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Combined omega-3 fatty acids, aerobic exercise and cognitive stimulation prevents decline in gray matter volume of the frontal, parietal and cingulate cortex in patients with mild cognitive impairment

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ABSTRACT

Previous studies in older adults suggested beneficial effects of omega-3 fatty acid (FA) supplementation, aerobic exercise, or cognitive stimulation on brain structure and function. However, combined effects of these interventions in patients suffering from mild cognitive impairment (MCI) are unknown. Using a randomized interventional design, we evaluated the effect of combined omega-3 FA supplementation, aerobic exercise and cognitive stimulation (target intervention) versus omega-3 FA supplementation and non-aerobic exercise (control intervention) on cognitive function and gray matter volume in patients with MCI. Moreover, we analyzed potential vascular, metabolic or inflammatory mechanisms underlying these effects. Twenty-two MCI patients (8 females; 60–80 years) successfully completed six months of omega-3 FA intake, aerobic cycling training and cognitive stimulation (n = 13) or omega-3 FA intake and non-aerobic stretching and toning (n = 9). Before and after the interventions, cognitive performance, magnetic resonance imaging of the brain at 3 T (n = 20), intima-media thickness of the internal carotid artery and serum markers of glucose control, lipid and B-vitamin metabolism, and inflammation were assessed. Intervention-related changes in gray matter volume of Alzheimer's disease (AD)-related brain regions, i.e., frontal, parietal, temporal and cingulate cortex were examined using voxel-based morphometry of high resolution T1-weighted images.

After the intervention period, significant differences emerged in brain structure between groups: Gray matter volume decreased in the frontal, parietal and cingulate cortex of patients in the control intervention, while gray matter volume in these areas was preserved or even increased after the target intervention. Decreases in homocysteine levels in the target intervention group were associated with increases in gray matter volume in the middle frontal cortex ($p = 0.010$). No significant differences in cognitive performance or other vascular, metabolic and inflammatory parameters were observed between groups. This pilot study provides preliminary evidence that omega-3 FA intake combined with aerobic exercise and cognitive stimulation prevents atrophy in AD-related brain regions in MCI patients, compared to omega-3 FA intake plus the control condition of stretching and toning. These promising findings should now be validated in a larger interventional trial.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with devastating impact on daily activities and independent living (Sperling et al., 2011). No effective pharmacological treatment has been identified to date (Eshkoor et al., 2015). In this situation, non-pharmacological interventions like nutritional supplementation (Gomez-Pinilla, 2008; Hooijmans et al., 2012), physical activity (Colcombe et al., 2006; Erickson et al., 2011) and cognitive stimulation (Rebok et al., 2014; Sitzer et al., 2006) receive increasing attention, although no definite conclusions can be drawn so far. For example, in healthy older adults, a previous interventional trial demonstrated beneficial effects of omega-3 FA intake on brain structure and function (Witte et al., 2014a), while others did not observe significant positive effects (Dangour et al., 2010; van de Rest et al., 2008). In patients with

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mild cognitive impairment (MCI) but not in those with dementia, omega-3 FA supplementation showed a significant improvement in short- and long-term memory function, and global cognition (Chiu et al., 2008; Freund-Levi et al., 2006; Lee et al., 2013). Moreover, there are currently no published studies investigating the effect of omega-3 FA on brain structure in MCI patients.

Exercise intervention in healthy older adults has frequently been demonstrated to increase cognitive function (Albinet et al., 2010; Erickson et al., 2011; Ruscheweyh et al., 2011), yet other trials showed minimal or no effects (Blumenthal and Madden, 1988; Madden et al., 1989). Notably, several imaging studies provide evidence that exercise training increases volume of brain regions that are vulnerable to age-related and disease-related atrophy, i.e., hippocampus, frontal, temporal and cingulate cortex, and induces neuroprotective cascades, such as increased concentration of serum brain-derived neurotrophic factor (BDNF) (Colcombe et al., 2006; Erickson et al., 2011; Ruscheweyh et al., 2011). First evidence also points towards beneficial effects of physical exercise intervention on cognitive performance (Suzuki et al., 2012) and gray matter structure (Suzuki et al., 2013) in MCI patients; however, large-scale studies are still missing.

Regarding cognitive stimulation, first interventional trials in humans have suggested beneficial effects on memory functions, attention, speed, and reasoning in healthy older adults (Ball et al., 2002; Rebok et al., 2014) and MCI patients (Forster et al., 2011; Kurz et al., 2009), a finding not replicated in other studies though (Slegers et al., 2009; Vidovich et al., 2015). Moreover, memory training led to increased gray matter volume in supramarginal, entorhinal, inferior temporal and inferior frontal regions in healthy adults and patients with subjective memory complaints (Engvig et al., 2014). In sum, lifestyle interventions as described above seem promising for healthy aging; however, the exact impact is still a matter of debate.

Studies that compared assessment of single lifestyle factors with assessment of combined lifestyle patterns found higher predictive power of the latter for cognitive performance (Floel et al., 2008; Kraft, 2012; Shea and Remington, 2015). Therefore, combined interventional approaches might exert synergistic effects on brain structure and function, and thus exceed the impact of each individual intervention, a hypothesis so far mainly derived from animal models (Chytrova et al., 2010; Wu et al., 2008). Omega-3 FAs constitute more than 30% of the membrane phospholipid composition, regulating membrane structure, fluidity and signal-transduction (Gomez-Pinilla, 2008). Moreover, omega-3 FAs modulate gene expression patterns that influence homocysteine/B-vitamin pathways (Huang et al., 2013), activate energy-generating mechanisms involved in glucose and lipid metabolism (Jump, 2002), and facilitate BDNF-mediated synaptic plasticity (Akbar et al., 2005). Physical activity is also known to lower plasma homocysteine (Randeva et al., 2002; Vincent et al., 2003), to regulate glucose homeostasis (Boule et al., 2005) and to enhance BDNF release, promoting synaptic plasticity, cell survival and proliferation (Erickson et al., 2011). Both omega-3 FA and physical activity have been shown to beneficially modulate dopamine production and D2 receptor function (Davis et al., 2010; Speelman et al., 2011), ß-amyloid deposition (Oksman et al., 2006; Yuede et al., 2009) and anti-inflammatory pathways (Kiecolt-Glaser et al., 2012; Pinto et al., 2012). Hence, given these partly overlapping but also divergent molecular and cellular associations that may optimize integrity of neuronal cell membranes and myelin sheaths, and promote neurogenesis and brain plasticity, combining both interventions may show additive or multiplicative benefits on brain function and structure in the aging brain (Bamidis et al., 2014). Furthermore, these interventions would then optimize the neural substrate necessary for cognitive stimulation to induce long-term changes in cognitive performance and brain structure. Supporting this hypothesis, it has been shown in humans that combined exercise and cognitive stimulation intervention leads to a greater improvement of cognitive function (Fabre et al., 2002) and stronger neuroplastic effects measured via electroencephalogram (Styliadis et al., 2015) compared to each single intervention and a control condition; but see discussion in Shatil (2013) and Leckie et al. (2014). Moreover, functional connectivity changes are induced by combined cognitive, psychological, and physical intervention in healthy older adults compared to low control cognitive stimulation (Zheng et al., 2015).

So far, the effects of a combined approach of nutritional supplementation, physical activity and cognitive stimulation have not been tested in patients with MCI. Our main objective was to investigate if a combined lifestyle intervention would exceed the beneficial effects of a single lifestyle intervention on brain structure and function. Based on our previous positive results for omega-3 FA supplementation alone (Witte et al., 2014a), we aimed to boost these effects with the addition of aerobic physical exercise and cognitive stimulation. In a pilot study we evaluated the effects of a combination of all three intervention strategies on brain structure and function in patients with MCI, compared to omega-3 FA supplementation plus the control condition of stretching and toning. These analyses represent the primary outcome of the study. To further elucidate underlying mechanisms, we conducted exploratory analyses and evaluated markers of atherosclerosis, i.e., intima-media thickness of the internal carotid artery, and serum markers of glucose control, lipid and B-vitamin metabolism, and inflammation.

