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RESEARCH ARTICLE

Anti-*N*-homocysteine-protein autoantibodies are associated with impaired cognition

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Abstract

Introduction: Elevated homocysteine (Hcy) and related metabolites accelerate Alzheimer's disease. Hcy-lowering B vitamins slow brain atrophy/cognitive decline in mild cognitive impairment (MCI). Modification with Hcy-thiolactone generates autoimmunogenic *N*-Hcy-protein. We tested a hypothesis that anti-*N*-Hcy-protein autoantibodies predict cognition in individuals with MCI participating in a randomized, double-blind, placebo-controlled VITACOG trial of B vitamins.

Methods: Participants with MCI (n = 196, 76.8 years old, 60% women) were randomly assigned to receive a daily dose of folic acid (0.8 mg), vitamin B_{12} (0.5 mg), and B_6 (20 mg) (n = 98) or placebo (n = 98) for 2 years. Cognition was analyzed by neuropsychological tests. Brain atrophy was quantified in a subset of patients (n = 167) by magnetic resonance imaging. Anti *N*-Hcy-protein auto-antibodies were quantified by enzyme-linked immunosorbent assay. Associations among anti-*N*-Hcy-protein autoantibodies, cognition, and brain atrophy were examined by multiple regression analysis.

Results: At baseline, anti-*N*-Hcy-protein autoantibodies were significantly associated with impaired global cognition (Mini-Mental State Examination [MMSE]), episodic memory (Hopkins Verbal Learning Test-revised), and attention/processing speed (Map Search). At the end of the study, anti-*N*-Hcy-protein autoantibodies were associated with impaired global cognition (MMSE) and attention/processing speed (Trail Making A). In the placebo group, baseline anti-*N*-Hcy-protein autoantibodies predicted, independently of Hcy, global cognition (Telephone Inventory for Cognitive Status modified [TICS-m]; MMSE) and attention/processing speed (Trail Making A) but not brain atrophy, at the end of study. B-vitamin treatment abrogated association of anti-*N*-Hcy-protein autoantibodies with cognition.

Discussion: These findings suggest that anti-*N*-Hcy-protein autoantibodies can impair functional (attention/processing speed and global cognition), but not structural (brain atrophy), aspects of cognition. Anti-*N*-Hcy-protein autoantibodies are a new factor associated with impaired cognition, which could be ameliorated by B vitamins.

KEYWORDS
anti-*N*-Hcy-protein autoantibodies, brain atrophy, cognition, homocysteine, mild cognitive impairment

1 | INTRODUCTION

Dementia is a major public health issue in aging modern societies. As of 2017, dementia affects 50 million individuals worldwide and continues to increase.[1] Brain atrophy, a feature of neurodegeneration, is occurring during normal aging but is more pronounced in mild cognitive impairment (MCI) and is further accelerated in Alzheimer's disease (AD).[2] As no effective treatment for dementia is available, identification of modifiable risk factors that affect cognitive decline and brain atrophy is important for the development of preventive interventions and treatments.[3,4] Reducing cognitive decline and brain atrophy is likely to slow the conversion of individuals with MCI to AD.[2]

MCI is defined as "cognitive decline greater than that expected for an individual's age and education level but that does not interfere notably with activities of daily life."[5] The prevalence of MCI is about 16% in individuals over 70 years old.[6,7] Half the MCI cases will develop dementia within 5 years after diagnosis.[5] Thus, there is an urgent need to identify biomarkers of cognitive decline and elucidate their mechanisms of action, which has important public health policy implications.

Elevated plasma total homocysteine (tHcy), that is, hyperhomocysteinemia (HHcy) is a risk factor for dementia and AD.[8] HHcy is very common in elderly individuals and is mostly caused by low B vitamin status and reduced renal function.[9,10]

Although Hcy is not a genetically coded amino acid, proteins do contain Hcy residues. One mechanism by which Hcy can become a constituent of proteins involves modification of protein lysine residues by Hcy-thiolactone.[11] The modification alters proteins' structure/function[12,13] and generates auto-immunogenic[14] and pro-inflammatory[15] *N*-homocysteinylated proteins (*N*-Hcy-protein).[11]

In humans, anti-*N*-Hcy-protein autoantibodies are associated with stroke and coronary artery disease.[11,14,15] However, it is not known whether anti-*N*-Hcy-protein autoantibodies are associated with cognition and how B vitamins could affect this association. The present work was undertaken to examine whether anti-*N*-Hcy-protein autoantibodies predict cognition in individuals with MCI. We accomplished this aim by studying relationships between anti-*N*-Hcy-protein autoantibodies quantified by enzyme-linked immunosorbent assay and various measures of cognition quantified by neuropsychological testing. We also studied how these relationships are modified by B vitamin supplementation.

