Preventing Alzheimer's disease-related gray matter atrophy by B-vitamin treatment

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Is it possible to prevent atrophy of key brain regions related to cognitive decline and Alzheimer's disease (AD)? One approach is to modify nongenetic risk factors, for instance by lowering elevated plasma homocysteine using B vitamins. In an initial, randomized controlled study on elderly subjects with increased dementia risk (mild cognitive impairment according to 2004 Petersen criteria), we showed that high-dose B-vitamin treatment (folic acid 0.8 mg, vitamin B6 20 mg, vitamin B12 0.5 mg) slowed shrinkage of the whole brain volume over 2 y. Here, we go further by demonstrating that B-vitamin treatment reduces, by as much as seven fold, the cerebral atrophy in those gray matter (GM) regions specifically vulnerable to the AD process, including the medial temporal lobe. In the placebo group, higher homocysteine levels at baseline are associated with faster GM atrophy, but this deleterious effect is largely prevented by B-vitamin treatment. We additionally show that the beneficial effect of B vitamins is confined to participants with high homocysteine (above the median, 11 μmol/L) and that, in these participants, a causal Bayesian network analysis indicates the following chain of events: B vitamins lower homocysteine, which directly leads to a decrease in GM atrophy, thereby slowing cognitive decline. Our results show that B-vitamin supplementation can slow the atrophy of specific brain regions that are a key component of the AD process and that are associated with cognitive decline. Further B-vitamin supplementation trials focusing on elderly subjets with high homocysteine levels are warranted to see if progression to dementia can be prevented.

structural neuroimaging | hippocampus | causal modeling | degeneration | clinical trial

The prevention of Alzheimer's disease (AD) is a major public health challenge, but several promising therapies targeting β-amyloid have failed in late-stage clinical trials (1). An alternative approach is to modify nongenetic risk factors and to treat people at risk of developing dementia before they develop the major symptoms (2, 3). Many cross-sectional and prospective studies have shown that raised levels of plasma total homocysteine (tHcy) are associated with cognitive impairment, AD, or vascular dementia (4–9), but randomized, controlled trials of tHcy-lowering treatment using B-vitamin supplementation have shown inconsistent results on cognitive function (10, 11). Factors such as dosage, vitamin combination, duration of treatment and the population treated possibly account for some of the discrepancies (10, 11). Another factor that may explain the discrepancies in these trials is baseline tHcy concentration: for instance, it has been found that subjects with modestly raised tHcy experience a beneficial effect of B-vitamin treatment on cognitive decline (12, 13).

For trials with the aim of slowing progression of cognitive decline, the usual design includes neuropsychological assessments. Unfortunately, such testing is subject to short-term fluctuations, practice effects and intra-/interrater variability. In contrast, structural neuroimaging provides a robust way of assessing changes of a longer-term nature, including the impact of the treatment (14–16). Accordingly, we recently showed, in a first study on the same subjects as included here, that B-vitamin treatment slowed the shrinkage of the whole brain over 2 y and that there was an interaction between treatment and tHcy at baseline (17).

Since the measure of overall brain size used in the initial study was nonspecific, key questions remain to be elucidated: (*i*) are B vitamins effective in preventing the atrophy of those gray matter (GM) regions specifically affected by the AD neurodegenerative process, (*ii*) does this effect differ according to baseline tHcy, (*iii*) does the reduction of atrophy with B vitamins occur in regions linked to cognitive and clinical outcomes, and (*iv*) can GM atrophy provide a biological intermediary in determining the causal chain of events linking B-vitamin treatment to changes in cognition?

To answer these questions, we used the data from a randomized, controlled trial (VITACOG) assessing the effect of B-vitamin treatment (folic acid 0.8 mg/d, vitamin B12 0.5 mg/d, vitamin B6 20 mg/d) on elderly volunteers with memory complaint who fulfilled the Petersen criteria for mild cognitive impairment (MCI) (18) over a period of 24 mo. MRI scans at baseline and the second time point from 156 subjects, 76 receiving placebo and 80 receiving B vitamins (mean age: 76 and 77 y, respectively; Table 1), were suitable for analysis. Local GM volume at baseline and after 24 mo was assessed with the optimized voxel-based morphometry (VBM) protocol using FSL (FMRIB Software Library) (19–21). Placebo and B-vitamin groups did not differ in baseline GM volume [minimum family-wise error (FWE), $P = 0.33$; *SI Methods*].

