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Vitamin B-12 concentration, memory performance, and hippocampal structure in patients with mild cognitive impairment[1,2]

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ABSTRACT

Background: Low-normal concentrations of vitamin B-12 (VitB12) may be associated with worse cognition. However, previous evidence has been mixed, and the underlying mechanisms remain unclear.

Objective: We determined whether serum VitB12 concentrations within the normal range were linked to memory functions and related neuronal structures in patients with mild cognitive impairment (MCI).

Design: In a cross-sectional design, we assessed 100 amnestic MCI patients (52 women; age range: 50–80 y) with low- and high-normal VitB12 concentration (median split: 304 pmol/L) for memory functions with the use of the Auditory Verbal Learning Test. MRI was performed at 3 tesla (n = 86) for the estimation of the volume and microstructure of the hippocampus and its subfields as indicated by the mean diffusivity on diffusion-weighted images. With the use of a mediation analysis, we examined whether the relation between VitB12 and memory performance was partially explained by volume or microstructure.

Results: MCI patients with low-normal VitB12 showed a significantly poorer learning ability (P = 0.014) and recognition performance (P = 0.008) than did patients with high-normal VitB12. Also, the microstructure integrity of the hippocampus was lower in patients with low-normal VitB12, mainly in the cornu ammonis 4 and dentate gyrus region (P = 0.029), which partially mediated the effect of VitB12 on memory performance (32–48%). Adjustments for age, sex, education, apolipoprotein E e4 status, and total homocysteine, folate, and creatinine did not attenuate the effects.

Conclusions: Low VitB12 concentrations within the normal range are associated with poorer memory performance, which is an effect that is partially mediated by the reduced microstructural integrity of the hippocampus. Future interventional trials are needed to assess whether supplementation of VitB12 may improve cognition in MCI patients even in the absence of clinically manifested VitB12 deficiency. This trial was registered at clinicaltrials.gov as NCT01219244. *Am J Clin Nutr* 2016;103:1045–54.

Keywords: episodic memory, hippocampus, MCI, mean diffusivity, vitamin B-12

INTRODUCTION

Impaired nutrition may contribute to the incidence of age-associated diseases including mild cognitive impairment (MCI)[13] and Alzheimer disease (AD) (1). Vitamin B-12 (VitB12) deficiency (<150 pmol/L) has been linked with increased risk of neuropathy, demyelination, cognitive decline, and dementia in addition to well-described hematologic and gastrointestinal symptoms as shown in numerous case-control (2–4) and epidemiologic (5) studies. Low concentrations of serum VitB12 and its active fraction holotranscobalamin result in an increase of total homocysteine (tHcy) and methylmalonic acid (MMA); folate deficiency leads to elevated tHcy concentrations (6, 7) and physiologic alterations that have also been associated with cognitive decline (2, 4, 7, 8). Already low-normal concentrations of VitB12 (150–300 pmol/L), which are particularly present in older adults (6), may indicate deficits in VitB12 metabolism (9) and cause neurologic symptoms even before the presence of hematologic abnormalities (10). At VitB12 concentrations >300 pmol/L, MMA (11), tHcy (8), and DNA (12) disruptions may be efficiently

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[2] Supplemental Material and Supplemental Tables S1–S5 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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[13] Abbreviations used: AD, Alzheimer disease; $ANCOVA_{RM}$, repeated-measures ANCOVA; apoE, apolipoprotein E; BDNF, brain-derived neurotrophic factor; CA, cornu ammonis; cB12, combined indicator of vitamin B-12 status; DG, dentate gyrus; DTI, diffusion tensor imaging; DV, dependent variable; ICV, intracranial volume; IV, independent variable; MCI, mild cognitive impairment; MD, mean diffusivity; MMA, methylmalonic acid; MV, mediator variable; SNP, single nucleotide polymorphisms; tHcy, total homocysteine; VitB12, vitamin B-12.

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reduced. Evidence from human cross-sectional (13, 14) and longitudinal (7, 15) studies have suggested that VitB12 status in the clinically normal range is positively correlated with cognitive function. However, some studies did not find associations (16, 17). Low VitB12 concentrations have also been associated with an accelerated rate of whole-brain atrophy (18) and insufficient myelination, leading to damage of the white matter (3, 19, 20). High-dose B-vitamin treatment is able to slow down gray-matter atrophy in AD-related regions (21, 22). In contrast, Scott et al. (23) showed no linkage between VitB12 status and the brain structure.

Studies on the association between all 3 variables (i.e., VitB12, memory performance, and brain structure) in MCI patients, who are a high-risk group for AD, are lacking (21, 24). Furthermore, it is unknown whether low VitB12 status is independently associated with the brain structure as well as cognitive scores or if a decline in the brain structure and connectivity mediates a loss of cognitive abilities.

Taken together, studies on the effects of low-normal VitB12 and associated B-vitamin metabolism on the brain structure and related cognitive functions in humans are still scarce and not unequivocal. Because of the high and increasing numbers of patients who suffer from MCI (25) and the high prevalence of low-normal VitB12 concentrations that affect up to 20% of the older population (>60 y of age) (26), these questions are also of high clinical interest. Therefore, we compared memory performance and the volume and microstructure of the hippocampus and its subfields in MCI patients with low-normal serum VitB12 concentrations with those in MCI patients with high-normal serum VitB12 concentrations. Furthermore, we used a simple mediation analysis to evaluate pathways that may link these variables. In addition, we assessed how these associations would be modulated if a combined score of VitB12 status (cB12), which was derived from serum VitB12, holotranscobalamin, tHcy, and MMA (27), was used instead of serum VitB12 only.