2 | METHODS

2.1 | Participants

We analyzed stored serum samples from participants with MCI who fulfilled the Petersen criteria[16] and had participated in a randomized controlled trial registered as VITACOG, ISRCTN 94410159 (http://www.controlled-trials.com).[17] Participants had a Mini-Mental State Examination (MMSE) score of > 24/30 and no evidence of dementia. Other participants' characteristics were collected at baseline and have been previously described, as was the study protocol.[17] Briefly, the study included individuals aged 77.6 ± 4.8 (n = 266, 60.7% female), some of whom (n = 168) had magnetic resonance imaging (MRI) scans at baseline and at 24.3 ± 0.7 months of follow-up. Participants were randomly assigned to the B vitamin treatment and placebo groups. Each participant received a daily oral TrioB Plus supplement tablet (folic acid, 0.8 mg; vitamin B_{12} - cyanocobalamin, 0.5 mg; vitamin B_6 - pyridoxine HCl, 20 mg) or a placebo tablet for an average of 2 years. Baseline characteristics of participants for whom serum samples were available for the anti-*N*-Hcy-protein autoantibody assays (B vitamin group, n = 99; placebo group, n = 99), are listed in Table S1 and Table S2 in supporting information. At baseline, 15.6% to 21.2% participants had stroke, transient ischemic attack, or MRI infarct; 5.1% to 10.1% had diabetes; and 8.2% to 8.1% had myocardial infarction (in the treatment and placebo group, respectively); 51.5% to 49.5%, 29.9.6% to 37.4%, and 16.5% to 19.2% participants used cardiovascular disease (CVD) drugs, aspirin, and B vitamins, respectively. Blood samples were collected at baseline and at the 24-month follow-up. All participants gave written informed consent. The study was carried out according to the principles of the Declaration of Helsinki and was approved by the Oxfordshire National Health Service Research Ethics Committee (COREC 04/Q1604/100).

2.2 | MRI scans

High-resolution T1-weighted images were acquired at baseline and at 2-year follow-up on a 1.5T Sonata MRI system (Siemens Medical Systems) and analyzed as previously described.[17,18]

2.3 | Cognitive testing

At baseline and follow-up, neuropsychological tests were carried out by trained research nurses and psychologists blind to patient's clinical dementia rating, as previously described in the trial protocol;[17] test scores are shown in Table S2. The tests are representative of cognitive domains affected in MCI: global cognition (MMSE;[19] Telephone Inventory for Cognitive Status modified [TICS-m][20]), episodic memory (Hopkins Verbal Learning Test-revised [HVLT]),[21] attention/processing speed (Map Search[22] and Trail Making A[23]). Attention/processing speed (Symbol Digits Modalities Test [SDMT]), executive function (Trail Making B, CLOX), semantic memory (Graded Naming, Category Fluency). The CANTAB Paired Associate Learning (PAL) and Spatial Recognition Memory (SRM) tests were also used as outcome measures.[24]

2.4 | Anti-*N*-Hcy-protein autoantibody assays

Anti-*N*-Hcy-protein autoantibodies were quantified as previously described.[14,25] Briefly, wells of a PoliSorp 96-well plate (Thermo Fisher Scientific) were coated with *N*-Hcy-albumin, blocked with bovine serum albumin, and human serum was added and allowed to bind overnight at 4°C. After removing unbound material, bound human immunoglobulin (Ig)G was quantified with goat anti-human IgG conjugated with horseradish peroxidase (Millipore-Sigma) and the 3, 3′,5,5′-tetramethylbenzidine/H_2O_2 peroxidase substrate. After quenching with H_2SO_4, A_{492} was recorded using a microplate reader. Each data point was obtained from triplicate measurements. Non-specific IgG binding was corrected for by subtracting A_{492} for unmodified albumin controls.

2.5 | Metabolite analyses

Values for plasma tHcy and other variables were obtained from analyses reported previously.[17]

2.6 | Genotyping

PON1 Q192R genotype was established by enzymatic activity measurements;[26] *MTHFR C677T*, *TCN C776G*, *COMT V158M*, and *BDNF V66M* genotypes were established by standard methods using polymerase chain reaction. Allele frequencies are listed in Table S1.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed the literature using PubMed resources, meeting abstracts/presentations. While an autoimmune response against homocysteine-modified proteins is not yet as widely studied as against other proteins, there are several publications describing the involvement of the anti-homocysteine-protein autoantibodies in stroke, heart disease, uremia, lupus, and neural tube defects. These relevant citations are appropriately cited.
2. **Interpretation**: Our findings provide support to a hypothesis describing pathologic role of anti-homocysteine-protein autoantibodies in the CNS. This hypothesis also applies to nonclinical and clinical findings in other body systems.
3. **Future directions**: The manuscript proposes a framework for the generation of new hypotheses and for additional studies aimed at discovery and further understanding of: (a) genetic and biochemical factors that affect generation of anti-homocysteine-protein autoantibodies and cognition; (b) the prevention of cognitive impairment by B-vitamins; and (c) the relationship between the cognitive domain-specific outcomes and region-specific brain damage.