Results

We first sought to establish whether the two treatment groups lost GM over the 2-y period. Atrophy was found in both placebo and B-vitamin groups in similar areas encompassing medial temporal, lateral temporoparietal, and occipital regions, as well as the anterior and posterior cingulate cortex (Fig. 1). Direct comparison between groups revealed a significant effect of treatment: subjects receiving B vitamins showed a significant reduction of atrophy

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Conflict of interest statement: A.D.S. is named as inventor on three patents held by the University of Oxford on the use of B vitamins to treat Alzheimer's disease or mild cognitive impairment (US6008221, US6127370, and PCT/GB2010/051557); H.R. is named as inventor on Patent PCT/GB2010/051557. Under the University of Oxford's rules, they could benefit financially if the patents are exploited.

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Data deposition: The preprocessed structural imaging scans used in this paper can be found at www.fmrib.ox.ac.uk/analysis/VB/.

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Table 1. Demographics and plasma measures at baseline for subjects undergoing the FSL-VBM analysis

Variable	Placebo (n = 76)	B vitamins (n = 80)	*P* value
Age, y	76 ± 4	77 ± 5	0.10
Sex, F/M	49/27	47/33	0.47
Time between scans, d	744 ± 24	740 ± 25	0.26
Education, y	14.5 ± 3	14.5 ± 3.5	0.47
ApoE: ε4/no ε4	25/51	20/60	0.30
Baseline plasma levels			
Vitamin B6, μmol/L	84 ± 28	86 ± 40	0.70
Folate, nmol/L	29 ± 18	29 ± 18	0.54
Vitamin B12, pmol/L	347 ± 99	356 ± 167	0.68
tHcy, μmol/L	11.4 ± 3.1	11.8 ± 3.6	0.47
Creatinine, μmol/L	96 ± 17	96 ± 18	0.97
Baseline cognitive scores			
CDR-SOB	1.1 ± 0.7	1.1 ± 0.7	0.85
MMSE	28.5 ± 1.5	28.5 ± 1.5	0.75
HVLT-R (delayed recall)	7.3 ± 3.1	8.1 ± 2.8	0.09
Category fluency (animals)	20.3 ± 4.8	20.4 ± 5.0	0.89

ApoE, apolipoprotein E; CDR-SOB, clinical dementia rating, sum of boxes; MMSE, Mini-mental state examination; HVLT-R, Hopkins verbal learning test, revised. Unless otherwise defined, values are mean ± SD.

compared with the placebo group in posterior brain regions including bilateral hippocampus and parahippocampal gyrus, retrosplenial precuneus, lingual and fusiform gyrus, as well as in the cerebellum (Table 2). These regions are among those most affected in AD, and also in MCI subjects who later convert to AD (14, 15, 22–24). In the regions showing significant treatment effect, the average loss of GM over 2 y was 3.7% (±3.7) in the placebo group compared with only 0.5% (±2.9) in the B-vitamin group (Fig. 2 and Fig. S1).

As previously shown (4, 17, 25–27), higher plasma tHcy levels are related to smaller global brain volume and white matter volume, smaller amygdala and hippocampus, and faster reduction in