METHODS

Study participants

In total, 100 patients (age range: 50–80 y) with MCI were recruited in Berlin (from the memory clinic of the Department of Neurology of the Charité University Hospital and a neurology specialist practice) and in Frankfurt am Main (Institute of General Practice), Germany. MCI patients (amnestic; single domain and multiple domains) were diagnosed according to Mayo criteria that were based on subjective cognitive complaints and objective memory impairment in standardized tests [performing ≥1.5 SDs below the age- and education-specific norm in relevant subtests (Total Word List and Delayed Recall Word and Figures) of the Consortium to Establish a Registry for Alzheimer's Disease-Plus test battery (28)], relatively preserved general cognition, no impairment in activities of daily living, and no dementia (25). At the time of the study, patients were not treated with VitB12 or folic acid supplements and were also not treated with medication approved for the treatment of dementia of the AD type [i.e., with acetylcholinesterase inhibitors or memantine (29)]. Exclusion criteria comprised severe untreated medical, neurologic, or psychiatric disease, brain pathologies identified in the MRI scan, and nonfluent German-language abilities. Psychiatric comorbidity was monitored with the use of the Beck's Depression Inventory (30) and the State-Trait Anxiety Inventory (31). Detailed sample characteristics of the included participants are summarized in **Table 1**. The study was approved by the Ethics Committee of the Charité University Hospital Berlin, Germany and was conducted 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 of the trial. This trial was registered at clinicaltrials.gov as NCT01219244.

Assessment of blood variables and single nucleotide polymorphism genotyping

All subjects underwent venous blood sampling after fasting overnight for ≥10 h. Serum concentrations of VitB12 and folate were measured with the use of an immunoassay and electrochemiluminescent technology (Cobas 8000 Analyzer; Roche Diagnostics), holotranscobalamin was measured with the use of a chemiluminescent microparticle immunoassay (Architect System i 2000SR Analyzer; Abbott), and serum creatinine was measured with the use of an enzymatic hydrogen peroxide color reaction (Cobas 8000 Analyzer; Roche Diagnostics). Plasma tHcy was analyzed with the use of HPLC (ClinRep HPLC Complete Kit, Recipe), and MMA was analyzed with the use of liquid chromatography with mass spectrometry (ClinMass Complete Kit, Recipe). Holotranscobalamin and MMA were not assessed in 4 patients because of an insufficient blood sample volume. DNA was extracted from whole blood with the use of a blood minikit (Qiagen) and stored at −80°C until analysis. The genotyping of the single nucleotide polymorphism (SNP) brain-derived neurotrophic factor (BDNF) rs6265, catechol-O-methyltransferase rs4680, apolipoprotein E (apoE) rs429358, and rs7412 that have been previously implicated in episodic memory performance (33–35) were performed with the use of the iPLEX Gold Sequenom MassARRAY system (Sequenom) and a predesigned Taqman assay (applied biosystems) at the laboratory of Dan Rujescu (University of Halle), after procedures that have been described previously (36). The genotyping of BDNF SNPs was not possible for one patient because of degraded DNA.

Assessment of memory functions

Learning and memory performances of all MCI patients were tested with the use of the German version of the Rey Auditory Verbal Learning Test (37). Patients were asked to learn a list of 15 words within 5 immediate recall trials followed by a 30-min delayed recall and delayed recognition test. Learning ability was defined as the sum of words learned in all 5 trials (maximum: 75 words); delayed recall represented the total number of remembered words after 30 min (maximum: 15 words). For delayed recognition, MCI patients were asked to recognize the 15 original words presented within 35 distractor words subsequent to the delayed recall tests (number of correctly recognized words minus false positives; maximum: 15 words). All testing was conducted by trained staff members according to standard procedures.

TABLE 1
Characteristics of MCI patients with low- and high-normal serum VitB12 concentrations with the inclusion of cardiovascular disease risk factors and potential confounders[1]

	VitB12 concentration, pmol/L		
Characteristic variable	Low (<304)	High (≥304)	*P*
VitB12, pmol/L	235.0 ± 42.0 (153–303)[2]	462.7 ± 123.0 (306–934)	<0.001[3]
Holotranscobalamin, pmol/L	65.9 ± 30.0 (11–128)	104.9 ± 25.1 (55–128)	<0.001[4]
Total (women), *n*	50 (23)	50 (29)	0.230[5]
Age, y	69 ± 7.8 (50–80)	69 ± 7.8 (50–80)	0.711[4]
Education, y	14.9 ± 3.8 (8–29)	15.3 ± 3.4 (6–23)	0.618[4]
BMI, kg/m^2	25.5 ± 3.0 (19–32)	26.3 ± 3.8 (19–37)	0.255[4]
Right handedness,[6] %	83.1 ± 32.7 (−50–100)	82.3 ± 24.1 (−10–100)	0.430[3]
Systolic blood pressure, mm Hg	140.0 ± 18.4 (107–177)	143.9 ± 18.9 (112–188)	0.344[4]
Smoking, pack-years	6.2 ± 10.7 (0–40)	9.2 ± 15.4 (0–60)	0.785[3]
Physical activity, kcal/wk	3497.4 ± 3005.8 (103–13,034)	3114.1 ± 2241.3 (321–8090)	0.868[3]
Beck's Depression Index, score	9.3 ± 5.9 (0–21)	10.1 ± 5.8 (0–21)	0.519[4]
State-Trait Anxiety Inventory X1 score	38.8 ± 8.7 (20–59)	40.0 ± 10.0 (22–69)	0.541[4]
Mini-Mental State Examination score	28.2 ± 1.7 (24–30)	28.3 ± 1.3 (24–30)	0.698[4]
Total homocysteine, μmol/L	17.0 ± 6.6 (3–31)	14.6 ± 7.1 (6–39)	0.019[3,*]
Methylmalonyl acid, μmol/L	0.24 ± 0.1 (0.11–0.50)	0.15 ± 0.04 (0.08–0.28)	<0.001[3]
Folate, ng/mL	9.8 ± 3.9 (4–20)	11.6 ± 5.0 (4–20)	0.042[4,*]
Creatinine, mg/dL	0.94 ± 0.21 (0.6–1.6)	0.86 ± 0.16 (0.5–1.3)	0.077[3]
Apolipoprotein_rs429358 and rs7412, e4−:e4+, *n*	22:28	29:21	0.161[5]
BDNF_rs6265, Val/Val:Met carriers (Met/Met), *n*	33:16 (1)	38:12 (0)	0.382[5]
COMT_rs4680, Val/Val:Met carriers (Met/Met), *n*	10:40 (16)	13:37 (14)	0.476[5]