2.7 | Statistical analysis

Normality of a variable distribution was tested with the Shapiro-Wilk statistic. For normally distributed variables, mean ± standard deviation (SD) was calculated. For non-normally distributed variables, medians were calculated. An unpaired two-sided *t* test was used for comparisons between two groups of variables with normal distribution. A Mann-Whitney rank sum test was used for comparisons between two groups of non-normally distributed variables. Associations between dependent (anti-*N*-Hcy-protein autoantibodies, cognition measures, brain atrophy) and independent variables were examined by Pearson's correlations and multiple linear regression. Statistical software packages PSPP, version 1.0.1 and Statistica, version 13 (TIBCO Software Inc.) were used. Probability values were two-sided and P value $< .05$ was considered statistically significant.

3 | RESULTS

3.1 | Determinants of anti-*N*-Hcy-protein autoantibodies at baseline

For the 196 participants with MCI, mean age at baseline was 76.6 years and 63.7% were women. Baseline serum anti-*N*-Hcy-protein autoantibodies levels varied from 0.013 to 1.418 A_{450} and tended to be higher in men (n = 71) than in women (n = 125), with median values 0.170 and 0.162 A_{492}/min, respectively (Table 1). Levels of tHcy showed a similar variation with age and sex.

Associations between baseline anti-*N*-Hcy-protein autoantibodies and baseline independent variables are presented in Table 2. In univariate analysis, hemoglobin (Hb: $\beta = 0.23$, $P = .001$) and global cognition (MMSE: $\beta = 0.15$, $P = .037$) were significantly associated with baseline anti-*N*-Hcy-protein autoantibodies, while cysteine (Cys: $\beta = 0.13$, $P = .076$) and stroke ($\beta = -0.10$, $P = .164$) tended to be associated.

In multiple regression analysis Cys, Hb, and stroke were significant determinants of anti-*N*-Hcy-protein autoantibodies at baseline (adjusted $R^2 = 0.08$, Model 1). Addition of neuropsychological measures in several cognitive domains improved the significance and predictive value of Model 1. Specifically, global cognition (MMSE; Models 3 and 5), verbal episodic memory (HVLT-TR, Total Recall score; Model 4), and attention/processing speed (Map Search; Models 2, 4, and 5) were significantly associated with anti-*N*-Hcy-protein autoantibodies (adjusted $R^2 = 0.09$ to 0.12; Table 2).

There was no association between baseline anti-*N*-Hcy-protein autoantibodies and other cognitive measures examined at baseline: episodic memory (HVLT-DR Delayed Recall, PAL Total Errors, CANTAB SRM), attention/processing speed (SDMT, Trail Making B, CLOX), attention/speed (Trail Making A), semantic memory (Graded Naming, Category Fluency), and global cognition (TICS-m).

TABLE 1 Baseline anti-*N*-Hcy-protein autoantibodies and tHcy levels in participants with MCI

Variable	Men (n = 71)		Women (n = 125)		*P* value
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	
Anti-*N*-Hcy-protein autoantibodies, A_{492}/min	0.238 ± 0.240	0.170 (0.01–1.42)	0.205 ± 0.176	0.162 (0.00–1.42)	.265
tHcy, μM	12.3 ± 3.7	11.5 (5.5–31.1)	11.5 ± 3.8	10.8 (7–23.4)	.171
Age, years	76.7 ± 4.5	76.3 (70–93)	76.6 ± 5.2	75.9 (70–87)	.945

Abbreviations: Hcy, homocysteine; MCI, mild cognitive impairment; SD, standard deviation; tHcy, total homocysteine.

TABLE 2 Baseline determinants of anti-*N*-Hcy-protein autoantibodies

Variable (n = 180–196)	Pearson correlation		Multiple regression[a]									
			Model 1		Model 2		Model 3		Model 4		Model 5	
	β	*P*	β	*P*	β	*P*	β	*P*	β	*P*	β	P
tHcy_1	0.07	.307										
Cys_1	0.13	.076	0.18	.025	0.17	.028	0.16	.040	0.19	.015	0.16	.041
Hb_1	0.23	.001	0.27	.001	0.30	.000	0.27	.001	0.30	0.000	0.28	.001
Stroke_1	−0.10	.164	−0.15	.047	−0.17	.031	−0.15	.057	−0.16	.038	−0.18	.020
MMSE_1	0.15	.037			0.15	.063	0.15	.048			0.19	.012
HVLT-TR_1	0.08	.295			0.13	.106			0.15	.020[b]		
Map search_1	−0.07	.331			-0.20	.016			−0.16	.041[c]	−0.18	0.028
1 – baseline			F = 3.89, *P* = .002, Adjusted R^2 = 0.08		F = 3.95, *P* = .000, Adjusted R^2 = 0.12		F = 3.93, *P* = .001 Adjusted R^2 = 0.09		F = 3.96, *P* = .000 Adjusted R^2 = 0.11		F = 4.09, *P* = .000 Adjusted R^2 = 0.11	

[a]Adjusted for age and sex.
[b]*P* = .05 in a model without Map Search.
[c]*P* = .117 in a model without HVLT-TR.
Abbreviations: Cys, cysteine; Hb, hemoglobin; HVLT-TR, Hopkins Verbal Learning Test Total Recall; MMSE, Mini-Mental State Examination; tHcy, total homocysteine.