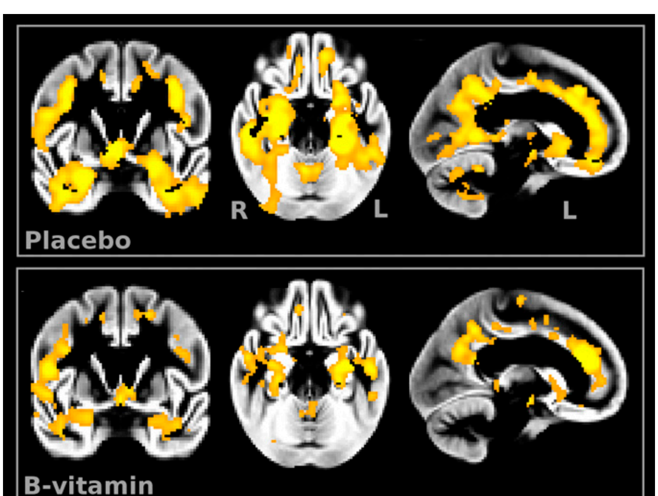

Fig. 1. Regional loss of GM volume in placebo and B-vitamin groups. Placebo- and B-vitamin–treated groups showed significant reduction of GM volume over the 2-y period in similar regions (in yellow, $P < 0.001$ FWE-corrected for multiple comparisons, overlaid onto the average of all 156 GM volume images; Montreal Neurological Institute (MNI) coordinates in mm: $x = -12$, $y = 4$, $z = -18$). The extent and significance of volume loss appeared markedly greater in the placebo group compared with the B-vitamin group and is confirmed by direct statistical comparison (text and Fig. 2).

overall brain size. Participants were divided into two groups according to baseline tHcy: those with measurements below and above the median (11.06 μmol/L). We investigated the interplay between treatment and baseline tHcy. First, we tested the effect of baseline tHcy on atrophy in the placebo and B-vitamin groups separately. Participants with high tHcy in the placebo group had greater GM atrophy compared with those with low tHcy (Fig. S2). In contrast, in the subjects receiving B vitamins, there was no difference in atrophy between participants with low and high baseline tHcy, even when looking at the entire distribution of t values over the brain [median t(placebo) = 0.53; median t(B vitamin) = −0.04]. Second, we tested the effect of treatment on atrophy in subjects with high and low tHcy levels separately. No significant effect of B-vitamin treatment was found in the participants with low baseline tHcy (minimum FWE, $P = 0.35$). However, B-vitamin treatment had a marked beneficial effect in reducing GM atrophy over 2 y in those with high tHcy [from 5.2% (±3.4) down to 0.6% (±2.1); Fig. 3]. In the high tHcy group, B vitamins reduced atrophy in similar regions as seen in the total group, and it also extended to anterior regions including the anterior cingulate cortex and piriform cortex, as well as prefrontal areas (Fig. 3, Fig. S3, and Table 2). A formal interaction between treatment status and baseline tHcy also proved significant (Fig. S4).

To establish which regions of the brain were associated with cognitive decline, we performed voxelwise linear regression analyses between change in GM volume and change in neuropsychological scores over time on all 156 participants. More precisely, we explored the relationship between GM loss and decline in measures of global function (CDR-SOB and MMSE), decline in episodic memory (HVLT-R), and decline in semantic memory (category fluency). Regression analyses revealed significant association of GM loss with worsening of CDR-SOB and MMSE scores, which was most pronounced bilaterally in the amygdalohippocampal complex and entorhinal cortex. At an uncorrected threshold (voxelwise $P < 0.01$), decreases in HVLT-R delayed recall and category fluency were associated with increased GM loss in the left hippocampus and entorhinal cortex (Fig. S5). These GM regions involved in cognitive decline also showed a reduction of atrophy with B-vitamin treatment in subjects with high tHcy levels (Fig. S6).

We finally sought to determine the role of tHcy and brain atrophy in mediating the influence of B-vitamin treatment on cognitive changes in participants with higher tHcy levels, as those were the only subjects to benefit from the treatment. We modeled treatment and change in plasma concentrations of tHcy and relevant B vitamins, imaging, and neuropsychological measures over the 2-y period as a directed acyclic graph. We found that the optimal Bayesian network explaining our data suggested the following chain of events: treatment led to a change in vitamin B12 and folate plasma concentrations, with only vitamin B12 appearing to play a role in modifying tHcy levels; changes in tHcy levels caused a change in GM atrophy, which, in turn, led to a modification of the CDR-SOB (Fig. 4).