Material and methods

Study participants

Patients (aged 60–80 years) with MCI were recruited in Berlin (memory clinic of the Department of Neurology of the Charité University Hospital and Neurology specialist practice) and Frankfurt am Main (Institute of General Practice), Germany. MCI patients (amnestic; single and multiple domain) were diagnosed according to Mayo criteria based on a subjective cognitive complaint and an objective memory impairment in standardized tests (performing at least 1.5 SD below age- and education-specific norm in relevant subtests (Total Word List, Delayed Recall Word/Figures, MMSE) of the CERAD-Plus test battery (Morris et al., 1989)), relatively preserved general cognition, no impairment in activities of daily living, and no dementia (Petersen et al., 1999). Exclusion criteria comprised severe untreated medical, neurological or psychiatric disease and brain pathologies identified in the magnetic resonance imaging (MRI) scan, no right-handedness (Oldfield, 1971), non-fluent German language abilities, BMI <18 kg/m^2 or >35 kg/m^2 and intake of dietary supplements containing fish oil before starting the trial. Furthermore, patients with attendance less than 50% of physical training sessions and self-reported misses of capsule intake >5 times/week over the entire intervention period were excluded from analysis. Psychiatric comorbidity was monitored using the Beck's Depression Inventory (BDI; (Kuhner et al., 2007)) and the State-Trait Anxiety Inventory (STAI X1; (Laux et al., 1981)).

Seventy-two MCI patients were screened for study eligibility by telephone, of which 27 failed to meet inclusion criteria. The remaining 45 were invited for baseline medical examination and MRI assessment. From this group, 10 patients had to be excluded either due to a pathological MRI finding ($n = 1$) or due to comorbidities ($n = 9$; cardiac arrhythmia, Parkinson's disease, Polycythemia vera, depression). Nine patients (target intervention $n = 6$; control intervention $n = 3$) did not complete the intervention due to time constraints ($n = 9$). In total, 26 MCI patients completed the current study. Of these, four patients (target intervention $n = 2$; control intervention $n = 2$) had to be excluded because they did not meet the criteria for minimal attendance (i.e., 50%) of the exercise/sham training over the six months, leaving 22 patients for per-protocol analysis (see flowchart Fig. 1). Dropouts ($n = 23$) and successful completers ($n = 22$) did not differ with regard to baseline assessments (all ps > 0.05).

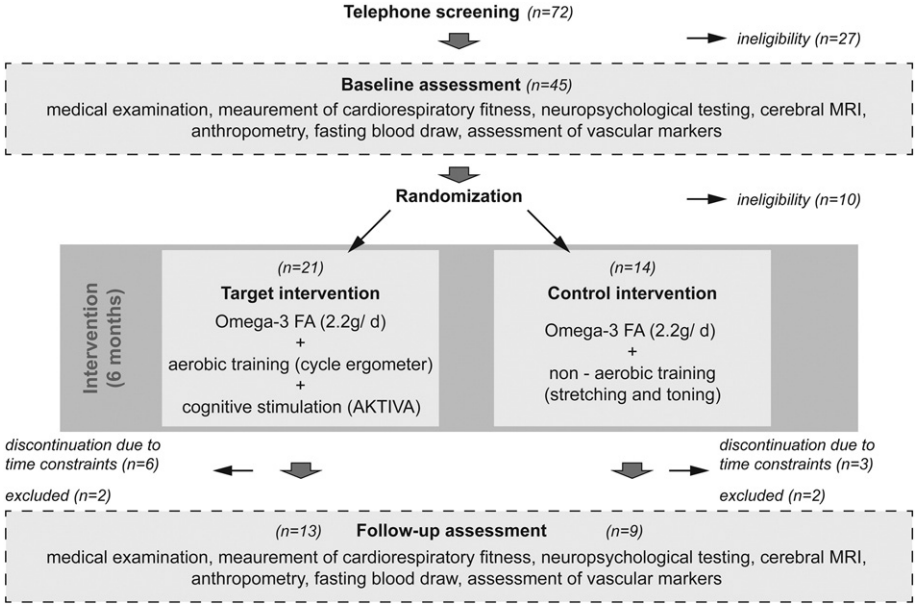

Fig. 1. Study flow chart. In total, 72 MCI patients were screened, of which 45 were invited for baseline assessment. 35 patients met the inclusion criteria and were randomized to a target intervention group (n = 21) comprising omega-3 fatty acids intake, aerobic exercise and cognitive stimulation or to a control intervention group (n = 14) with omega-3 FA intake, stretching and toning training. 26 MCI patients completed follow-up assessment. Nine patients did not complete participation due to time constraints and four patients were excluded from final analysis because they did not attend physical training sessions regularly. Thus, twenty-two patients successfully completed the intervention (target intervention, n = 13; control intervention, n = 9). Before and after the intervention period, patients underwent a standardized medical examination, including measurement of cardiorespiratory fitness, neuropsychological testing, cerebral magnetic resonance imaging (MRI), anthropometry, assessment of vascular markers and fasting blood draw for detection of serum parameters and genetic status. AKTIVA = active cognitive stimulation–prevention in the elderly/"*Aktive Kognitive Stimulation–Vorbeugung im Alter*", BMI = body mass index, FA = fatty acid.

Study design

During baseline assessments, patients underwent a standardized medical examination, assessment of cardiorespiratory fitness, neuropsychological testing, structural MRI of the brain, as well as fasting blood sampling and assessments of anthropometric data, carotid intima media thickness (CIMT) and body fat (baseline assessment; see Fig. 1). Patients were randomized to an omega-3 FA plus aerobic exercise and cognitive stimulation condition (target intervention; n = 13) or an omega-3 FA plus stretching and toning condition (control intervention; n = 9).

MCI patients of both groups received supplementation capsules with 2200 mg long-chain omega-3 FA per day (4 capsules comprising 1320 mg eicosapentaenoic acid (EPA), 880 mg docosahexaenoic acid (DHA) and additional 15 mg vitamin E) for six months, instructed to follow a regular intake before or at a main meal. Capsules were provided by Via Vitamine, Oberhausen, Germany.

The physical training sessions lasted for 45 min twice a week over six months for both the target and control intervention and were instructed and supervised by trained exercise leaders. Patients in the target intervention started the exercise training with a four-minute warm-up at an intensity of 40% of the intensity achieved at the anaerobic threshold during baseline graded exercise test. The anaerobic threshold was estimated according to the V-slope method that is highly reliable and validated even if age-defined target heart rate is not achieved during exercise testing (Beaver et al., 1986). The intensity was gradually increased (max 30 W/min) up to 80% of the intensity at anaerobic threshold (training target intensity). Duration of training at target intensity started at 20 min, and was gradually increased to 30 min over the course of 4 weeks. Each training session was followed by a cool-down for 6 min at 40% intensity. Training with 80% intensity was maintained (heart rate controlled) throughout the study to improve cardiorespiratory fitness, similar to Colcombe et al. (2004) and Ruscheweyh et al. (2011). During training sessions, electrocardiography and heart rate were monitored continuously and blood pressure was measured every 2 min (Customed software, Ottobrunn). Patients were encouraged to cycle with their training target intensity (based on 80% of their heart rate at anaerobic threshold), as determined during baseline exercise testing. In the control intervention, patients carried out a non-aerobic stretching and toning training (intensity at or below 50% of the intensity at anaerobic threshold), including muscle mobilization and strengthening, muscle-toning workouts using dumbbells or resistance bands, balance and coordination training, designed for older individuals (Colcombe et al., 2004; Ruscheweyh et al., 2011). All stretching and toning sessions started and ended with a five-minute warming up and cooling down by stretching.