3.2 | Baseline anti-*N*-Hcy-protein autoantibodies determine cognition at the end of study—The placebo group

Multiple regression analysis for the placebo group in models including baseline tHcy, age, sex, neuropsychological test score, and atrophy rate revealed that baseline anti-*N*-Hcy-protein autoantibodies were associated with cognition in three domains at the end of study: general cognition (TICS-m, MMSE), and attention/speed (Trail Making A). There was no association between anti-*N*-Hcy-protein autoantibodies and several other cognitive domains examined at the end of study: episodic memory (HVLT-TR, HVLT-DR, PAL Total Errors, CANTAB SRM), executive function (SDMT, Trail Making B, CLOX), attention/processing speed (Map Search), and semantic memory (Graded Naming, Category Fluency).

3.2.1 | Global cognition–TICS-m

Variables that significantly determined general cognition score with the TICS-m test at the end of the study included baseline anti-*N*-Hcy-protein autoantibodies (β = −0.19, *P* = .040), baseline TICS-m score (β = 0.34, *P* = .001), rate of brain atrophy (β = −0.43, *P* = .000), and age (β = −0.28, *P* = .004; Table 3, Model 2; adjusted R^2 was 0.50). Although TICS-m was associated with tHcy in univariate analysis (β = −0.39, *P* = .000), it was not associated with tHcy in multiple regression analysis (Model 3; β = −0.12, *P* = .245). Notably, tHcy did not affect the association of anti-*N*-Hcy-protein autoantibodies with the TICS-m score (Table 3, Model 4 vs. Model 2). Because a higher score on the TICS-m test indicates better cognition, these findings suggest that higher levels of anti-*N*-Hcy-protein autoantibodies have a detrimental effect on general cognition while tHcy does not.

3.2.2 | Global cognition–MMSE

Variables that significantly determined global cognition score in the MMSE test at the end of the study included baseline MMSE score (β = 0.30, *P* = .004), rate of brain atrophy (β = −0.31, *P* = .003), and age (β = −0.29, *P* = .009; Table 4, Model 5; adjusted R^2 was 0.36). Baseline anti-*N*-Hcy-protein autoantibodies tended to be associated with MMSE score (Model 6: β = −0.18, *P* = .071; adjusted R^2 was 0.37).

TABLE 3 Baseline determinants of cognition at the end of study–Placebo group

Variable (n = 82–112)	Global memory: TICS-m_2									
	Pearson correlation		Multiple regression[a] Model 1		Model 2		Model 3		Model 4	
	β	P	β	P	β	P	β	P	β	P
Anti-*N*-Hcy_1	0.01	.937			−0.19	.040			−0.19	.040
tHcy_1	−0.39	.000					−0.12	.245	−0.11	.382
Atrophy_rate	−0.51	.000	−0.40	.000	−0.43	0.000	−0.35	.001	−0.38	.001
TICS-m_1	0.44	.000	0.32	.000	0.34	.001	0.31	.000	0.33	.000
Age_1	−0.34	.000	−0.28	.002	−0.28	.004	−0.24	.014	−0.24	.021
1 – baseline 2 – end of study			F = 12.11, P = .000, Adjusted R^2 = 0.49		F = 9.48, P = .000, Adjusted R^2 = 0.50		F = 10.08, P = .000, Adjusted R^2 = 0.49		F = 8.48, P = .000, Adjusted R^2 = 0.49	

[a]Adjusted for sex and *MTHFR677CT*, *TCN776CG*, *COMT V158M* genotypes.
Abbreviations: Hcy, homocysteine; TICS-m, Telephone Inventory for Cognitive Status modified.

TABLE 4 Baseline determinants of cognition at the end of study–Placebo group

Variable (n = 82–112)	Global cognition: MMSE_2									
	Pearson correlation		Multiple regression[a] Model 5		Model 6		Model 7		Model 8	
	β	P	β	P	β	P	β	P	β	P
Anti-*N*-Hcy_1	−0.10	.346			−0.18	.071			−0.18	.073
tHcy_1	−0.29	.002					0.02	.821	−0.10	.488
Atrophy rate	−0.36	.001	−0.31	.003	−0.31	.004	−0.26	.045	−0.26	.041
MMSE_1	0.35	.000	0.30	.004	0.32	.003	0.31	.003	0.33	.002
Age_1	−0.29	.002	−0.29	.009	−0.31	.005	−0.24	.048	−0.27	.032
1 – baseline 2 – end of study			F = 7.32, P = .000, Adjusted R^2 = 0.36		F = 6.52, P = .000, Adjusted R^2 = 0.37		F = 6.31, P = .000, Adjusted R^2 = 0.35		F = 5.71, P = .000, Adjusted R^2 = 0.36	

[a]Adjusted for sex, *PON1 Q192R*, and *BDNF V66M* genotypes.
Abbreviations: Hcy, homocysteine; MMSE, Mini-Mental State Examination; tHcy, total homocysteine.