Discussion

Here, in an unbiased, voxel-based analysis, we have demonstrated that B-vitamin treatment, by lowering mean plasma tHcy levels (by 29%; *SI Methods*), markedly reduces GM atrophy in regions particularly susceptible to AD. The loss of total GM volume over the 2-y period (2.0% in the placebo group, 1.4% in the B-vitamin group; *SI Methods*) was comparable to yearly measures of the overall brain atrophy rate obtained in our previous study (1.08% and 0.76%, respectively) (17). The regions significantly benefiting from the treatment thus show less atrophy relative to the total GM volume loss in the B-vitamin group (0.5% vs. 1.4%), whereas the same regions in the placebo group show an accelerated atrophy compared with the whole GM (3.7% vs. 2.0%), suggesting this set of regions is highly

Table 2. MNI coordinates of regions showing reduced loss of GM with B-vitamin treatment

Cortical region	Side	MNI (mm) *x*	*y*	*z*	Local maximum *t*
Medial temporal areas					
Hippocampus	L	−40	−30	−14	4.23
Hippocampus	R	40	−20	−14	4.03
Parahippocampal gyrus	L	−22	−32	−14	3.80
Parahippocampal gyrus	R	20	−40	−12	3.66
Entorhinal cortex	L	−26	−12	−44	3.47
Entorhinal cortex	R	20	−22	−28	3.20
Other visuospatial areas					
Retrosplenial precuneus/ventral posterior cingulate	L	−6	−40	2	3.39
Retrosplenial precuneus/ventral posterior cingulate	R	12	−46	−2	3.16
Lingual gyrus	L	−22	−56	−6	2.70
Lingual gyrus	R	18	−52	−2	3.61
Fusiform gyrus (temporal) (only high tHcy subjects)	L	−40	−8	−44	4.53
Fusiform gyrus (temporal)	R	42	−12	−32	4.28
Fusiform gyrus (temporo-occipital)	R	34	−44	−18	3.33
Fusiform gyrus (occipital)	L	−24	−82	−8	3.66
Lateral occipital cortex	L	−42	−76	2	3.37
Lateral occipital cortex (only high tHcy subjects)	R	40	−74	−4	4.33
Inferior parietal lobule (supramarginal gyrus)	R	52	−42	36	3.21
Inferior parietal lobule (angular gyrus)	R	64	−48	16	2.87
Other temporal areas					
Middle temporal gyrus	R	60	−18	−6	3.09
Inferior temporal gyrus	L	−50	−60	−14	4.16
Inferior temporal gyrus	L	−48	−46	−28	4.44
Inferior temporal gyrus	R	50	−20	−28	3.93
Cerebellum					
Cerebellum (crus I)	R	42	−48	−32	3.94
Cerebellum (crus II)	R	40	−52	−44	3.92
Cerebellum (IX)	L	−16	−50	−46	3.82
Cerebellum (VIIIb)	L	−20	−42	−48	4.05
Anterior areas (only high tHcy subjects)					
Straight gyrus	—	4	44	−26	3.58
Piriform cortex	L	−28	14	−14	4.52
Piriform cortex	R	34	10	−14	2.94
Striatum	L	−18	14	−2	2.86
Striatum	R	6	4	−2	2.96
Pars triangularis	L	−48	28	14	3.07
Middle frontal gyrus (BA46)	R	40	46	8	4.33
Superior frontal gyrus (BA9)	R	24	36	32	3.41
Dorsal anterior cingulate (BA32)	—	4	46	8	2.38
Ventral anterior cingulate (BA24)	R	12	12	48	3.39
Ventral anterior cingulate (BA24)	L	−14	24	32	2.64

Common local peaks (unless specified otherwise) of the significant clusters ($P < 0.05$ FWE-corrected) showing reduced GM atrophy in the B-vitamin group in contrast with the placebo group for all MCI 156 subjects (Fig. 2) and for subjects with high tHcy level (Fig. 3). Local maximum *t* values correspond to Fig. 3. BA, Brodmann area.