In addition to aerobic exercise training, MCI patients of the target intervention participated in cognitive stimulation. The program called AKTIVA (active cognitive stimulation–prevention in the elderly: *Aktive Kognitive Stimulation–Vorbeugung im Alter*; here adapted for MCI patients) is an approach to enhance cognitive activity in everyday life via encouraging the use of cognitively stimulating leisure activities and memory strategies, and conveying a positive attitude towards aging, disease, and self-perception. For detailed training description of stimulation regimen see Supplementary information and Tesky et al. (2014, 2011). The sessions lasted for 90 min starting in the 4th week of the intervention, and comprised 12 group sessions and one individual coaching session, including detailed advice on how to initiate cognitively stimulating activities at home. In order to determine the frequency of these home-based activities during the intervention period, the participants were required to complete daily activity protocols.

Following the intervention, baseline measurements were repeated (follow-up assessment; see Fig. 1).

The study was approved by the Ethics Committee of the Charité University Hospital Berlin, Germany, and was in accordance with the declaration of Helsinki. All subjects gave informed written consent before participation in the study and received a small reimbursement at the end.

Compliance of omega-3 fatty acid intake and physical exercise

The number of remaining omega-3 FA capsules was counted after 12 and 24 weeks, and patients completed a questionnaire on capsule intake at the end of the study. In addition, changes in omega-3 index (von Schacky and Harris, 2007) served as a measure of omega-3 FA intake. Patients were instructed not to change their dietary habits throughout the intervention. Participation in physical training and cognitive stimulation was monitored and controlled by the training leaders. Changes in physical fitness were measured by peak oxygen consumption (VO_2 peak) at baseline and follow-up visit.

Omega-3 index

Erythrocyte membrane fatty acid compositions were assessed at baseline and after six months. Blood samples were collected and immediately centrifuged, and the erythrocyte fraction was stored at −80 °C until analysis. One sample of the target intervention group had to be excluded due to technical problems. The omega-3 index (von Schacky and Harris, 2007) was defined as the percentage of EPA (C20:5n-3) plus DHA (C22:6n-3) of total fatty acid areas. In addition, the percentage of arachidonic acid (C20:n4–6) was assessed, using a gas chromatograph (HP 5890 Series II with Autosampler). Analyses were performed by Omegametrix Laboratory, Martinsried, Germany.

Assessment of cardiorespiratory fitness

At baseline and follow-up aerobic fitness was evaluated by graded maximal exercise testing on a cycling ergometer (Ergoline ergoselect 100, Bitz, Germany) in the exercise laboratory of the Department of sport science at the Humboldt University, Berlin, Germany. Participants completed a standardized step incremental cycle ergometer test (25 W increments every 3 min). Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured directly by spiroergometry (Jaeger Oxycon, Germany). Heart rate, blood pressure and electrocardiography were monitored continuously and recorded every 3 min at the end of each intensity increment by a physician and a technical assistant. Maximum effort of the elderly patients was defined by the following two criteria: age-defined target heart rate (i.e., heart rate > 85% of predicted maximum heart rate (211 − (0.8 × age)) (Tanaka et al., 2001)) and a respiratory exchange ratio ≥ 1.0 (RER = VCO_2 peak/VO_2 peak) suggesting an adequate level of intensity for older adults (Barnes et al., 2003). Mean VO_2 of the highest complete performance level achieved by the participants was used as indicator of cardiorespiratory fitness, expressed as peak oxygen consumption normalized by body mass (VO_2 peak, ml/kg ∗ min^{-1}) (Burns et al., 2008). Participants exercised until exhaustion or signs of cardiac or respiratory distress (Gibbons et al., 2002).

Neuropsychological assessment

A trained psychometrician administered a neuropsychological test battery including the German version of the auditory verbal learning test (AVLT), forward and backward digit spans, verbal fluency (semantic and phonemic), trail making test (TMT) part A and B, and STROOP Color-Word test (Lezak, 2004; van de Rest et al., 2008). In the AVLT, patients had to learn a list of 15 words within 5 immediate recall trials (sum of words learned in all 5 trials correspond to learning ability), followed by a delayed recall (correct remembered words after 30 min) and recognition trial (recognition of the 15 original words presented within 35 distractor words). Parallel versions were used to avoid test–retest effects. To assess cognitive function of different domains, test scores were z-transformed and averaged to create composite scores for executive function, memory performance, sensorimotor speed, and attention, according to van de Rest et al. (2008) and Witte et al. (2014a). Composite scores were calculated as follows: executive function = [z phonemic fluency + z semantic fluency − z TMT ((part B − part A) / part A) − z STROOP (part C − (part A + part B) / 2)] / 4; memory = [z AVLT learning + z AVLT delayed recall + z AVLT recognition + z digit span backward] / 4; sensorimotor speed = [−z TMT part A − z STROOP part A − z STROOP part B] / 3; attention = z digit span forward. Mood during testing was assessed by the positive and negative affect schedule (PANAS; (Krohne et al., 1996)). The mini-mental status examination (MMSE; (Folstein et al., 1975)) was administered as a measure of global cognition.

Magnetic resonance image (MRI) acquisition

MRI scanning was conducted at baseline and follow-up using a 3 Tesla Siemens Trio system with a 12-channel head coil at the Berlin Center for Advanced Neuroimaging. High resolution T1-weighted scans (3D Magnetization Prepared Rapid Acquisition with Gradient Echoes (MPRAGE); TR = 1900 ms, TE = 2.52 ms, 192 sagittal slices, voxel-size of 1.0 × 1.0 × 1.0 mm^3, flip angle = 9°) were acquired. Image preprocessing and analysis were done using the software package FSL 4.1 (www.fmrib.ox.ac.uk/fsl). Two subjects could not receive MR scanning due to metallic implants (target intervention n = 1, control intervention n = 1), leaving 20 for analysis.

For voxel-wise analysis of changes in gray matter volume, we used a customized longitudinal version of voxel-based morphometry (VBM; (Good et al., 2001)), implemented in FSL (Douaud et al., 2009). For detailed overview of VBM procedure, see Supplementary information, Fig. S1. According to Witte et al. (2014a), structural images were first brain-extracted and gray matter-segmented using the FSL tools BET and FAST (1). Then we calculated an average "halfway"-transformation per subject based on the T1-images of time points 1 and 2 and created a halfway-space image as initial, subject-specific template (2). Per subject, the gray matter images of time points 1 and 2 were then co-registered to this subject-specific T1-template using rigid-body transformation, and averaged (3). The averaged mean-gray matter images of all subjects were then co-registered to a 2 × 2 × 2 MNI gray matter template using non-linear transformations, and averaged. Next, we created an initial study-specific gray matter template by taking the mean of this averaged mean-gray matter image and its right-left-flipped copy, to avoid potential bias due to lateralization effects (4). Subsequently, all mean-gray matter images were co-registered to the initial study-specific gray matter template using nonlinear registrations, and averaged. A second study-specific template was then created by taking the mean of this averaged mean-gray matter image and its right-left flipped copy (5). Subsequently, all gray matter-images of time points 1 and 2 were registered to the second study-specific template, by combining the rigid-body transform of the respective time point to the subject-specific template with the subsequent nonlinear transform of the mean-gray matter image to the second study-specific template. To adjust for local contraction or enlargement due to the non-linear component of the transformation, the resulting spatially aligned gray matter images were multiplied by the Jacobian determinant of the warp field. Before fed into voxel-wise statistics, a smoothing kernel of sigma = 3 was applied (6).

Anthropometric data and blood parameters

Anthropometric measures included weight, height, body mass index (BMI) and body fat (percentage, measured using bioelectrical impedance analysis, B.I.A. 2000-M, Pöcking, Germany). Patients also reported their physical activity and other lifestyle habits using the Freiburger physical activity questionnaire designed and validated by Frey et al. (1999). All subjects underwent venous blood sampling after fasting overnight of at least 10 h. Serum levels of triacylglycerides, total cholesterol, high-to-low density lipoprotein (HDL-to-LDL) ratio, total homocysteine, vitamin B12, folate, glycated hemoglobin A1c (HbA1c) as long-term measure of glucose, insulin, leptin, BDNF, insulin-like

growth factor 1 (IGF-1), high-sensitive C-reactive protein (hsCRP), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) were assessed (for details, see Supplementary information). All parameters were analyzed by IMD Laboratory, Berlin, Germany.