[1]*$P < 0.05$. BDNF, brain-derived neurotrophic factor; COMT, catechol-*O*-methyltransferase; MCI, mild cognitive impairment; VitB12, vitamin B-12.
[2]Mean ± SD; range from minimum to maximum in parentheses (all such values).
[3–5]Group comparisons were performed with the use of the following tests: [3]Mann-Whitney-*U* test, [4]unpaired *t* test, [5]chi-square test.
[6]Refers to the use of the right hand in daily activities and was measured according to the Edinburgh Handedness Inventory (32).

MRI acquisition

MRI scanning was conducted with the use of a 3-tesla Siemens Trio system (Siemens AG) with a 12-channel head coil at the Berlin Center for Advanced Neuroimaging. High-resolution 3-dimensional T1-weighted scans (magnetization prepared rapid acquisition with gradient echoes; repetition time: 1900 ms; echo time: 2.52 ms; 192 sagittal slices; voxel-size of 1.0 × 1.0 × 1.0 mm^3; flip angle: 9°), and diffusion-weighted spin-echo echo-planar imaging scans (repetition time: 7500 ms; echo time: 86 ms; 61 axial slices; voxel size of 2.3 × 2.3 × 2.3 mm^3; 64 directions with a *b* value of 1000 s/mm^2 and 10 directions with a *b* value of 0 s/mm^2) were acquired. Image preprocessing and the analysis were done with the use of the software packages FSL 4.1 (Analysis Group, FMRIB; www.fmrib.ox.ac.uk/fsl) and FreeSurfer 5.3 (Laboratory for Computational Neuroimaging, http://surfer.nmr.mgh.harvard.edu/). Fourteen subjects had to be excluded from the image analysis because of the inaccessibility of MRI scans ($n = 2$ metallic implants; $n = 12$ because of logistic problems).

Hippocampal volume

An automated hippocampal subfield segmentation was carried out according to Van Leemput et al. (38) to extract individual volumes of cornu ammonis (CA) fields 1–4 (CA1–CA4), dentate gyrus (DG), subiculum, and the hippocampus tail. Therefore, high-resolution T1-weighted images were registered to standard space of the Monteal Neurological Institute by rigid-body transformation before segmentation with the use of the FSL 4.1 software package. Cortical and subcortical reconstructions and volumetric segmentation, including of the hippocampus, were performed with the use of the FreeSurfer 5.3 software package. Briefly, processing included motion correction, intensity normalization, and skull stripping with the use of a watershed algorithm [more technical details are described in Fischl et al. (39)]. Individual hippocampus volumes were adjusted for the intracranial volume (ICV) according to previous studies (40, 41) with the use of the following formula:

$$\text{Adjusted volume} = \text{raw volume } b \times (\text{ICV mean ICV}) \quad (1)$$

The coefficient *b* represents the slope of the regression of a region-of-interest volume on the ICV. Results of the region-of-interest segmentation were superimposed on anatomic images and visually inspected to exclude a misregistration or erroneous region-of-interest identification.

Hippocampal microstructure

The microstructure of the hippocampus was assessed by the mean diffusivity (MD), which was estimated with the use of diffusion tensor imaging (DTI) in line with previous studies (40, 41). Therefore, a tensor model was fitted to the motion-corrected DTI data at each voxel to create individual 3-dimensional maps of fractional anisotropy and the MD. Individual T1-weighted images were co-registered to the fractional anisotropy maps (more suitable for correct registration than MD maps are) with the use of rigid-body transformations. The transformation matrices obtained were used to transform masks of the total hippocampus and hippocampus subfields (derived by segmentation with the use of the FreeSurfer 5.3 software package) to the MD maps for the

extraction of mean individual hippocampal MD values. Higher MD values are considered to reflect the faster diffusion of water molecules in the tissue because of a loss of membrane integrity or synaptic contacts and, thus, are considered to be inverse measures of the integrity of neuronal cell bodies, axons, and dendrites in cerebral gray matter (40, 41).