The MMSE score was associated with tHcy in univariate analysis (β = −0.29, P = .002) but not in multiple regression analysis (Table 4, Model 7). Notably, tHcy did not affect the association between anti-*N*-Hcy-protein autoantibodies and the MMSE score (Model 8). Because a higher score on the MMSE indicates better cognitive outcome, these findings suggest that anti-*N*-Hcy-protein autoantibodies tend to have a detrimental effect on global cognition while tHcy does not.

3.2.3 | Attention/processing speed–Trail Making A

Multiple regression analysis showed that variables that significantly determined the attention/processing speed score in the Trail Making A test at the end of study were: baseline anti-*N*-Hcy-protein autoantibodies (β = −0.99, P = .039), baseline tHcy (β = 0.26, P = .033), baseline Trail Making A score (β = 0.22, P = .014), rate of brain atrophy (β = −0.54, P = .000), and *COMT V158M* genotype (β = −0.19, P = .025; Table 5, Model 12; adjusted R^2 was 0.53).

Trail Making A score was associated with tHcy in both univariate (β = 0.45, P = .000) and multiple regression analysis (Table 5, Model 11: β = 0.26, P = .022; adjusted R^2 was 0.45). However, models with anti-*N*-Hcy-protein autoantibodies (Model 10, R^2 = 0.51; Model 8, R^2 = 0.53) had higher R^2 values than models with (Model 11, R^2 = 0.45) or without tHcy (Model 9, R^2 = 0.42). Notably, tHcy did not affect the significance of the association of anti-*N*-Hcy-protein autoantibodies with the Trail Making A score but slightly increased the adjusted R^2 to 0.53 (Model 12 vs. Model 10). These findings suggest that anti-*N*-Hcy-protein autoantibodies and tHcy have independent detrimental effects on the attention/processing speed domain of cognition (higher score in the Trail Making A test indicates worse cognitive outcome).

3.3 | B vitamin treatment modifies effects of anti-*N*-Hcy-protein autoantibodies on cognition

Effects of B vitamin treatment on anti-*N*-Hcy-protein autoantibodies and associated variables are shown in Table S2. As previously reported, there was a significant 25% reduction in the tHcy levels at the end of study in the B vitamin (8.9 ± 2.2 vs. 11.8 ± 3.4, P = 7.E-13) but not in the placebo group (13.1 ± 4.7 vs. 21.1 ± 4.1, P = .112), indicating efficacy of

TABLE 5 Baseline determinants of cognition at the end of study–Placebo group

Variable (n = 99–133)	Attention/processing speed: Trail Making A_2									
	Pearson correlation		Multiple regression[a]							
			Model 9		Model 10		Model 11		Model 12	
	β	P	β	P	β	P	β	P	β	P
Anti-*N*-Hcy_1	0.07	.474			0.19	.034			0.18	.039
tHcy_1	0.45	.000					0.26	.022	0.26	.033
Atrophy rate_2	0.55	.000	0.49	.000	0.54	.000	0.39	0.000	0.41	.000
Trail Making A_1	0.42	.000	0.22	.016	0.26	.005	0.19	.035	0.22	.014
Age_1	0.34	.000	0.22	.035	0.24	.015	0.09	.359	0.15	.134
COMT V158M	−0.12	.216	-0.19	.034	−0.19	.035	−0.17	.051	−0.19	.025
1 – baseline 2 – end of study			F = 12.45, *P* = .000, Adjusted R^2 = 0.42		F = 12.43, *P* = .000, Adjusted R^2 = 0.51		F = 11.92, *P* = .000, Adjusted R^2 = 0.45		F = 12.00, *P* = .000, Adjusted R^2 = 0.53	

[a]Adjusted for sex.
Abbreviations: Hcy, homocysteine; tHcy, total homocysteine.

the B vitamin treatment. There was also a significant increase in anti-*N*-Hcy-protein autoantibodies at the end of study in the placebo group (0.239 ± 0.217 vs. 0.185 ± 0.128 A_{412}, *P* = .034) that was abrogated in the B vitamin group (0.250 ± 0.251 vs. 0.270±0.230 A_{412}, *P* = .559). Hemoglobin levels were reduced at the end of study both in the B vitamin (13.3 ± 1.3 vs. 13.7 ± 1.3, *P* = .012) and placebo (13.5 ± 1.2 vs. 13.8 ± 1.2, *P* = .055) groups.

Global cognition (MMSE score) was significantly decreased in the placebo group at the end of study (27.7 ± 2.3 vs. 28.2 ± 1.5, *P* = .035); this decrease was prevented by the B vitamin treatment (27.8 ± 2.2 vs. 28.1 ± 1.8, *P* = .267; Table S2). Measures of cognition in verbal episodic memory (HVLT-TR and HVLT-DR scores), attention/processing speed (Map Search and Trail Making A scores) did not differ between the end of study and baseline both in the B vitamin and placebo groups.