SNP genotyping

The single nucleotide polymorphisms (SNP) BDNF rs6265, catechol-O-methyltransferase (COMT) rs4680, apolipoprotein E (APOE) rs429358 and rs7412 have been previously implicated in episodic memory performance (Corder et al., 1993; Egan et al., 2003; Witte and Floel, 2012) and ApoE4 allele carrier status is associated with higher risk of AD (Farrer et al., 1997), potential confounding factors if unevenly distributed between intervention groups in our small cohort of patients with MCI. DNA was extracted from whole blood using a blood mini-kit (Qiagen, Hilden, Germany) and stored at −80 °C until analysis. Genotyping of the SNPs above were performed on a Sequenom® MassARRAY iPLEX, Taqman Assay at the laboratory of Prof. Dr. Dan Rujescu (University of Halle, Germany) following procedures described previously (O'Dwyer et al., 2012).

Carotid intima media thickness

Intima Media Thickness of the distal right and left common carotid artery was determined according to Witte et al. (2014b). CIMT of the far vessel wall was semi-automatically measured with the B-mode duplex ultrasound transducer positioned 1 cm proximal to the carotid bulb using a commercially available standardized real-time measurement method (Esaote Mylab25Gold, Cologne, Germany). CIMT was defined as the distance between the characteristic echoes of the lumen–intima interface and the media–adventitia interface. Mean values (measured in μm) were created by performing three CIMT measurements of each side. Two subjects had to be excluded due to data loss (target intervention n = 1, control intervention n = 1).

Statistical analyses

Before data analysis, normal or near-normal distribution and homogeneity of variances were tested by the Kolmogorov–Smirnov test and the Levene's Test. Accordingly, parametric and non-parametric tests were calculated. Level of significance was set at $\alpha < 0.05$. SPSS 22.0 (PASW, SPSS; IBM, Armonk, NY) was used for the analysis. Initially, all analyses conducted were unadjusted. In a second step, age and sex were entered as covariates.

At baseline, demographic characteristics, cardiovascular and genetic risk factors, and global cognitive function were compared between groups using independent t-tests, Mann–Whitney U-test, or chi-squared-test, as indicated. Changes over intervention time in physical fitness, omega-3 FA, cognitive performance, anthropometry, vascular parameters, self-reported physical activity, mood, and blood serum parameters were evaluated using paired t-tests or Wilcoxon signed-rank test, as appropriate. To detect differences between groups with regard to changes over time in selected variables, we performed repeated-measures analysis of variance ($ANOVA_{RM}$) with "time" as a repeated factor (baseline versus follow-up) and "group" as a between-subject factor (target versus control intervention). Correction for multiple comparison was applied for detection of significant changes in cognitive performance, as primary study outcome, using a Bonferroni threshold of $\alpha = 0.05/4$ cognitive subtests = 0.0125. Imaging results were corrected for multiple comparisons using an alpha level of 0.005 and a threshold-free cluster enhancement (TFCE) approach (details see below). Due to the exploratory nature of further analyses regarding potential underlying mechanisms, corrections for multiple comparisons were not applied when evaluating anthropometric measures, vascular parameters, physical activity and fasting serum parameters.

Intervention effects on gray matter volume were compared voxel-wise between the groups using permutation-based non-parametric inference called "Randomise", an FSL tool (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/Randomise) that has shown to be highly accurate in studies with small sample sizes (Anderson and Robinson, 2001). For a powerful and hypothesis-driven analysis of structural changes in AD at-risk patients we created a mask consisting of AD-related brain regions, in line with Dickerson et al. (2009) and McDonald et al. (2009). The AD-related regions of interest were defined on probabilistic maps of the brain structural Harvard/Oxford Atlas (thresholded at 0.5 and binarized), including the frontal (cortical regions anterior to the precentral gyrus), temporal (hippocampus, superior, middle and inferior temporal regions), parietal (supramarginal and angular gyrus, superior parietal lobule and precuneus) and cingulate cortex, according to Villeneuve et al. (2014). In a second step we conducted VBM analysis using a whole-brain gray matter mask to validate the results obtained when using the AD-related brain mask.

Randomise output data were thresholded using TFCE (Smith and Nichols, 2009) and minimum cluster size was set to 10 voxels to eliminate single outliers with suspiciously high t-values. Given our small sample size, results were considered significant for $\alpha < 0.005$, according to previous studies (Martinez et al., 2013; Yi et al., 2015). To detect associations between changes in fasting serum parameters, cognitive performance and gray matter volume after the six months intervention, we ran bivariate correlations; Pearson or Spearman's rank test according to distribution of the data.

Results

Baseline characteristics

At baseline, both intervention groups were comparable with regard to age, sex, education, cardiovascular risk factors, physical activity, MMSE scores (all ps > 0.05; Table 1) and allelic variant frequency of memory-associated gene polymorphisms (APOE e4-, BDNF- and COMT-SNP carrier status; p > 0.05; see also Supplementary information Table S2).

Cardiorespiratory fitness and omega-3 index

Omega-3 index significantly increased, i.e., significantly higher proportions of DHA and EPA were measured in the erythrocyte membranes, in both intervention groups (paired t-test, all ps < 0.05; Table 2), indicating high compliance with omega-3 FA capsule intake. Moreover, a significant decrease of arachidonic acid was observed in both groups (paired t-test, all ps < 0.05; Table 2), further supporting a regular omega-3 FA intake (Burns et al., 2007; Healy et al., 2000) (for detailed results see Supplementary information).

We observed a trend-wise interaction effect of group by time on physical fitness (VO_2 peak), measured during graded maximal exercise testing ($ANOVA_{RM}$, $F_{(1,\ 20)} = 2.08$, $p = 0.166$). At baseline, physical fitness was similar between both groups (unpaired t-test, $t_{(19)} = 0.72$, $p = 0.478$). VO_2 peak decreased by 2.1% after six months in patients of the control intervention, whereas patients of the target intervention showed an increase of 4.5% in VO_2 peak (trend, paired t-test, $t_{(12)} = -1.72$, $p = 0.117$; Table 2). Note that all patients performed up to a RER ≥ 1.0, suggesting an adequate level of intensity during fitness testing. However, 31% of patients in the target intervention and 22% in the control intervention did not reach the target heart rate, reflecting partial inadequacy of maximal effort during fitness testing that may cause an underestimation of VO_2 peak (Thompson et al., 2009). Together, aerobic exercise training increased cardiorespiratory fitness in contrast to non-aerobic training, but changes were not significant.

Table 1
Baseline characteristics of MCI patients dependent on group.

Characteristic parameters	Target intervention	Control intervention	p
VO_2peak [ml/min * kg]	22.1 ± 4.9 (17–33)	23.7 ± 5.8 (17–33)	0.478[a]
n (Women) [n]	13 (4)	9 (4)	0.512[c]
Age [years]	70 ± 7.2 (60–80)	70 ± 5.2 (61–76)	1.000[b]
Education [years]	16.1 ± 4.1 (11–24)	16.5 ± 2.9 (12–21)	0.812[a]
Body mass index (BMI) [kg/m^2]	25.4 ± 3.5 (19–32)	24.3 ± 2.8 (21–30)	0.242[b]
Right-handedness [%]	80.8 ± 21.1 (50–100)	85.0 ± 23.4 (40–100)	0.442[b]
Systolic blood pressure [mm Hg]	147.0 ± 16.4 (122–181)	152.7 ± 36.6 (107–208)	0.624[a]
Smoking [pack years]	9.6 ± 16.1 (0–50)	11.7 ± 15.4 (0–40)	0.972[b]
Physical activity [kcal/week]	3442.0 ± 2500.0 (572–7899)	3615.2 ± 3247.9 (371–10,706)	0.973[b]
Beck's Depression Index (BDI) [score]	7.1 ± 5.5 (0–20)	7.9 ± 6.4 (0–20)	0.752[a]
State-Trait Anxiety Inventory-X1 [score]	36.4 ± 7.8 (26–51)	40.4 ± 11.7 (26–55)	0.354[a]
Mini mental state examination (MMSE) [score]	28.5 ± 1.1 (26–30)	27.9 ± 1.7 (26–30)	0.291[a]

Data expressed as mean ± SD (range; min–max); group comparisons were performed using: [a] unpaired t-test, [b] Mann − Whitney − U-test and [c] Chi-square test.