Statistical analyses

General

VitB12 concentrations ≥150 and <300 pmol/L have generally been defined as being in the low-normal range, and concentrations ≥300 pmol/L have been defined as being in the high-normal range (8, 42, 43). In the current study, the median VitB12 concentration was shown to be 304 pmol/L, which was very close to the boundary between low- and high-normal concentrations. As a primary analysis, the MCI cohort was split on the median of the serum VitB12 concentration in a group with low-normal (153–303 pmol/L) and a group with high-normal (304–934 pmol/L) VitB12 concentrations. Subsequently, group means were compared for baseline characteristics, memory performances, and hippocampal structures. In addition, we calculated a combined indicator of vitamin B-12 status (cB12), which included measures of serum VitB12, holotranscobalamin, tHcy, and MMA according to Fedosov et al. (27) to exclude VitB12 deficiency in the MCI cohort and to more fully account for all 4 markers of VitB12 metabolism (**Supplemental Material**). Moreover, in a secondary explorative analysis, we assessed how these associations would be modulated by the use of a cB12 score (derived from all 4 biomarkers) as an independent variable (IV). Therefore, we split the cB12 at the median by obtaining 2 groups with low-adequate VitB12 status and high-adequate VitB12 status in line with our primary analysis. Thus, a comparison of memory performances and hippocampus brain structures between VitB12 groups were similar in both primary and secondary analyses. To detect associations between intrinsically linked serum VitB12, holotranscobalamin, folate, tHcy, and MMA, we ran bivariate correlations (Spearman rank). All variables were tested for normal distribution (unimodal, |skewness| <1). Accordingly, parametric or nonparametric tests were calculated as appropriate. A 2-sided level of significance was set at $\alpha = 0.05$. No adjustment for multiple testing was applied. In case of missing values, patients were excluded from the particular analysis. SPSS 22.0 software (PASW, SPSS; IBM) was used for the analysis.

Baseline characteristics

Demographic characteristics and cardiovascular and genetic risk factors were compared between groups with the use of independent *t* tests, the Mann-Whitney *U* test, or the chi-square-test as indicated (Table 1).

Memory and hippocampus structure

To assess between-group differences in episodic memory performance, we first conducted a repeated-measures ANCOVA ($ANCOVA_{RM}$) that included all 3 Auditory Verbal Learning Test subtasks in one model, which was followed by separate 1-factor univariate ANCOVAs for the single subtasks. In a subsequent exploratory analysis of the potential underlying mechanisms that link VitB12 concentrations with memory performance, we conducted ANCOVAs to assess potential differences in structural measures of the total hippocampus and its subfields. Initially, all analyses were conducted unadjusted. Subsequently, apoE e4 status, tHcy, folate, and creatinine, which were variables that showed a difference between VitB12 groups, were entered together with age, sex, and years of education as covariates. All factors are known to be associated with the VitB12 concentration, cognition, and brain structure. With such adjusted regression models, we also aimed to study whether potential effects were independent or might have been mediated by the elements of one-carbon metabolism such as tHcy and folate (14, 18). We report unstandardized regression coefficients (referred to as βs) ± SEs that were interpreted as average differences between groups, which were also determined after adjustment for confounders. To examine the relation between age and VitB12 on cognitive outcomes or the hippocampus structure, we calculated a regression model with an additional interaction term for the VitB12 concentration and age.

Mediation analysis

The relation between a predictor and an outcome can be mediated in total or in part by a mediator variable (MV), which has been conceptualized as a mechanism through which the predictor influences the outcome (44). To test the hypothesis that the MD, which is indicative of microstructural integrity, of the hippocampus CA4 and DG subfields mediates the influence of the VitB12 concentration on memory outcomes, the multiple mediation macro called INDIRECT of Preacher and Hayes (45) was used in the SPSS program for a simple mediation analysis, which was similar that used in previously established studies (41, 46). This toolbox allowed for the evaluation of the indirect effect between the IV and the dependent variable (DV) through a mediator (MV) while taking covariates (sex, age, education, apoE e4 status, tHcy, folate, and creatinine) into account. A bootstrapping resampling strategy was used with 5000 bootstrap samples for testing the hypotheses of mediation. In the current study, path *a* represents the direct effect of the IV (VitB12) on the MV (MD of CA4 and DG), and path *b* indicates the effect of the MV on the DV (learning ability and recognition). Path *c* denotes the total effect of the IV (plus MV) on the DV, whereas path *c′* shows the direct effect of the IV (minus MV) on the DV. Here, we report only models that met the criteria for mediation; there were significant associations ($P < 0.05$) of paths *a*, *b*, and c and nonsignificant associations of path *c′*. A bias-corrected 95% CI was calculated to determine the contribution of the mediator (indirect effect; path $a \times b$), which reached significance when the interval range excluded zero. Unstandardized and standardized coefficients are reported that show the relations between the single variables.

RESULTS

In total, 100 MCI patients were included into the study and, according to VitB12 concentrations, were stratified into a low-normal–VitB12 group that included 50 patients (23 women; mean age: 69 y) and into a high-normal–VitB12 group that included 50 patients (29 women; mean age: 69 y). VitB12 groups did not differ significantly with regard to age, sex, education,

cardiovascular disease risk factors, and allelic variant frequency of memory-associated gene polymorphisms (apoE e4-, BDNF-, and catechol-*O*-methyltransferase–SNP carrier status) (all $P > 0.05$). However, concentrations of holotranscobalamin ($P < 0.001$) and folate ($P = 0.042$) were significantly lower, and tHcy ($P = 0.019$) and MMA ($P < 0.001$) were significantly higher, in the group with low-normal VitB12, which, to some extent, were attributable to a significant positive correlation between VitB12 and holotranscobalamin (Spearman correlation: 0.601; $P < 0.001$) and VitB12 and folate (Spearman correlation: 0.269; $P = 0.007$) and a significant negative correlation between VitB12 and tHcy (Spearman correlation: −0.279; $P = 0.005$) and VitB12 and MMA (Spearman correlation: −0.465; $P < 0.001$). Furthermore, serum creatinine concentrations tended to be higher in low-normal–VitB12 patients than in the high-normal–VitB12 patients ($P = 0.077$) (Table 1). All patients displayed cB12 scores that were calculated from the biomarkers serum VitB12, holotranscobalamin, tHcy, and MMA within the low-normal (−1.5 to −0.5) or adequate (−0.5 to +1.5) range of VitB12 status, which was defined according to Fedosov et al. (5), and showed no symptoms of clinically overt VitB12 deficiency, indicating the absence of this condition in the current cohort (Supplemental Material). cB12 groups, which were obtained after a median split, did not differ significantly with regard to patient characteristics except for folate and creatinine concentrations (**Supplemental Table S1**).

