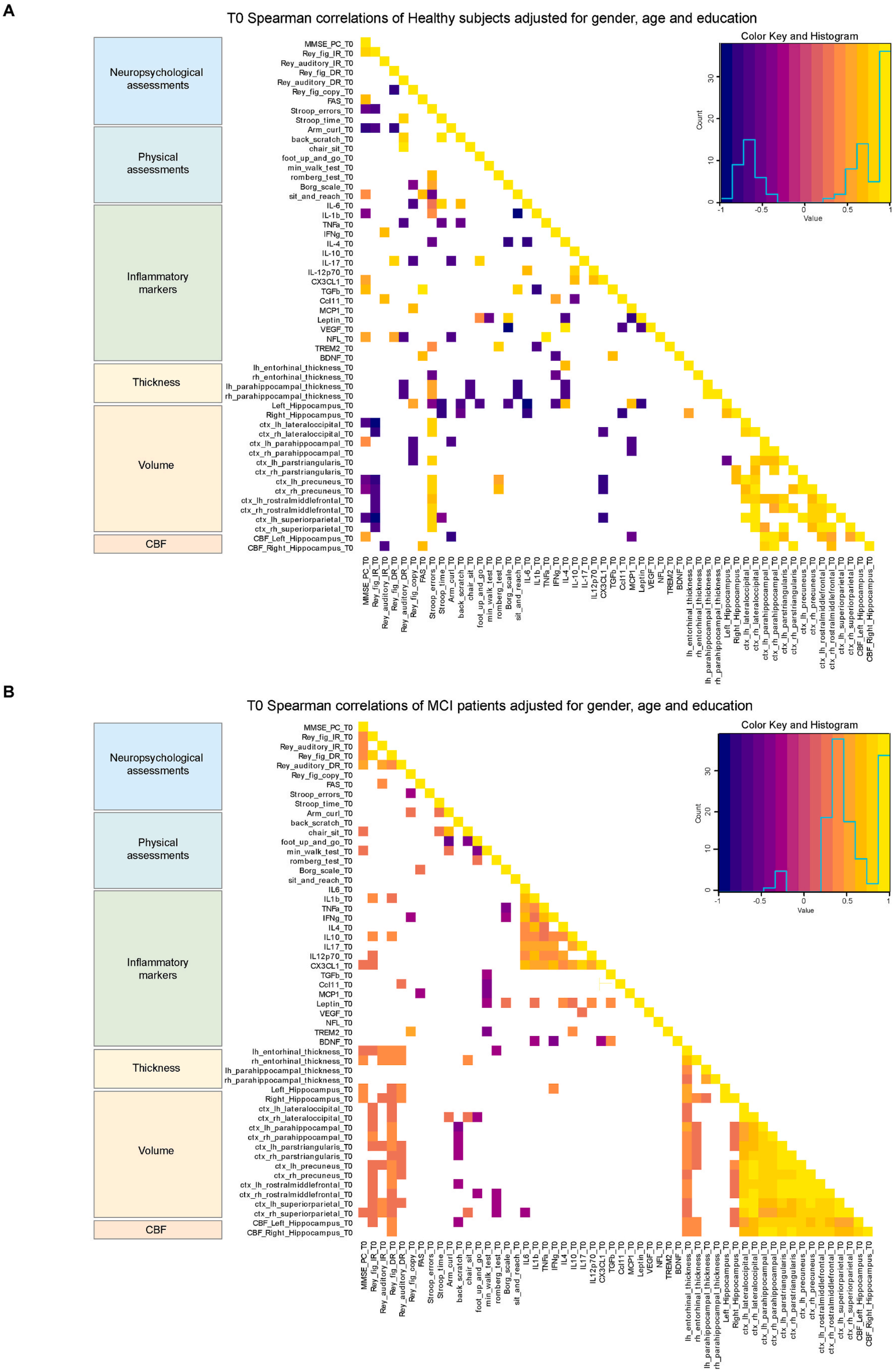
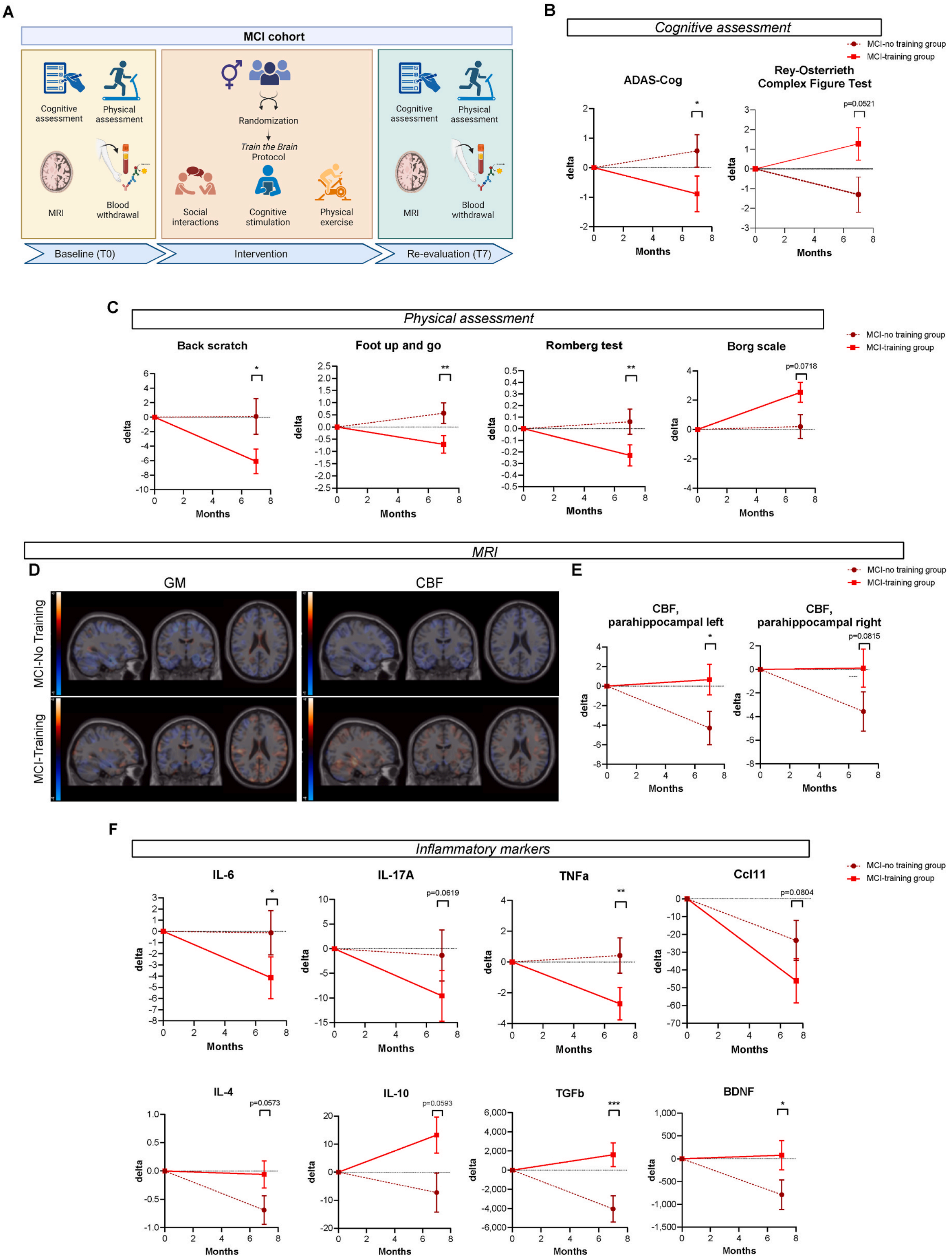