Cognitive performance

Mean baseline scores of all cognitive domains were comparable between intervention groups ($p > 0.220$), however, note that performance of patients of the control intervention was slightly better compared to the patients of the target intervention. After the intervention, we found no changes in executive function, memory, sensorimotor speed and attention in both groups after correction for multiple-comparisons (Table 3). Adjustment for age and sex did not change these results.

Gray matter volume

Longitudinal voxel-based morphometry within AD-related brain regions showed differential changes in gray matter volume in areas of the frontal, parietal, and cingulate cortex of MCI patients after target and control intervention (TFCE, $p < 0.005$; Fig. 2; Table 4).

Specifically, we observed a decrease in gray matter volume within the middle frontal cortex (−5.0%), superior frontal cortex (−6.1%), frontal pole (−5.0%), angular cortex (−4.6%), precuneus (−2.5%) and posterior cingulate cortex (−5.1%) of MCI patients after control intervention. In contrast, patients in the target intervention showed an increase in gray matter volume in the middle frontal cortex (+2.1%), frontal pole (+3.5%), angular cortex (+1.8%), precuneus (+7.0%) and posterior cingulate cortex (+0.28%); and almost preserved volume in the superior frontal cortex (−0.5%), see Fig. 3. However, the smallest cluster within the precuneus did not survive the adjustment for age and sex (see Supplementary information Table S3). No effects over time could be observed for other AD-related brain regions, i.e., temporal cortex and no effects were seen for the inverse contrast (control intervention > target intervention). These results remained largely unchanged when performing VBM analysis using a whole brain gray matter mask. Differences to the analysis with the AD-related brain mask encompassed an additional cluster in the paracingulate area (cluster size 12 voxels; 0.002; $x = 8$, $y = 44$, $z = 22$), and a reduction in the overall cluster size, most likely due to lower statistical power.

Together, omega-3 FA supplementation combined with aerobic exercise and cognitive stimulation preserved and partially improved gray matter volume in frontal, parietal and posterior cingulate cortices, whereas atrophy was seen in the control intervention group.

Changes in anthropometry, vascular parameters, physical activity and fasting serum parameters

Exploratory analysis of vascular, metabolic or inflammatory markers revealed different changes between groups over time for homocysteine ($ANOVA_{RM}$, $F_{(1, 20)} = 5.27$, $p = 0.031$), with a significant decrease in total homocysteine concentration in patients of the target intervention (paired t-test, $t_{(12)} = 2.80$, $p = 0.016$), but not of the control intervention ($p = 0.367$). Adjustment for age and sex did not attenuate the effect ($ANOVA_{RM}$, $F_{(1, 20)} = 6.35$, $p = 0.021$). High-dose omega-3 FA supplementation reduced triacylglyceride concentration in both groups, in line with previous studies (Skulas-Ray et al., 2011; Witte et al., 2014a). For all other parameters, no significant differential changes between groups over time were observed (see Table 5).

Associations between changes in physical fitness, serum parameters, cognitive performance and gray matter volume

Changes in omega-3 index, EPA and DHA concentration were not significantly associated with changes in cognitive functions and gray matter volume (all ps > 0.05). Changes in physical fitness (VO_2peak) were associated with increases in mean gray matter volume of the significant cluster in the angular cortex (Pearson correlation, $r = 0.505$, $p = 0.023$), while no significant associations emerged between changes in physical fitness and cognitive functions (all ps > 0.05). In the control

Table 2
Changes in physical fitness, omega-3 and -6 FA concentrations of MCI patients dependent on group.

	Target intervention (n = 13)			Control intervention (n = 9)		
	BL	FU	p-Value	BL	FU	p-Value
VO_2 peak [ml/min * kg]	22.1 ± 4.9	23.1 ± 4.4	0.177[a]	23.7 ± 5.8	23.2 ± 4.8	0.585[a]
Omega-3 index [%]	6.4 ± 1.6	12.5 ± 2.1	<**0.001**[a]	5.4 ± 1.8	12.4 ± 1.4	<**0.001**[a]
EPA [%]	1.0 ± 0.3	4.2 ± 1.1	<**0.001**[a]	0.8 ± 0.3	4.1 ± 1.0	<**0.001**[a]
DHA [%]	5.4 ± 1.4	8.4 ± 1.2	<**0.001**[a]	4.6 ± 1.5	8.3 ± 0.8	<**0.001**[a]
Arachidonic acid [%]	15.2 ± 1.2	12.2 ± 1.4	<**0.001**[a]	14.9 ± 1.1	11.2 ± 1.3	<**0.001**[a]

Data expressed as mean ± SD. Significant results are highlighted in bold ($p < 0.05$). BL = baseline, DHA = docosahexaenoic acid; FU = follow-up; EPA = eicosapentaenoic acid.
[a] Paired t-test.

Table 3
Changes in cognitive performance of different domains in MCI patients dependent on group.

	Target intervention (n = 13)			Control intervention (n = 9)		
	BL	FU	p-Value	BL	FU	p-Value
Executive function	−0.09 ± 0.6	−0.23 ± 0.6	0.160[a]	0.12 ± 0.7	0.38 ± 0.6	**0.030**[a]
Memory	−0.20 ± 0.7	−0.12 ± 0.7	0.635[a]	0.21 ± 0.8	0.24 ± 0.8	0.775[a]
Sensorimotor speed	−0.10 ± 0.9	−0.01 ± 0.8	0.345[b]	0.15 ± 0.8	0.02 ± 1.0	0.314[b]
Attention	−0.22 ± 1.0	−0.36 ± 1.0	0.656[a]	0.37 ± 1.1	0.4 ± 0.8	0.839[a]

Composite scores of different cognitive domains expressed as mean z-score ± SD. Significant results are highlighted in bold ($p < 0.05$). BL = baseline, FU = follow-up.
[a] Paired t-test.
[b] Wilcoxon signed-rank test.

group only, the decrease in gray matter volume within the frontal pole was associated with worse performance in executive tasks (Pearson correlation, $r = 0.751$, $p = 0.032$). Decreases in homocysteine concentration over time were associated with higher mean gray matter volume of the significant cluster in the middle frontal cortex (Spearman correlation, $r = -0.706$, $p = 0.010$; Fig. 4). No significant associations emerged between other serum parameters and changes in cognitive functions and gray matter volume over time.