TICS-m score was significantly increased both in the B vitamin (26.9 ± 5.0 vs. 24.8 ± 2.9, *P* = .0001) and placebo groups (26.5 ± 4.4 vs. 25.0 ± 2.7, *P* = .001) at the end of study (Table S3 in supporting information). However, analysis of male and female subgroups showed that the effect of B vitamin treatment on cognition (TICS-m score) was sex-dependent. Specifically, in the male subgroup at the end of-study, the TICS-m score was increased in the B vitamin–treated (26.4 ± 3.9 vs. 24.5 ± 2.8, *P* = .013) but not in the placebo-treated (25.9 ± 5.3 vs. 25.2 ± 2.9, *P* = .445) group (Table S3). In contrast, in the female subgroup, the TICS-m score was increased both in the B vitamin–treated (26.6 ± 4.6 vs. 24.8 ± 2.9, *P* = .005) and in the placebo (26.7 ± 3.5 vs. 24.8 ± 2.6, *P* = .0002) groups.

Multiple regression analysis for the B vitamin treatment group in models including baseline tHcy, age, sex, neuropsychological test score, and atrophy rate revealed that the treatment abrogated the associations between baseline anti-*N*-Hcy-protein autoantibodies, attention/processing speed (Trail Making A), and global cognition (MMSE, TICS-m) at the end of study (Table 6). B vitamin treatment also abrogated the association of atrophy rate with the Trail Making A_2 score (β = 0.09, *P* = .389) and mitigated the association of atrophy rate with the TICS-m_2 score (β = −0.25, *P* = .029). In contrast, the association of atrophy rate with the MMSE_2 score was not affected by B vitamin treatment (β = −0.43, *P* = .000). The neuropsychological test scores in the B vitamin group at the end of study were still associated with the corresponding scores at baseline (Table 6).

3.4 | Baseline anti-*N*-Hcy-protein autoantibodies not associated with brain atrophy rate at the end of study

Previous studies have shown that tHcy is a major determinant of brain atrophy rate.[17] To determine whether anti-*N*-Hcy-protein autoantibodies could determine brain atrophy, we carried out multiple regression analysis in models including baseline anti-*N*-Hcy-protein autoantibodies, tHcy, age, sex, brain volume, and brain atrophy rate.

In the placebo group at the end of study, we found that anti-*N*-Hcy-protein autoantibodies were not associated with brain atrophy rate in a model without (β = −0.02, *P* = .858; R^2 was 0.09) or with tHcy (β = −0.03, *P* = .785; R^2 was 0.36). However, R^2 was higher in a model with baseline anti-*N*-Hcy-protein autoantibodies (R^2 = 0.36) than without (R^2 = 0.25). We confirmed that baseline tHcy was strongly associated with brain atrophy (β = 0.43, *P* = .000; R^2 was 0.25) in the placebo group, as previously reported.[17]

In the B vitamin group at the end of study, baseline anti-*N*-Hcy-protein autoantibodies were also not associated with the brain atrophy rate. The dependence of brain atrophy rate on baseline tHcy was abrogated by the B vitamin treatment (R^2 = −0.01), as previously reported.[17]

3.5 | Determinants of anti-*N*-Hcy-protein autoantibodies at the end of study

We also examined associations between the end-of-study anti-*N*-Hcy-protein autoantibodies and other end-of-study variables for the

TABLE 6 Baseline determinants of cognition at the end of study–B vitamin group

	Multiple regression					
	MMSE_2[a] Global cognition		TICS-m_2[b] Global cognition		Trail Making A_2[c] Attention/processing speed	
Variable	*β*	*P*	*β*	*P*	*β*	*P*
Anti-*N*-Hcy_1	0.02	.877	−0.05	.657	0.05	.626
Atrophy_rate_2	−0.43	.000	−0.25	.029	0.09	.389
MMSE_1	0.39	.001				
Trail Making A_1					0.59	.000
TICS-m_1			0.29	.012		
Age_1	−0.03	.783	−0.24	.025	−0.03	.756
1 – baseline 2 – end of study	F = 5.99, *P* = .000, Adjusted R^2 = 0.38		F = 4.19, *P* = .000, Adjusted R^2 = 0.29		F = 5.48, *P* = .000, Adjusted R^2 = 0.31	

[a]Adjusted for age, sex, tHcy, and *PON1 Q192R* genotype.
[b]Adjusted for sex, age, tHcy, and *MTHFR677CT*, *TCN776CG*, *COMT V158M* genotypes.
[c]Adjusted for age, sex, tHcy, and *COMT V158M* genotype.
Abbreviations: Hcy, homocysteine; MMSE, Mini-Mental State Examination; TICS-m, Telephone Inventory for Cognitive Status modified.