Fig. 3. Multiple correlations analysis at T0. Partial Spearman's rank correlations adjusted according to sex, age and education comparing each pair of variables at baseline. Only significant correlation values (p-value <0.05) are shown. **A.** Multiple correlations across Healthy subjects. **B.** Multiple correlations across MCI patients.



(caption on next page)

Fig. 4. The TTB intervention ameliorates inflammatory biomarkers while improving cognitive performance and brain perfusion in MCI patients. A. Representative cartoon showing the TTB intervention. **B.** Panel of cognitive assessment evaluations showing between-group mean differences at T7 in MCI-training vs MCI-no training, showing, from left to right: ADAS-Cog score, Rey Auditory Verbal Learning task and Rey Osterrieth Complex Figure tests, assessing verbal and visual memory functions, respectively. p-value from mixed-model repeated measures analyses, adjusted for baseline score, sex, age and years of education; *, $p < 0.05$; **p, <0.01; ***p, <0.001. **C.** Panel of physical assessments showing between-group mean differences at T7 in MCI-training vs MCI-no training, from left to right: back scratch, foot up and go, Romberg and Borg scale tests. p-value from mixed-model repeated measures analyses, adjusted for baseline score, sex, age and years of education; *, $p < 0.05$; **p, <0.01; ***p, <0.001. **D.** T-maps (t range = [-4, 4]), overlayed on T1w structural images in MNI space, obtained by paired *t*-test statistical analysis, comparing T0 and T7 (T7>T0), in the two subgroups of MCI (no-training, top panel; training bottom panel) for grey matter concentration (GM, left panel) and cerebral perfusion (CBF, right panel). Images in radiological convention. **E.** Between-group mean differences of CBF measurements at T7 in MCI-training vs MCI-no training in left and right parahippocampal regions. p-value from mixed-model repeated measures analyses, adjusted for baseline score, sex, age and years of education; *, $p < 0.05$; **p, <0.01; ***p, <0.001. **F.** Panel of blood measurements of inflammatory markers showing between-group mean differences at T7 in MCI-training vs MCI-no training, from up, left to down, right: Interleukin-6 (IL-6), Interleukin-17A (IL-17A), Tumor Necrosis Factor alpha (TNFα), Eotaxin (CCL11), Interleukin-4 (IL-4), Interleukin-10 (IL-10), Transforming Growth Factor beta (TGFβ), Brain- Derived Neurotrophic Factor (BDNF) (p-value from mixed-model repeated measures analyses, adjusted for baseline score, sex, age and years of education; *, $p < 0.05$; **p, <0.01; ***p, <0.001).