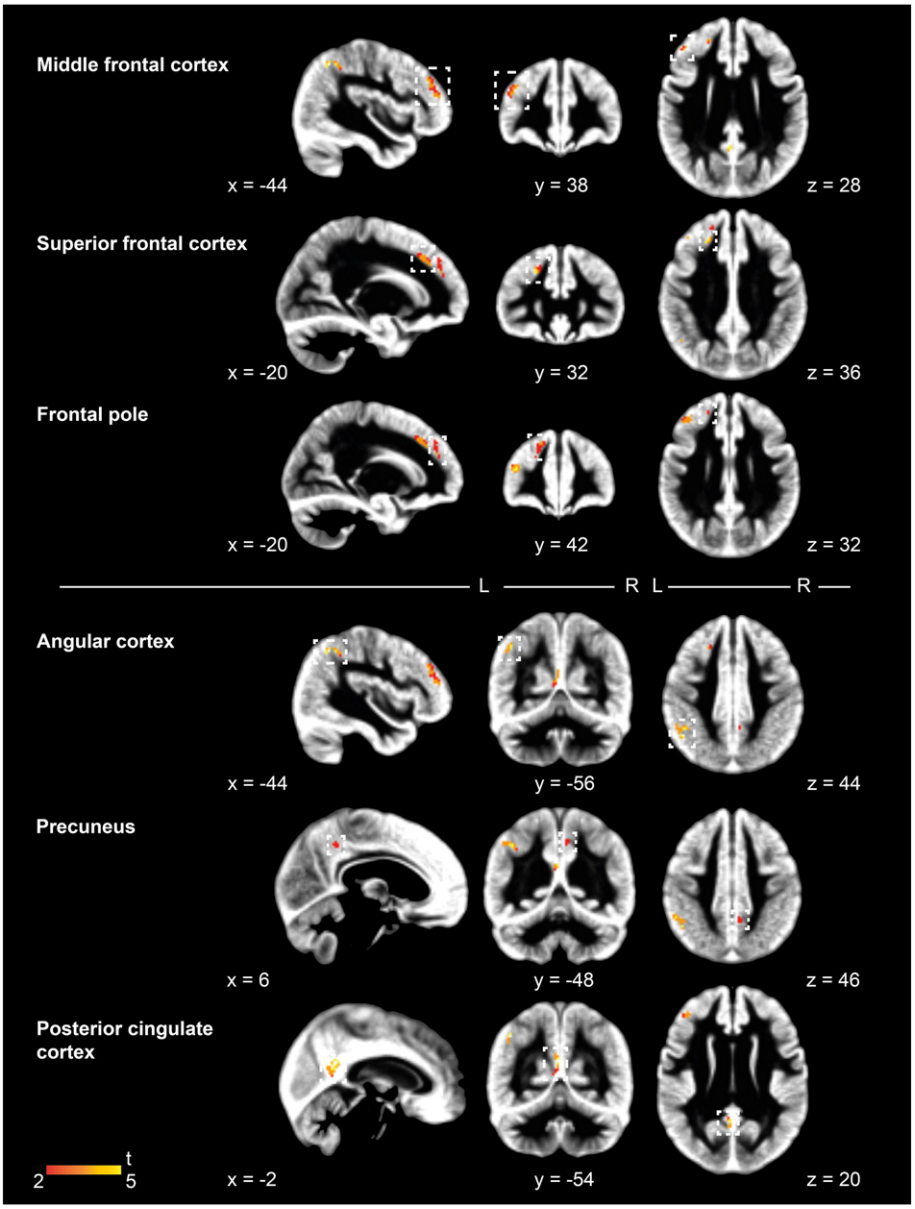

Fig. 2. Regional changes in cortical gray matter volume after six months of omega-3 FA intake, aerobic exercise and cognitive stimulation (target intervention) or of omega-3 FA intake, stretching and toning training (control intervention), compared by voxel-wise morphometry in AD-related brain regions using an AD-mask. Gray matter volume decreased in MCI patients of the control intervention, whereas it increased or remained constant in patients of the target intervention in frontal, parietal and posterior cingulate cortices. Color bar indicate t-values of significant voxels (target intervention > control intervention; TFCE, $p < 0.005$, cluster size ≥ 10). For better visualization we superimposed the t-map on the study-specific gray matter template. Images are displayed in neurological convention, coordinates in mm according to MNI space. R = right, L = left.

Table 4
Differential changes in gray matter volume dependent on group, as assessed by VBM in AD-related brain regions.

Target intervention > Control intervention			MNI coordinates (hot voxel)		
Brain area	No. of voxel[a]	p (hot voxel)	X	Y	Z
Frontal lobe					
Middle frontal cortex, left	159	<0.001	−44	38	28
Superior frontal cortex, left	39	<0.001	−20	32	36
Frontal Pole, left	36	0.001	−20	42	32
Parietal lobe + cingulate cortex					
Angular cortex, left	61	0.001	−44	−56	44
Precuneus, right	11	0.002	6	−48	46
Posterior cingulate cortex, left	51	0.002	−2	−54	20

TFCE, $p < 0.005$.
[a] Cluster <10 voxels are not shown.

Discussion

In this pilot study, we demonstrated for the first time that omega-3 FA intake combined with aerobic exercise and cognitive stimulation over six months led to reduced atrophy in AD-related brain regions of MCI patients, compared to omega-3 FA intake plus the control condition of stretching and toning. No significant group differences emerged for cognitive parameters over time.

Cognitive changes

In previous unimodal interventional trials, omega-3 FA supplementation improved global cognitive and executive performance (Chiu et al., 2008; Freund-Levi et al., 2006; Witte et al., 2014a) and physical activity interventions led to improvements in episodic (Ruscheweyh et al., 2011) and spatial memory (Erickson et al., 2011), and executive function (Albinet et al., 2010; Colcombe and Kramer, 2003). Similarly, cognitive stimulation improved memory and executive functions in older adults (Belleville et al., 2006; Buschert et al., 2011). Combined physical and cognitive activity demonstrated larger benefits on cognitive performance than each intervention alone (Anderson-Hanley et al., 2012; Maillot et al., 2012; Mortimer et al., 2012). In the present study, we did not find a significant improvement of cognition in the target intervention, nor any significant differential changes between groups, possibly due to the low number of patients included in the study. Alternatively, beneficial effects of omega-3 FA in both groups may have diluted the advantage of the combined intervention on cognitive measures.

Brain structural changes

The effects of combined approaches on brain structure are so far largely unknown, but growing evidence suggests that multimodal interventions may lead to additive or even superadditive effects (Bamidis et al., 2014; Holzschneider et al., 2012; Trachtenberg et al., 2002). Here, we demonstrated that gray matter atrophy rate in MCI patients was significantly reduced after combined intervention with omega-3 FA, aerobic exercise and cognitive stimulation compared to omega-3 FA supplementation plus the control condition of stretching and toning. Previous research already reported beneficial effects of single non-pharmacological interventions on gray matter structure particularly in AD-related brain regions (Bamidis et al., 2014). For instance, supplementation with omega-3 FA previously increased gray matter volume within the hippocampus, precuneus and temporal lobe in older healthy adults (Witte et al., 2014a). Furthermore, it has been demonstrated that aerobic physical exercise partially reduced gray matter atrophy or even improved volume of the hippocampus, frontal, temporal, parietal and cingulate cortex (Colcombe et al., 2006; Erickson et al., 2011; Ruscheweyh et al., 2011). Also, memory training increased cortical thickness within AD-related brain regions, i.e., the temporal lobe, supramarginal and entorhinal gyri, frontal and orbitofrontal cortex (Boyke et al., 2008; Engvig et al., 2010, 2014).

For a powerful hypothesis-driven analysis of structural changes in AD at-risk patients we used a defined mask consisting of AD-vulnerable brain regions as previously recommended by Dickerson et al. (2009) and McDonald et al. (2009). We found that gray matter volume was preserved and partially improved in the frontal, parietal and posterior cingulate cortex after six months combined omega-3 FA supplementation, aerobic exercise, and cognitive stimulation in contrast to the control intervention, indicating that the combined intervention influenced the disease-related trajectories in a more favorable way than omega-3 FA supplementation plus the control condition of stretching and toning.