VitB12 and memory performance

MCI patients with low-normal VitB12 concentrations showed significantly poorer memory performance [$ANCOVA_{RM}$; $F_{(1,97)} = 4.34$, $P = 0.016$], specifically in learning-ability scores ($P = 0.014$, Cohen's $d = 0.50$) and recognition scores ($P = 0.008$, Cohen's $d = 0.56$), and also a trend for lower delayed-recall scores ($P = 0.069$, Cohen's $d = 0.37$) in comparison with MCI patients with high-normal VitB12 concentrations. Adjustment for potential risk factors and confounders such as age, sex, education, apoE e4 status, tHcy, folate, and creatinine did not attenuate the effects on memory performance [$ANCOVA_{RM}$; $F_{(1,97)} = 3.49$, $P = 0.040$; P-learning = 0.027; P-recognition = 0.02] (**Table 2**). Furthermore, with regard to recognition performance but not learning ability, we showed a significant interaction between the VitB12 concentration and the age of patients [ANCOVA; $F_{(1,96)} = 5.74$, $P = 0.019$, Cohen's $d = 0.48$], which suggested that the observed association of the VitB12 concentration and recognition was more pronounced in older patients. With the use of the cB12, these associations remained significant except for learning ability (**Supplemental Table S2**).

VitB12 and hippocampal volume and microstructure

As regards the volume of the total hippocampus and its subfields, we observed no difference between patients with low-normal VitB12 concentrations and patients with high-normal VitB12 concentrations (**Supplemental Table S3**). For the hippocampal microstructure, MCI patients with low-normal VitB12 concentrations showed a significantly higher MD, which indicated worse hippocampal microstructural integrity (41) in the hippocampus subfields CA4 and DG ($P = 0.029$, Cohen's $d = 0.49$) than did patients with high-normal VitB12 concentrations. Adjustment for potential risk factors and confounders such as age, sex, education, apoE e4 status, tHcy, folate, and creatinine did not attenuate the effect for CA4 and DG ($P = 0.011$). Furthermore, a trend for a higher MD was seen within the hippocampus tail ($P = 0.081$, Cohen's $d = 0.39$) of MCI patients with low-normal VitB12 concentration, which reached significance after full adjustment for age, sex, education, apoE e4, tHcy, folate, and creatinine ($P < 0.05$). Moreover, after full adjustment, significant differences were also seen for the CA2/3 subfields ($P = 0.040$, Cohen's $d = 0.46$) (**Table 3**). In addition, with regard to CA4 and DG subfields, we showed a trend for an interaction between the VitB12 concentration and the age of the patients [ANCOVA; $F_{(1,82)} = 3.20$, $P = 0.077$, Cohen's $d = 0.39$], which suggested that the observed difference in the MD was more pronounced in older than in younger patients. These associations remained significant with the cB12 (**Supplemental Table S4**).

Mediation model analysis

A single-level mediation analysis indicated a significant indirect effect of the VitB12-group difference in learning ability performance ($\beta = 2.46$; 95% CI: 0.38, 5.12) and recognition memory performance ($\beta = 1.00$; 95% CI: 0.22, 2.07) through the MD of the hippocampus CA4 and DG subfields in MCI patients. This mediation effect remained significant after full adjustment for age, sex, education, apoE e4, tHcy, folate, and creatinine (**Figure 1**, **Table 4**). The mediator (path $a \times b$) explained 48% of the total effect (path c) of the VitB12 concentration on learning ability and 32% on recognition memory performance. More specifically, the association between a low-normal VitB12 concentration and poorer learning and recognition performances was in

TABLE 2
Episodic memory performance in MCI patients with low- and high-normal serum VitB12 concentrations adjusted for confounders ($n = 100$)[1]

	VitB12 concentration[2]		Effect of VitB12 between groups[3]	
Memory variable	Low ($n = 50$)	High ($n = 50$)	Unadjusted	Adjusted
Learning ability	40.74 ± 10.24 (20–60)	45.64 ± 9.40 (27–68)	4.90 ± 1.96 (0.014*)	4.20 ± 1.87 (0.027*)
Delayed recall	6.18 ± 4.12 (0–14)	7.56 ± 3.35 (0–14)	1.38 ± 0.75 (0.069)	1.07 ± 0.73 (0.146)
Recognition memory	6.54 ± 6.82 (−11 to 15)	9.68 ± 4.62 (−7 to 15)	3.14 ± 1.17 (0.008**)	2.86 ± 1.21 (0.020*)

[1]Univariate ANCOVA was used for group comparisons with adjustment for age, sex, education, apoE e4 status, folate, and creatinine. apoE, apolipoprotein E; MCI, mild cognitive impairment; tHcy, total homocysteine; VitB12, vitamin B-12.

[2]All values are mean ± SD numbers of words; ranges from minimum to maximum in parentheses.