4. Discussion

Efforts to treat Alzheimer's disease (AD) in its advanced stages have largely been unsuccessful. New monoclonal antibodies targeting amyloid, such as lecanemab and donanemab, showed modest clinical benefits and carried significant risks (van Dyck et al., 2023; Sims et al., 2023). While these drugs may represent a step forward in the treatment of Alzheimer's disease, the core public health message remains unchanged: addressing modifiable risk factors remains essential to preserving brain health and reducing dementia risk across the lifespan (Livingston et al., 2020, 2024).

Mild cognitive impairment (MCI) represents a critical window for preventive strategies, particularly those aimed at delaying progression to dementia (Pini et al., 2016; Zacková et al., 2021). In this primary study, we replicated the Train the Brain (TTB) multidomain intervention in a new cohort of MCI subjects and examined its impact not only on cognitive and physical outcomes but also on a broad set of immune biomarkers. We confirm that the TTB intervention, which combines physical and cognitive training in a social setting, significantly improves cognitive performance and cerebral perfusion in MCI participants (Train the Brain Consortium, 2017). Importantly, we show that changes in an array of immune mediators, selected as the most robust indicators of immune polarization for adaptive and innate responses, correlated with cognitive and physical improvement. Thus, the intervention modulates specific peripheral immune markers, suggesting that immune pathways may play an active role in mediating these cognitive benefits. These molecules can therefore be exploited to monitor the effects of the training and better define responsive target aging populations for precision risk reduction.

In line with the concept of "inflammaging"—a low-grade, sterile inflammatory state characteristic of aging individuals (Franceschi et al., 2018; Cribbs et al., 2012) —pro-inflammatory cytokines tend to rise inconsistently across healthy older adults. In cognitively Healthy individuals, the observed inverse correlations between pro-inflammatory cytokines (e.g., IL-6, IL-17A) and performance on memory tasks likely reflect low-grade immune activation associated with physiological aging. The negative association between IL-6 and hippocampal volume, for example, aligns with known links between systemic inflammation and hippocampal atrophy in elderly populations (Marsland et al., 2008; Sudheimer et al., 2014). These patterns suggest that even in the absence of cognitive impairment, subtle immune-cognitive interactions are detectable and may precede overt decline.

While increased levels of individual pro-inflammatory cytokines in MCI have been previously reported (Guerreiro et al., 2007), our data demonstrate that these cytokines are not acting in isolation, but rather form a tightly interconnected network (Schmidt-Morgenroth et al., 2023). This suggests that MCI is characterized by a distinct immune signature—marked by coordinated amplification of inflammatory signals—that diverges from the more variable immune landscape observed in healthy aging. Specifically, we identified a complex network of immune mediators, including positively correlated pro- and anti-inflammatory cytokines (IL-6, IL-17A, IL-12p70, IFNγ, TNFα, IL-4, and IL-10), alongside inversely correlated factors such as IFNγ, TGFβ, and MCP-1/CCL2. The emergence of strong, coordinated positive correlations among pro-inflammatory cytokines indicates a more systemic and dysregulated immune profile. The correlation between IL-10 and improved memory performance (Rey–Osterrieth test) is particularly notable, as it suggests a potential compensatory anti-inflammatory response in individuals with better preserved cognition. Similarly, the associations between soluble TREM2 and memory outcomes may reflect microglial activation states linked to early neurodegenerative processes.

Notably, the TTB protocol disrupts the tightly correlated immune network observed at baseline in MCI subjects and produces a shift in this immune profile. Specifically, we observed an upregulation of key anti-inflammatory cytokines - including IL-10 and TGF-β as well as IL-4, the most abundant Th2-polarizing cytokine-, stabilization of BDNF levels, and reduction of TNFα, a cytokine associated with Th1 polarization. These changes occurred alongside improved cognitive and physical outcomes. suggesting that the intervention may partially work by restoring immune balance (Garetto et al., 2015; Martini et al., 2019). The reduction in pro-inflammatory signaling along with the observed stabilization or improvement in memory and executive functions, underscore the potential of targeted cognitive and physical training to modulate neuroinflammatory pathways.