vulnerable to the possible underlying pathological process. Some of the GM regions that benefit the most from the B-vitamin treatment (hippocampus, parahippocampal gyrus, inferior parietal lobule and retrosplenial cortex) are all part of a "parieto–medial temporal pathway" involved in visuospatial learning and spatial long-term memory (28). Notably, these regions, together with the fusiform and inferior temporal gyrus, which overall show the most significant reduction of atrophy by B vitamins in participants with high tHcy levels, are also among the best to discriminate between healthy aging and MCI, and all show the greatest atrophy rate in the progression from MCI to AD (23, 29, 30). We also found some significant differences within the cerebellum. This result might seem surprising at first, but there is now clear anatomical and functional evidence for the role of the cerebellum in cognitive and behavioral functions, and particularly for autobiographical memory and working memory (31, 32). Accordingly, our strongest results in the cerebellum are localized to regions that are known to be connected to the medial temporal lobe and prefrontal cortex (31, 33). As part of the default mode network and of the network defined by an encoding memory paradigm, these cerebellar regions have also been shown to be altered in healthy subjects with an increased risk for dementia (34).

When splitting the groups according to the overall baseline tHcy median value, we further showed that the treatment was significantly beneficial only to participants with higher levels of tHcy, in line with the cognitive outcomes shown in this population (13). In these participants with high tHcy levels, there was again a clear difference in loss of GM between regions significantly benefiting from vitamin B and the entire GM: less atrophy in the B-vitamin group (0.6% vs. 1.6%), accelerated atrophy in the placebo group (5.2% vs. 2.7%). In the B-vitamin group, we found no GM atrophy difference between those with