[3]All values are βs (unstandardized regression coefficients) ± SEs; P values in parentheses. *$P < 0.05$, **$P < 0.01$.

TABLE 3
Microstructures of the total hippocampus and hippocampal subfields that are dependent on VitB12 concentrations were measured with the mean diffusivity, which was estimated with the use of diffusion tensor imaging and adjusted for confounders (n = 86)[1]

	VitB12 concentration		Effect of VitB12 between groups	
Mean diffusivity	Low (n = 41)	High (n = 45)	Unadjusted	Adjusted
Total hippocampus	1.23 ± 0.13 (1.06–1.61)	1.20 ± 0.10 (1.01–1.41)	−2.70 ± 2.45 (0.274)	−3.14 ± 2.05 (0.130)
CA1	1.19 ± 0.18 (0.92–1.63)	1.16 ± 0.17 (0.86–1.67)	−2.78 ± 3.73 (0.458)	−3.37 ± 3.54 (0.344)
CA2/3	1.29 ± 0.20 (0.99–1.91)	1.24 ± 0.14 (1.00–1.52)	−4.95 ± 3.70 (0.185)	−6.68 ± 3.19 (0.040*)
CA4 and DG	1.16 ± 0.18 (0.85–1.84)	1.09 ± 0.11 (0.93–1.37)	−7.08 ± 3.19 (0.029*)	−8.13 ± 1.13 (0.011*)
Subiculum	0.95 ± 0.08 (0.82–1.24)	0.94 ± 0.06 (0.82–1.15)	−0.72 ± 1.60 (0.655)	−0.81 ± 1.69 (0.632)
Hippocampus tail	1.20 ± 0.20 (0.94–1.83)	1.13 ± 0.13 (0.94–1.51)	−6.42 ± 3.63 (0.081)	−7.47 ± 3.39 (0.031*)

[1]Note that higher mean diffusivity refers to worse microstructural integrity. Fourteen subjects were excluded from the mean diffusivity analysis because of missing MRI scans. A univariate ANCOVA was used for group comparison with adjustment for age, sex, education, apoE e4 status, tHcy, folate, and creatinine. apoE, apolipoprotein E; CA, cornu ammonis; DG, dentate gyrus; tHcy, total homocysteine; VitB12, vitamin B-12.

[2]All values are means ± SDs (10^{-3} mm^2/s); ranges from minimum to maximum in parentheses.

[3]All values are βs (unstandardized regression coefficients) ± SEs (10^{-5}); P values in parentheses. *$P < 0.05$.

part mediated by a worse integrity of the hippocampus CA4 and DG microstructure. A significant mediation by other hippocampus subfields was not shown. The effect of the cB12 on recognition performance was likewise mediated by the hippocampus CA4 and DG microstructure (**Supplemental Table S5**).

DISCUSSION

In the current study, we showed that VitB12 in the low-normal range (≥150 and <304 pmol/L) was associated with poorer memory performance that was partially mediated through its negative effect on the microstructure of CA4 and DG subfields in MCI patients. VitB12 deficiency was excluded in all patients on the basis of a combined indicator of the 4 biomarkers serum VitB12, holotranscobalamin, tHcy, and MMA (i.e., cB12). All associations remained significant after correction for sex, apoE e4 status, tHcy, folate, and creatinine. The association of VitB12 and memory performance was more pronounced in older patients; however, adjustment for age did not attenuate the observed effects. In a secondary analysis, the observed associations were largely confirmed with the use of the cB12. Our findings complement those of previous studies that suggested that low-normal VitB12

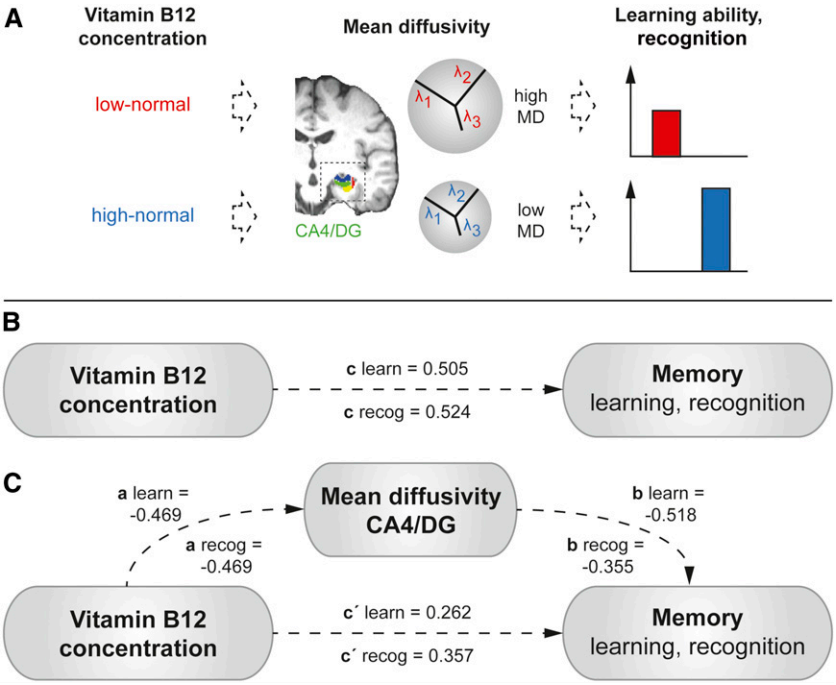

FIGURE 1 Schematic overview of the results of the single-level mediation analysis (n = 86). (A) Low-normal VitB12 concentrations are associated with poorer learning ability and recognition performance in MCI patients, which are partially mediated by a reduced microstructural integrity (MD*-1) of the hippocampal CA4 and DG subfields. (B) c denotes the total effect of the VitB12 concentration on memory performance. (C) a denotes a direct effect of VitB12 to the MD of CA4 and DG; b denotes the direct effect of the MD of CA4 and DG on memory performance; and c' denotes a direct effect of the VitB12 concentration on memory performance with the MD of CA4 and DG controlled for; an indirect effect of the VitB12 concentration on memory performance through the MD of CA4 and DG can be calculated by the multiplication of paths a and b. Dashed arrows indicate associative and no causal relations. MD = ($\lambda 1 + \lambda 2 + \lambda 3$) ÷ 3. CA, cornu ammonis; DG, dentate gyrus; learn, learning ability; MD, mean diffusivity; recog, recognition; VitB12, vitamin B-12.