Of note, IL-10, which inhibits microglial production of pro-inflammatory cytokines and promotes neuronal survival and adult neurogenesis (Perez-Asensio et al., 2013; Pereira et al., 2015), emerged as a particularly promising biomarker. IL-10 increases after training (Marafon et al., 2024) and correlates with improvements in both short- and long-term memory performance. These findings suggest that the early upregulation of IL-10 following the physical and cognitive training applied in a social setting may contribute causally to the observed cognitive improvements. Thus, IL-10 emerges as a mechanistic mediator of intervention efficacy, also representing a promising peripheral biomarker for monitoring response to training in at-risk populations.

Although not significantly correlated with cognitive tasks, the other identified molecular pathways may also contribute to the beneficial effects of the TTB intervention. TGF-β, a soluble cytokine produced—alongside IL-10—by anti-inflammatory regulatory T lymphocytes (Sakaguchi et al., 2009), is known for its neuroprotective properties, including the promotion of synaptogenesis and neuronal plasticity (Su et al., 2023). Also, IL-4 plays a protective role in aging by promoting anti-inflammatory responses and supporting tissue repair, which helps counteract chronic inflammation and neurodegeneration commonly seen in older adults (Gomez-Nicola and Boche, 2015). Similarly, BDNF is well recognized for both its anti-inflammatory potential (Charlton et al., 2023; Gao et al., 2022) and its ability to enhance neuroplasticity (Sale et al., 2014). Collectively, these correlation patterns reinforce the concept that the training intervention not only improves clinical measures but also orchestrates a beneficial rebalancing of immune and neurotrophic pathways, which may contribute to slowing or reversing aspects of MCI progression.

Our data also highlight a prominent role for CCL11, a chemokine previously associated with accelerated aging (Ivanovska et al., 2020), which significantly decreases following the TTB intervention. Although, unlike IL-10, CCL11 did not show a direct correlation with cognitive performance in this cohort, prior findings from our group demonstrated that elevated CCL11 levels suppress training-induced neuroplasticity signaling in aged mice, potentially by impairing hippocampal neurogenesis (Scabia et al., 2021). This suggests that the reduction of CCL11 may also play a causal role in mediating the cognitive benefits observed with the TTB protocol.

In conclusion, this study not only confirms the TTB protocol as an effective, non-invasive intervention to delay cognitive decline but also identifies specific immune pathways that may serve as early peripheral biomarkers of pathological aging and indicators of training efficacy. Moreover, our findings suggest that the cognitive improvements observed may be mediated, at least in part, by the early activation of regulatory T cells and the subsequent enhancement of anti-inflammatory immune responses.

4.1. *Limitations of the study*

This study has limitations. The control group of cognitively healthy individuals was small, leading to reduced statistical power for analysis comparing inflammatory biomarkers between MCI and cognitively healthy participants (power 0.20) thus limiting our ability to generalize immune comparisons across populations. The MCI sample, though sufficient for initial analyses, was reduced following randomization and intervention allocation, and some participants dropped out before post-intervention assessments. These issues limited within-subject comparisons and prevented analysis of sex-based effects. Larger, longitudinal studies will be essential to validate these findings and clarify the role of immune biomarkers in predicting or mediating training response.

CRediT authorship contribution statement

Genni Desiato: Writing – original draft, Data curation, Investigation, Conceptualization. **Paolo Bosco:** Formal analysis, Methodology, Data curation. **Simona Cintoli:** Investigation, Conceptualization, Data curation. **Laura Biagi:** Investigation, Methodology, Data curation. **Chiara Braschi:** Investigation. **Chiara Del Nero:** Investigation, Data curation. **Immacolata Minichiello:** Investigation, Data curation. **Marianna Noale:** Software, Formal analysis, Methodology, Data curation. **Elisa Faggiani:** Methodology, Investigation. **Alessandro Rossi:** Formal analysis, Methodology, Data curation. **Davide Pozzi:** Supervision, Writing – original draft. **Marinos Kallikourdis:** Supervision, Writing – original draft. **Lorenza Pratali:** Investigation, Methodology. **Stefania Maggi:** Supervision, Conceptualization, Data curation. **Gloria Tognoni:** Methodology, Supervision, Conceptualization. **Nicoletta Berardi:** Supervision, Writing – original draft. **Lamberto Maffei:** Investigation. **Alessandro Sale:** Supervision, Funding acquisition, Investigation, Conceptualization. **Michela Tosetti:** Investigation, Supervision, Funding acquisition. **Michela Matteoli:** Writing – original draft, Methodology, Conceptualization, Writing – review & editing, Supervision, Investigation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2025.101062.

Data availability

The authors do not have permission to share data.

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