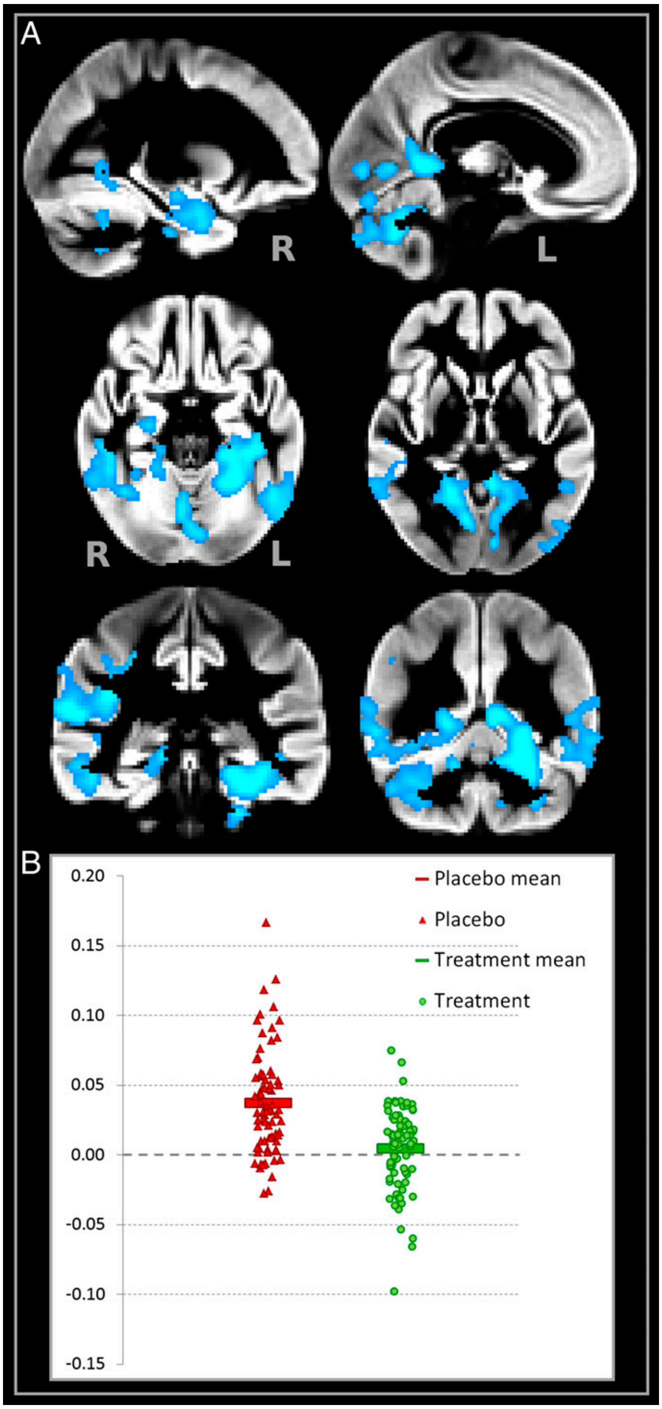

Fig. 2. B-vitamin treatment significantly reduces regional loss of GM ($P <$ 0.05 FWE-corrected). (*A*) Brain regions in blue demonstrate where B-vitamin treatment significantly reduces GM loss over the 2-y period ($x = 26$ and -8, $y = -34$ and -52, $z = 0$ and -16). All blue areas correspond to regions of significant loss in placebo and known to be vulnerable in AD (Fig. 1). (*B*) Percentage of GM loss for the 156 participants over the 2-y period, averaged across those brain regions that showed significant effect of B vitamins: placebo group (red triangles) had an average loss of 3.7% (±3.7), whereas the B-vitamin group (green circles) showed a loss of 0.5% (±2.9).