TABLE 4
Mediation of VitB12 group differences in learning ability (models A and B) and recognition performance (models C and D) through the hippocampal microstructure of CA4 and DG subfields (n = 86)[1]

Effect	Coefficient ± SE (mediation, %)	t	P	95% CI (bias corrected)
Model A				
a (IV − MV)	−0.0001 ± 0.00	−2.22	0.029	—
b (MV − DV)	−35,026.9 ± 6383.6	−5.49	<0.001	—
c [(IV − DV) + MV]	5.16 ± 2.16	2.38	0.019	—
c' [(IV − DV) − MV]	2.68 ± 1.92	1.40	0.166	—
$a \times b$ (IV − MV − DV)	2.46 ± 1.21 (48)	—	—	0.38, 5.12
Model B				
a (IV − MV)	−0.0001 ± 0.00	−2.61	0.011	—
b (MV − DV)	−25,242.7 ± 7064.8	−3.57	0.006	—
c [(IV − DV) + MV]	5.14 ± 2.09	2.47	0.016	—
c' [(IV − DV) − MV]	3.08 ± 2.03	2.47	0.133	—
$a \times b$ (IV − MV − DV)	2.07 ± 1.01 (40)	—	—	0.44, 4.45
Model C				
a (IV − MV)	−0.0001 ± 0.00	−2.22	0.029	—
b (MV − DV)	−14,092.0 ± 4147.2	−3.40	0.001	—
c [(IV − DV) + MV]	3.14 ± 1.29	2.44	0.017	—
c' [(IV − DV) − MV]	2.15 ± 1.25	1.72	0.089	—
$a \times b$ (IV − MV − DV)	1.00 ± 0.46 (32)	—	—	0.22, 2.07
Model D				
a (IV − MV)	−0.0001 ± 0.00	−2.62	0.011	—
b (MV − DV)	−12,017.9 ± 4832.6	−2.49	0.015	—
c [(IV − DV) + MV]	3.38 ± 1.37	2.46	0.016	—
c' [(IV − DV) − MV]	2.40 ± 1.39	1.73	0.088	—
$a \times b$ (IV − MV − DV)	0.98 ± 0.48 (29)	—	—	0.24, 2.19

[1]In model A, the IV was VitB12, the MV was MD of CA4 and DG, and the DV was learning ability; not adjusted. In model B, the IV was VitB12, the MV was MD of CA4 and DG, and the DV was learning ability; fully adjusted. In model C, the IV was VitB12, the MV was MD of CA4 and DG, and the DV was recognition; not adjusted. In model D, the IV was VitB12, the MV was MD of CA4 and DG, and the DV was recognition; fully adjusted. A single-level mediation analysis (INDIRECT) was conducted, which was unadjusted (models A and C) and adjusted for age, sex, education, apoE e4, tHcy, folate, and creatinine (models B and D) with the use of a bootstrapping strategy with 5000 resamples. Unstandardized coefficients with SE, t, and P values are depicted in the table. In the models, a denotes the direct effect of VitB12 to MD of CA4 and DG; b denotes the direct effect of MD of CA4 and DG on memory performance; c denotes the total effect of VitB12 on memory performance; c' denotes the direct effect of VitB12 on memory performance with MD of CA4 and DG controlled for; and $a \times b$ denotes the indirect effect of VitB12 on memory performance through MD of CA4 and DG (percentage of path c). The significance of the mediator (indirect effect) was determined by the bias-corrected 95% CI excluding zero. apoE, apolipoprotein E; CA, cornu ammonis; DG, dentate gyrus; DV, dependent variable; IV, independent variable, MD, mean diffusivity; MV, mediator variable; tHcy, total homocysteine; VitB12, vitamin B-12.