In fact, our finding that in the control intervention, gray matter atrophy rate was ~4% within six months in AD-vulnerable regions, a value in the upper range or even slightly above previous longitudinal studies with MCI and AD patients (Chetelat et al., 2005; Douaud et al., 2009; Jack et al., 2004; McDonald et al., 2009), possibly due to the large

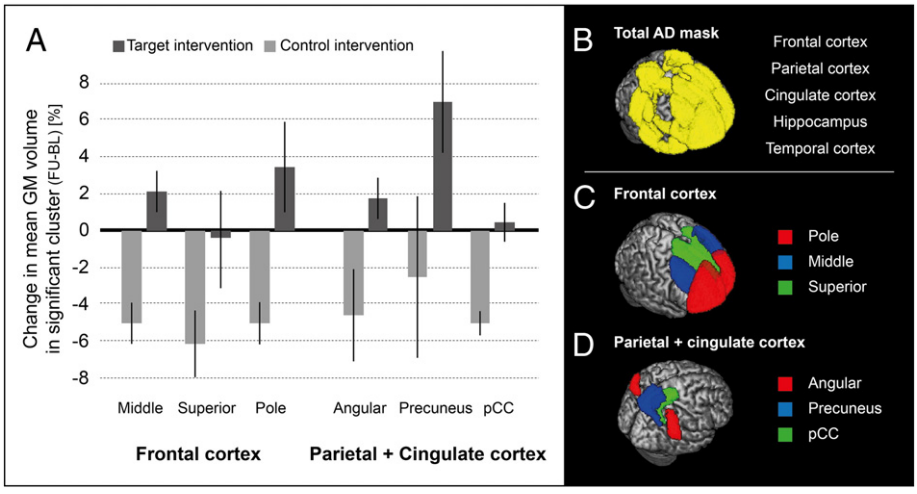

Fig. 3. A decline in cortical gray matter volume in MCI patients receiving omega-3 FA supplementation, stretching and toning training (control intervention) contrasted to reduced atrophy or even increase of gray matter volume in patients participating in six months omega-3 FA intake, aerobic exercise and cognitive stimulation (target intervention) (A). FSL-VBM analysis was conducted using a brain mask consisting of AD-vulnerable regions, i.e., the hippocampus, frontal, parietal, cingulate and temporal cortex (B). Significant changes of gray matter volume were observed within the frontal lobe (C; superior and middle frontal cortex and frontal pole), the parietal lobe (D; angular cortex and precuneus) and in the posterior cingulate cortex (D). Error bars indicate standard error of mean. BL = baseline, FU = follow-up, GM = gray matter, pCC = posterior cingulate cortex.

Table 5
Changes in anthropometry, vascular markers, physical activity and fasting serum parameters of MCI patients dependent on group.

	Target intervention (n = 13)			Control intervention (n = 9)		
	BL	FU	p-Value	BL	FU	p-Value
Weight [kg]	72.6 ± 10.2	71.9 ± 9.2	0.145[a]	68.7 ± 12.6	68.9 ± 12.5	0.738[a]
Body mass index [kg/m^2]	25.4 ± 3.5	25.3 ± 3.0	0.411[a]	24.3 ± 2.8	24.5 ± 2.6	0.310[b]
Body fat [%]	27.1 ± 6.1	25.5 ± 7.3	0.233[a]	28.0 ± 4.6	29.1 ± 5.6	0.548[a]
Positive PANAS [score]	30.9 ± 6.6	30.4 ± 6.8	0.343[a]	30.9 ± 7.3	31.7 ± 6.4	0.714[a]
Negative PANAS [score]	13.3 ± 4.9	12.1 ± 2.5	0.382[a]	14.8 ± 5.3	13.7 ± 4.6	**0.026**[b]
Systolic blood pressure [mm Hg]	147.0 ± 16.4	139.7 ± 19.4	0.167[a]	152.7 ± 36.6	143.2 ± 21.0	0.302[a]
Diastolic blood pressure [mm Hg]	89.5 ± 6.4	89.2 ± 9.7	0.917[a]	86.7 ± 12.0	84.9 ± 6.4	0.436[a]
CIMT left [μm]	645.6 ± 74.2	678.1 ± 117.3	0.411[a]	742.6 ± 132.1	700.0 ± 114.0	0.546[a]
CIMT right [μm]	665.5 ± 116.0	692.6 ± 112.8	0.889[a]	681.4 ± 106.6	667.3 ± 120.6	0.956[a]
Physical activity [kcal/week]	3442.0 ± 2500.0	4192.9 ± 2499.7	0.388[b]	3615.2 ± 3247.9	3526.2 ± 2655.8	0.515[b]
Triacylglycerides [mg/dl]	110.3 ± 47.8	83.0 ± 30.5	**0.011**[a]	100.9 ± 36.8	78.1 ± 30.9	**0.005**[a]
Total cholesterol [mg/dl]	210.2 ± 27.4	208.9 ± 41.1	0.887[a]	227.8 ± 27.7	222.9 ± 24.0	0.176[a]
LDL-to-HDL ratio	2.1 ± 0.7	2.0 ± 0.8	0.293[a]	2.4 ± 0.9	2.2 ± 0.7	0.186[a]
Homocysteine [μmol/l]	15.9 ± 7.0	12.5 ± 3.8	**0.016**[a]	17.6 ± 3.6	19.9 ± 6.4	0.367[a]
Vitamin B12 [pg/ml]	509.4 ± 255.8	555.0 ± 290.7	0.972[b]	395.0 ± 135.7	396.9 ± 127.5	0.944[a]
Folate [ng/ml]	9.7 ± 3.1	8.9 ± 3.8	0.605[a]	9.4 ± 4.3	9.6 ± 5.0	0.874[a]
HbA1c [%]	6.0 ± 0.4	6.0 ± 0.5	0.918[b]	5.8 ± 0.4	5.7 ± 0.3	0.054[a]
Insulin [mU]	8.7 ± 3.5	8.2 ± 4.3	0.622[a]	7.8 ± 3.2	6.6 ± 3.5	0.276[a]
Leptin [ng/ml]	4.0 ± 2.2	3.3 ± 2.8	0.565[a]	8.0 ± 9.4	4.0 ± 3.9	0.051[b]
BDNF [pg/ml]	4670.8 ± 1538.9	3781.2 ± 1136.9	0.087[b]	4632.2 ± 1381.1	3803.6 ± 2168.0	0.134[a]
IGF-1 [ng/ml]	138.5 ± 28.6	134.7 ± 34.7	0.724[b]	151.2 ± 49.2	155.3 ± 47.4	0.779[b]
hsCRP [pg/ml]	0.7 ± 0.6	1.0 ± 1.1	0.169[b]	1.4 ± 2.1	2.0 ± 2.8	0.612[b]
TNF-α [pg/ml]	8.4 ± 1.6	8.7 ± 2.6	0.500[a]	7.9 ± 2.4	8.2 ± 2.2	0.528[b]
Interleukin-6 [pg/ml]	2.6 ± 2.2	2.2 ± 0.6	0.715[b]	8.8 ± 20.4	5.4 ± 9.3	0.655[b]

Data expressed as mean ± SD. Significant results are highlighted in bold ($p < 0.05$). BL = baseline, FU = follow-up.
[a] Paired t-test.
[b] Wilcoxon signed-rank test.

number of APOE e4 carriers (73%) (Manning et al., 2014) or biased by the small sample size, indicated that omega-3 FA supplementation plus the control condition of stretching and toning does not suffice to prevent atrophy in these regions in MCI patients. However, compared to our previous results from omega-3 FA intervention in healthy older adults (Witte et al., 2014a), we achieved beneficial effects on brain structure in partially overlapping brain regions with the combined multimodal intervention. Such a combined interventional approach may be necessary in patients with a higher vulnerability for brain atrophy, i. e., patients with MCI, particularly if APOE e4 allele carriers (Manning et al., 2014). Omega-3 FA supplementation may provide the basis for protection and increase of gray matter volume via physical activity and cognitive stimulation (Denis et al., 2013; Lim et al., 2005; Lynch et al., 2007), given that the latter beneficial effects rely on availability of sufficient metabolic substrates for training-induced changes in neuronal membranes and other parts of brain cytoarchitecture (Mahadik et al., 2001). Thus, providing omega-3 FA, essential for membrane integrity, synaptic function, neurochemistry, and neuronal protection (Crupi et al., 2013), may allow training to exert maximal benefits.