B vitamin and placebo groups (Table S4 in supporting information) and for the whole cohort (Table S5 in supporting information). In bivariate analysis for the B vitamin group, hemoglobin (Hb: $\beta = 0.28$, $P = .006$) was significantly associated with anti-*N*-Hcy-protein autoantibodies, while attention/processing speed (Map Search: $\beta = 0.20$, $P = .055$) tended to be associated (Table S4). Similar associations of anti-*N*-Hcy-protein autoantibodies with Hb and the Map Search score were observed in multiple regression analysis in a model also involving age, sex, and Cys ($P = .018$; adjusted R^2 was 0.09).

In bivariate analysis for the placebo group, HVLT-TR ($\beta = 0.20$, $P = .044$) and TICS-m ($\beta = 0.26$, $P = .009$) were significantly associated with anti-*N*-Hcy-protein autoantibodies (Table S4). In multiple regression analysis, none of the models that included TICS-m or HVLT-TR, and Cys, was significant ($P > .05$), although TICS-m was significant ($\beta = 0.27$, $P = .013$) and HVLT-TR tended to be significant ($\beta = 0.21$, $P = .052$).

In multiple regression analysis for the whole cohort at the end of study, anti-*N*-Hcy-protein autoantibodies were significantly associated with the treatment code ($\beta = 0.29$, $P = .002$), Hb ($\beta = 0.31$, $P = .001$), MMSE ($\beta = 0.20$, $P = .028$), and Trail Making scores ($\beta = 0.19$, $P = .036$); adjusted R^2 was 0.12 (Table S5).

4 | DISCUSSION

The present study of a well-characterized cohort of participants with MCI provides evidence suggesting that serum anti-*N*-Hcy-protein autoantibodies are an important factor accelerating cognitive decline. We also show that the detrimental effects of anti-*N*-Hcy-protein autoantibodies on cognition are prevented by a treatment with B vitamins for 2 years. Specifically, we found that in the placebo group, (1) measurements of serum anti-*N*-Hcy-protein autoantibodies at baseline allowed ascertainment of cognition in three measures at the end of study 2 years later: general or global cognition (TICS-m and MMSE scores), attention/processing speed (Trail Making A score); (2) effects of baseline serum anti-*N*-Hcy-protein autoantibodies on cognition were independent of tHcy; (3) baseline tHcy predicted attention/processing speed (Trail Making A score) but not global cognition (TICS-m and MMSE scores) at the end of study; (4) baseline serum anti-*N*-Hcy-protein autoantibodies were not associated with brain atrophy. In the B vitamin group, (5) baseline serum anti-*N*-Hcy-protein autoantibodies were not associated with global cognition (TICS-m and MMSE scores), or attention/processing speed (Trail Making A score) at the end of study. We also found that (6) baseline anti-*N*-Hcy-protein autoantibodies were associated with baseline MMSE and HVLT-TR scores while (7) the end-of-study anti-*N*-Hcy-protein autoantibodies were associated with the end-of-study MMSE and Trail Making A scores.

The original discovery of anti-*N*-Hcy-protein autoantibodies in a cohort of stroke patients and controls[14] suggested that these autoantibodies could affect the function of the central nervous system (CNS). This suggestion is further supported by a study in mice showing that anti-*N*-Hcy-protein autoantibodies are elevated in a mouse model of neural tube defects.[27] The present study, showing that baseline anti-*N*-Hcy-protein autoantibodies predict cognition in participants with MCI assessed 2 years later, adds another piece of evidence supporting the importance of these autoantibodies in the human CNS. Specifically, anti-*N*-Hcy-protein autoantibodies predicted outcomes in attention/processing speed (Trail Making A score), global cognition (MMSE score), and global memory (TICS-m score) but not in other more specific cognitive domains. Importantly, the present study also showed that B vitamin treatment for 2 years abrogated the negative effects of anti-*N*-Hcy-protein autoantibodies on three measures of cognition: Trail Making A, MMSE, and TICS-m scores.

The baseline serum anti-*N*-Hcy-protein autoantibodies exhibited a significant inter-individual variation in the participants (142-fold; from 0.01 to 1.42 A_{492}) compared to a more limited inter-individual variation in tHcy levels (Table 1). Somewhat lower inter-individual

variation (32-fold) has been observed in stroke,[14] coronary artery disease,[15,28,29] uremia,[30] systemic lupus erythematosus,[31] and rheumatoid arthritis,[32] human diseases also characterized by hyperhomocysteinemia.