contributes to memory dysfunction (47, 48), cognitive decline, and increased risk of AD (5, 7, 13–15). Interventional studies with B-vitamin supplementation have further supported a causal relation between VitB12 and cognitive function (49, 50). For instance, the supplementation of B vitamins (vitamin B-6, VitB12, and folate) resulted in an improvement of episodic memory and semantic memory even in subjects with high-normal baseline concentrations of VitB12 (332 pmol/L) (49), which pointed to positive effects of higher VitB12 concentrations that were already within the normal range. However, it could not be excluded that these effects were at least partially due to increases in folate or vitamin B-6 concentrations (49). In agreement with these findings, we observed better learning and recognition in patients with VitB12 concentrations >303 pmol/L than in patients with low-normal VitB12, which indicated that the current threshold for VitB12 supplementation (150 pmol/L) (42) in older adults may not be adequate, particularly when first cognitive deficits are present. However, other studies did not report detrimental effects of low VitB12 on memory performance (16, 51) or improved memory functions after supplementation (17, 52). Discrepant findings may have been due to the inclusion of individuals with conditions such as cancer or diabetes that led to higher VitB12 concentrations (51), study cohorts without evidence of an incipient cognitive decline [i.e., which are unlikely to have shown a decline in the placebo group over the study period (17, 52)], differences in test batteries used between studies (16, 51), or a short duration of treatment (52). As regards VitB12 and the hippocampal structure, previous animal (53) and human (18, 21) studies have shown that VitB12 deficiency resulted in neuronal degeneration and gray matter atrophy. To our knowledge, only 2 previous studies have investigated the MD in the brain with regard to low VitB12. The authors (3, 20) reported an increase in the MD in multiple white-matter tracts; however, they did not evaluate MD changes in gray-matter brain regions. Treatment with VitB12 most prominently slowed the rate of atrophy in AD-related brain regions, including the hippocampus, in humans (21) and may have prevented environmentally induced detrimental effects on the DG as has been shown in rodent models (54). In the current study, we observed significant group differences in the hippocampus microstructure but not in the volume, which were similar results to those of den Heijer et al. (40). These findings may have been attributable to a higher sensitivity of DTI to detect early or subtle changes in the hippocampus structure than is shown with volume measurements (40, 41, 55). We showed higher MD values in the hippocampus, which were most pronounced in

CA4 and DG subfields but also extended to CA2/3 subfields and the hippocampus tail, in MCI patients with low-normal VitB12. Hippocampus subfields differ in vulnerability because of their different anatomy and regional molecular profiles (56), thereby offering a plausible explanation for our findings of selective differences in the MD in specific subfields. Higher MD values are considered to be an inverse measure of the integrity of neuronal cell bodies, axons, and dendrites in cerebral gray matter (40, 41). Therefore, our findings substantially extend the current VitB12-associated brain morphologic findings by detailed diffusion-tensor imaging at 3 tesla. In the current study, the hippocampus microstructure was detrimentally affected by lower VitB12 and, thus, may have driven the poorer memory performance in MCI patients. With the performance of a simple mediation analysis (41, 46), we offered a neurobehavioral model in which the microstructural integrity of the CA4 and DG subfields mediated 32–48% of the VitB12-related performance in learning and recognition in MCI patients, whereas other hippocampus subfields showed no mediation effect. Thus, low VitB12 might provoke regional demyelination, impaired neurogenesis, and neurotransmitter signaling in these patients, which causes a loss of synaptic contacts (57, 58) and subsequent memory impairment even before showing severe atrophy (40). The DG is one of the unique regions in the brain in which adult neurogenesis possibly takes place throughout life, thereby indicating the necessity of correct DNA replication and nucleotide synthesis, which are mechanisms that are dependent on VitB12 (59). Therefore, CA4 and DG subfields may be more vulnerable to nutrition components such as low VitB12 than are adjacent subfields during the aging process. In support of this idea, a recent imaging study on hippocampus subfields (60) showed that the DG subfield had a stronger age-related decrease in the cerebral blood volume than did other hippocampus subfields, which possibly indicated a regional decrease in the neuronal metabolism and associated functions. The DG influences both short- and long-term memory performances (61), thereby supporting the mediation effect of CA4 and DG microstructure on both learning ability and recognition in the current study. To improve the specificity of VitB12 diagnostics, the use of additional biomarkers (i.e., holotranscobalamin, tHcy, and MMA) that reflect the metabolism of VitB12 may be advisable (6, 7). However, an elevated concentration of tHcy may be caused by both folate and VitB12 deficiency, and both increased MMA and tHcy could be a result of impaired renal function, and in addition, inconsistencies between these relations may be apparent under certain circumstances (e.g., in an ambulatory care setting) (27, 62–64). Therefore, a combined indicator (cB12) of all 4 biomarkers may increase the precision of VitB12 diagnostics and, therefore, was used in the current study to exclude VitB12 deficiency. Moreover, we showed that associations between the serum VitB12 concentration, which is by far the most-commonly used variable in clinical screening (65), and poorer memory performance emerged almost unchanged when with the use of the combined score instead of serum VitB12 concentrations.

Some limitations should be considered when interpreting our findings. First, our study had a cross-sectional design; therefore, it was difficult to draw definite conclusions about causalities. Second, because of the limitation in the DTI voxel size, we could not exclude partial volume effects through the segmentation of the small hippocampus subfields and possibly contamination by adjacent cerebral spinal fluid, particularly in atrophic hippocampus regions, which could have induced false high MD values. However, when we entered the hippocampus volume as a covariate in the statistical model, the MD remained significantly different between the VitB12 groups. Strengths of the study included the rather-large sample size of well-characterized MCI patients with a detailed brain-imaging protocol and the adjustment for multiple confounding risk factors for neurodegenerative disease including age, sex, education, apoE e4 status, tHcy, folate, and creatinine. VitB12 deficiency was excluded, and primary associations from serum VitB12 were largely confirmed with the use of the cB12.

In conclusion, our findings show, for the first time to our knowledge, that, even in the absence of VitB12 deficiency, a low-normal VitB12 status is associated with a worse microstructure in the CA4 and DG hippocampus subfields that partially mediate memory deficits in MCI patients. Moreover, despite their intrinsic linkage, we hypothesize that VitB12 mediates a tHcy- and folate-independent influence on neurocognitive function as indicated by similar results after adjustment for these confounders. The current results suggest that higher cutoffs for serum VitB12 deficiency (e.g., 300 pmol/L) and earlier supplementation in older adults, particularly those with incipient dementia, might be advisable, which is an assumption to be addressed in future randomized controlled intervention trials.

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