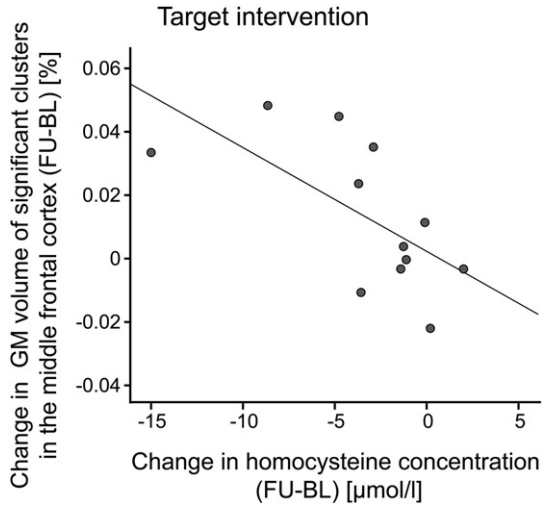

Fig. 4. Associations between changes in serum homocysteine and gray matter volume. The decrease in homocysteine concentration in patients receiving omega-3 FA supplementation, aerobic exercise and cognitive stimulation (target intervention) was significantly associated with reduced atrophy or even increase of gray matter volume within the significant cluster of the middle frontal cortex (Spearman correlation, $p = 0.010$). BL = baseline, FU = follow-up, GM = gray matter.

Target intervention-related increases in gray matter volume were found in the prefrontal cortex, most pronounced in the frontal pole, and in the precuneus. Volume changes in these areas are in line with previous findings on single interventional approaches, most notably, physical activity and cognitive stimulation interventions.

Physical activity has been shown to increase gray matter volume in prefrontal and cingulate cortex (Colcombe et al., 2006; Ruscheweyh et al., 2011), as well as temporal areas including the hippocampus (Colcombe et al., 2006; Erickson et al., 2011). An increase in physical fitness was associated with an increase in gray matter volume in the angular cortex in the current study. These changes are possibly mediated by an increase in global blood volume or activation of biochemical cascades (e.g., neurotrophin synthesis), and subsequently, new vascular and neuronal structures in areas that are most vulnerable to age-related atrophy (Bullitt et al., 2009; Hedden and Gabrieli, 2004; Thomas et al., 2012). Notably, only few intervention trials assessed the effects of physical activity on brain volume in AD at-risk patients, that is, MCI (Suzuki et al., 2013) underlining the importance of the current study.

Cognitive stimulation intervention may also specifically impact on the prefrontal cortex. The frontal pole is activated during tasks comprising social interactions, self-knowledge, person-knowledge, mentalizing, verification processes and internal action monitoring (Amodio and Frith, 2006; Tsujimoto et al., 2011). Such processes will be strengthened in groups with cognitive stimulation, such as AKTIVA that aimed to reflect and improve the attitude towards aging, disease, healthy lifestyle and self-perception of patients with MCI (Tesky et al., 2014; Tesky et al., 2011). In addition, learning of memory strategies in old age may be associated with changes in prefrontal areas that are implicated in memory selection processes (Fletcher and Henson, 2001). Moreover,

the precuneus is involved in internal representation through mental imagery, episodic memory retrieval, self-processing and consciousness (Cavanna and Trimble, 2006), implying in part functional connectivity with the frontal cortex (Zhang and Li, 2012). It is possible that such processes are activated through cognitive stimulation that focuses on self-awareness and coping with memory problems, as administered in the present study. In line with this hypothesis, structural changes in the precuneus have been found after mindfulness-meditation-training, indicating self-referential processing (Kurth et al., 2014).

Cognitive stimulation and physical activity may complement each other regarding their effects on gray matter volume (Chklovskii et al., 2004; Mahncke et al., 2006). Bamidis et al. (2014) hypothesized that in a multimodal intervention, physical exercise increases the potential for neuro-, synapto- and angiogenesis while cognitive exercise guides it. Cognitive stimulation may activate brain areas that are involved in specific tasks during enhanced neuroplasticity that in turn lead to functional integration of the newborn neurons and synapses into the respective brain networks (Geibig et al., 2012; Trachtenberg et al., 2002), a hypothesis supported by our region-specific effects on gray matter volume. Thus, the promising significant effects seen in this study on AD-related brain regions indicate a profound advantage of a combined intervention compared to single interventions.

Underlying mechanisms

In the current study, homocysteine, a risk factor for the development of atherosclerosis and neurodegenerative disorders (Brustolin et al., 2010), was decreased after six months combined intervention, whereas it remained unchanged in patients after control intervention. Decreases in homocysteine may be related to omega-3 FA supplementation, given that omega-3 FAs regulate the expression of genes encoding enzymes that are involved in homocysteine metabolism (Huang et al., 2013), but see mixed results in empirical studies (Beavers et al., 2008; Mohammadpour et al., 2013; Pooya et al., 2010; Tayebi-Khosroshahi et al., 2013). Moreover, exercise training is also known to decrease homocysteine concentrations (Randeva et al., 2002; Vincent et al., 2006; Vincent et al., 2003). Our finding of decreased homocysteine concentration in the target group after six months cycling training, in contrast to no changes after omega-3 FA supplementation plus the control condition of stretching and toning, supports the hypothesis that homocysteine changes seen in the present study might be due to increased physical activity. Additive effects of omega-3 FA and physical exercise may also be possible; however, this cannot be disentangled further due to the current study design. Although we did not find a significant increase in VO_2 peak in the target group (cycling), we believe that exercise training was conducted appropriately, given that exercise intensity was determined based on the anaerobic threshold that was reached by all patients during testing, independent of achieving age-defined target heart rate. In addition, exercise intensity was continuously adjusted during training intervention according to patient's performance (heart rate controlled) to maintain training intensity at 80% of the anaerobic threshold. However, insufficient exertion during exercise testing, leading to an underestimation of VO_2 peak (Thompson et al., 2009), at both baseline and follow-up intervention testing, might explain the lack of significant increase after the intervention period, and also the lack of stronger associations between changes in physical fitness and gray matter volume. Note though that a trend-wise increase of VO_2 peak still emerged after exercise intervention, an effect likely to be more pronounced in a larger sample.

Notably, we found that the decrease in homocysteine concentration was associated with increase of gray matter volume in the middle frontal cortex in patients of the target intervention, providing evidence for a potential mechanism underlying the effects of a combined intervention on brain structure. This is in line with a previous study that showed decreases in gray matter atrophy rate within the middle frontal cortex after homocysteine-lowering intervention (Douaud et al., 2013).

Limitations

First, the small number of MCI patients in both groups might have prevented us from observing significant changes in cognitive functions, serum, and vascular markers after the combined intervention. Surrogate markers such as gray matter volume might be more sensitive than behavioral outcomes to detect differences between a combined versus single intervention. Second, including an omega-3 FA supplementation control group instead of a placebo control group may have led to underestimation of target-intervention-related effects. However, the result of beneficial effects of a combined intervention on AD-related brain regions, compared to atrophy rates of ~4% in the single omega-3 FA supplementation group, strengthens the assumption of synergistic effects of nutritional supplementation with physical training and cognitive stimulation. Third, the increase in physical fitness after aerobic exercise intervention was not significant, either due to insufficient training intensity or due to the fact that several patients did not reach individual maximum performance during cardiorespiratory fitness testing. However, we found a significant association between improved physical fitness and increased regional gray matter volume, indicating intervention-driven effects. Moreover, previous studies likewise demonstrated beneficial effects on brain structure after increased physical activity, regardless of its intensity (Ruscheweyh et al., 2011). Fourth, we did not include additional groups with physical exercise intervention alone or placebo capsules only. Thus, we were not able to disentangle the specific contribution of individual interventions to the beneficial impact on brain structure. However, in line with recent studies in the field (Kivipelto et al., 2013) the primary focus of the present study was to investigate if a combined lifestyle intervention would exceed the beneficial effects of a single lifestyle intervention regarding brain function and structure, without aiming to disentangle in detail each individual contribution.

Conclusion

In the present study, we demonstrated that a combined intervention with omega-3 FA, aerobic exercise and cognitive stimulation significantly reduced gray matter atrophy in AD-related brain regions in patients with MCI, compared to omega-3 FA intake plus control condition of stretching and toning. These results extend previous findings on exercise or cognitive stimulation to a combined approach of dietary supplements, aerobic exercise and cognitive stimulation, and suggest a possible well-tolerated and widely applicable approach to prevent structural and possibly also functional decline in MCI. This hypothesis should now be tested in larger randomized-controlled trials.

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Conflict of interest

The authors declare no conflict of interest.

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