The present findings that anti-*N*-Hcy-protein autoantibodies predict outcomes in three cognitive tests (Trail Making A as well as MMSE and TICS-m, which has a higher memory component than MMSE), underscores the need for identification of factors affecting the levels of anti-*N*-Hcy-protein autoantibodies. In the present study, using multiple regression analysis, we found that stroke, two metabolic (Cys and Hb), and three neuropsychological (MMSE, HVLT-TR, and Map Search scores) factors were associated with anti-*N*-Hcy-protein autoantibodies at baseline (Table 2), while Hb and two measures of cognition (MMSE and Trail Making A scores) were also associated with anti-*N*-Hcy-protein autoantibodies at the end of study (Table S5). The molecular bases of these associations are not known and remain to be investigated. As these factors explained just 12% of the variability in anti-*N*-Hcy-protein autoantibodies, other determinants remain to be discovered. Possible candidates include genes coding for enzymes involved in the synthesis (methionyl-tRNA synthetase, MARS)[33] and turnover (paraoxonase 1, PON1[34]; bleomycine hydrolase, BLMH[35]; biphenyl hydrolase like, BPHL[36,37]) of Hcy-thiolactone, which participates in the generation of *N*-Hcy-proteins,[38] which in turn induce the formation of anti-*N*-Hcy-protein autoantibodies.[14]

It should be noted that tHcy was not identified as a determinant of anti-*N*-Hcy-protein autoantibodies in the present cohort of participants with MCI. Although surprising at first glance, this finding can be explained by factors downstream of tHcy, such as Hcy-thiolactone- and/or *N*-Hcy-protein-metabolizing enzymes or methionyl-tRNA synthetase gene (*MARS*) copy number, which can influence levels of anti-*N*-Hcy-protein autoantibodies by affecting the generation of *N*-Hcy-proteins in our cohort. Supporting this possibility are findings showing that increased *MARS* gene copy number elevates levels of *N*-Hcy-protein in colon cancer patients.[39,40] This, however, remains to be investigated in MCI in future studies.

Although anti-*N*-Hcy-protein autoantibodies predicted cognition in the attention/processing speed (Trail Making A score) and global cognition (TICS-m and MMSE scores) in the present study, these autoantibodies did not predict brain atrophy rate. As tHcy predicts the brain atrophy rate, these findings clearly show that each of the two variables linked to Hcy metabolism, anti-*N*-Hcy-protein autoantibodies and tHcy, have different effects on the human CNS. It appears that anti-*N*-Hcy-protein autoantibodies affect functional (attention/processing speed and global cognition), but not structural (brain atrophy), aspects of cognition. In contrast, tHcy affects the structural (brain atrophy) and only some functional aspects of cognition (attention/processing speed) independently of anti-*N*-Hcy-protein autoantibodies.

In the present study, the treatment with B vitamins for 2 years abrogated effects of anti-*N*-Hcy-protein autoantibodies on attention/processing speed (Trail Making A score) and global cognition (TICS-m and MMSE scores). At the same time, B vitamin treatment abrogated the dependence of attention/processing speed (Trail Making A score) on the rate of brain atrophy. However, B vitamin treatment did not eliminate the dependence of global memory (TICS-m and MMSE scores) on the rate of brain atrophy. Thus, although B vitamin treatment slows brain atrophy, it eliminates the dependence of cognition on the rate of brain atrophy only in the specific domains of attention and processing speed but not in the more general and memory-related domains (global cognition).

Anti-*N*-Hcy-protein autoantibodies can be beneficial by clearing damaged *N*-Hcy-proteins from the circulation. However, the autoantibodies can also be harmful when they form the antigen–autoantibody complex with *N*-Hcy-protein on the vascular endothelium, which would attract macrophages to the vascular wall. The macrophages will bind to the complex and digest it, causing damage to the vascular surface. If this process were to occur in brain vasculature, it would result in cognitive domain-specific outcomes depending on which brain region was damaged. Such scenario could account for the present findings showing that anti-*N*-Hcy-protein autoantibodies predict outcomes in attention/processing speed (Trail Making A score) and global cognition (MMSE and TICS-m scores) but not in other more specific cognitive domains.

The associations between anti-*N*-Hcy-protein autoantibodies and measures of cognition raise a question whether upstream metabolites on a pathway leading to the antibody response, such as Hcy-thiolactone and *N*-Hcy-protein,[11] can be directly associated with cognition, independently of the antibodies. However, this interesting possibility remains to be examined in future studies.

In conclusion, our findings provide the first experimental evidence suggesting that anti-*N*-Hcy-protein autoantibodies could play a role in the CNS by impairing cognition, independently of Hcy, in the general/memory-related (global cognition) as well as in the specific attention/processing speed domains in individuals with MCI. These findings also support a novel concept that anti-*N*-Hcy-protein autoantibodies are a risk factor for cognitive impairment, which can be prevented by B vitamins, thus highlighting a novel positive aspect of B vitamin treatment on the CNS.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

O. Włoczkowska quantified anti-*N*-Hcy protein autoantibodies and contributed to data analysis and writing the manuscript; J. Perła-Kaján

quantified serum PON1 activities and contributed to data analysis; A.D. Smith and H. Refsum were co-PIs of the VITACOG trial and provided samples and data from the VITACOG database; C. de Jager was responsible for the neuropsychological testing; H. Jakubowski designed the study, analyzed the results, and wrote the manuscript. All authors have read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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