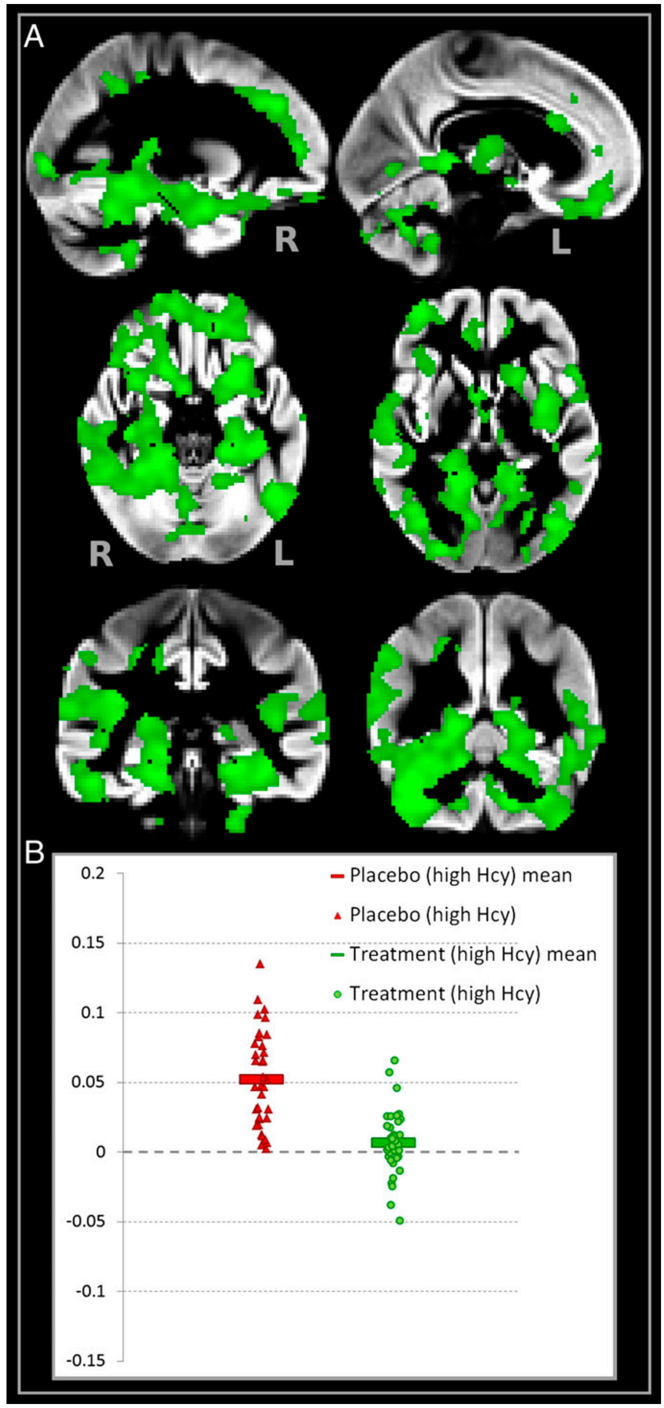

Fig. 3. B-vitamin treatment is only effective in participants with higher tHcy levels ($P < 0.05$ FWE-corrected). (*A*) Brain regions in green demonstrate where B-vitamin treatment significantly reduces GM loss in participants with high tHcy levels (>11.06 μmol/L) at baseline ($x = 26$ and -8, $y = -34$ and -52, $z = 0$ and -16). (*B*) Percentage of GM loss for each of the 77 participants with high tHcy level: placebo group ($n = 35$) showed an average loss of 5.2% (± 3.4) of GM volume over 2 y, whereas B-vitamin group (n =42) showed an average loss of 0.6% (±2.1).

low and high baseline tHcy, suggesting that taking B vitamins mimics the protective effect over time of having lower baseline tHcy. While vitamin B12 deficiency may lead to neurological and neuropsychiatric disorders, no participant had vitamin B12 deficiency at baseline in this study (<150 pmol/L). Our network analysis also suggests that changes in GM atrophy, while significantly related to a change in vitamin B12 levels, appear to be mediated via lowering of tHcy levels. The apparent lack of influence of changes in folate levels may be because, in populations

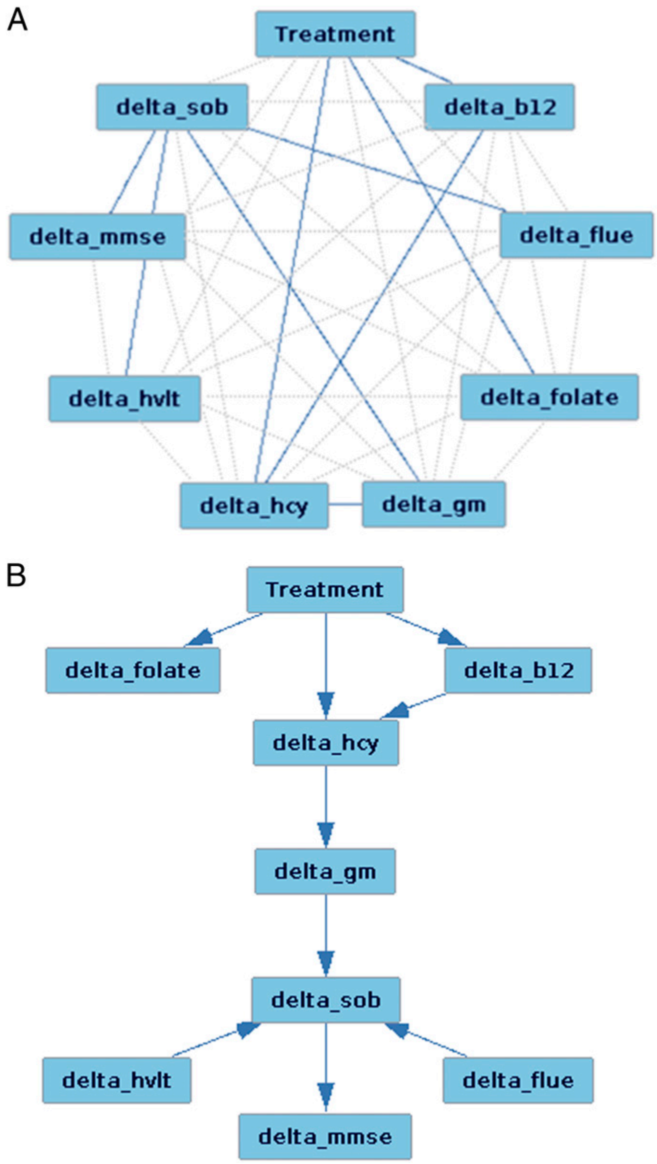

Fig. 4. Directed acyclic graph analysis of treatment and changes ("delta") in B vitamins, tHcy, GM volume, and cognitive performance over the 2-y period. Of all possible edges (gray, *A*), we found that the optimal Bayesian network explaining our data (with a very good model fit, χ^2 $P = 0.64$; *SI Methods*) identified nine statistical dependencies between variables (*A*, blue) and causality for each of these edges as presented in *B* (rearranged for clarity of display): treatment increases B vitamins' "delta" concentration, with B12 lowering tHcy "delta" concentration, which in turn slows grey matter atrophy ("gm"), which then delays cognitive decline over time ("sob" for CDR-SOB, "hvlt" for HVLT-R delayed recall, "flue" for category fluency).

with good folate status as here (mean: 29 nmol/L), the main determinant of tHcy levels is vitamin B12 status (35).

A plausible hypothesis to explain why lowering tHcy might decrease the rate of atrophy of the specific brain regions involved in AD is that the atrophy of such regions is related to the presence of neurofibrillary tangles (36). The deposition of neurofibrillary tangles is caused by the formation of phosphorylated τ, and raised tHcy concentrations lead to an increase in phosphorylated τ (37, 38). Thus, lowering tHcy may decrease levels of phosphorylated τ and thereby reduce the degree of regional GM atrophy. Another possible explanation might stem from the converging evidence of an impaired neuroregeneration seen in AD (39, 40). In the adult, neurogenesis occurs in the hippocampus and olfactory bulb, whereas evidence for it taking place in regions such as the prefrontal, posterior parietal, and inferior temporal cortex, as well as piriform cortex and amygdala, remain controversial (41). Epigenetic mechanisms, involving DNA and histone methylation, are important for neurogenesis (42). Raised tHcy is an inhibitor of methylation reactions and can inhibit the proliferation of neuronal cell precursors (43, 44). As one of the main brain functions of vitamin B12 is in methylation (43) and as B12 deficiency can also impair neuronal cell proliferation (45), it is thus conceivable that vitamin B12 helps to maintain adult neurogenesis in the very regions targeted by AD.

By using measures of GM structure to robustly assess the effect of B-vitamin supplementation and by characterizing the relationship between atrophy in specific GM regions and cognitive decline, we have been able to demonstrate the benefit of this treatment in those elderly subjects with high levels of tHcy. High-dose B-vitamin treatment could modify a key component of the disease process leading to AD: the atrophy of GM regions involved in the cognitive decline of the study participants. Importantly, we also show that B-vitamin treatment is of no benefit for those participants with low levels of tHcy. While our voxelwise, whole GM analysis was used to map the possible effect of treatment in an unbiased manner, it is also a limitation of this study in the context of a randomized controlled trial. First, such a voxelwise approach, as it encompasses data from ~200,000 voxels, is potentially more susceptible to Type I error compared with a region-of-interest approach, which is the method usually favored for clinical trials. Another limitation is that the results described here are parts of the secondary outcomes of the clinical trial as defined in our prespecified plan of data analysis (more specifically, of a regional brain volume changes analysis), while the primary endpoint of this trial was the impact of B vitamins on overall brain atrophy (17). We note, however, that the statistical analyses carried out in this study and presented in Figs. 1–3 were all determined a priori and were performed blind to the treatment group. Baseline comparisons, effect of ApoE ε4 on GM atrophy and causal Bayesian network analysis (Fig. 4) were carried out a posteriori. Finally, while our voxel-based analyses must be strictly regarded as post hoc in the context of the clinical trial, they were fully corrected for multiple comparisons with stringent control of Type I error (i.e. with the Family Wise Error rate, instead of, e.g., False Discovery Rate) using nonparametric resampling-based inference that makes only weak assumptions on the data; thus, they comply with best practice for confirmatory inference in neuroimaging (46).

MCI is common among elderly populations, with approximately 16% of those older than 70 y of age showing this syndrome (47). In population studies, it has been found that plasma tHcy concentrations increase with age. The proportion with concentrations >13 μmol/L, even in countries with mandatory folic acid fortification, ranges from 9% to 14% of those older than 60 y (48). Thus, a significant proportion of elderly subjects may be at risk for dementia as a result of elevated tHcy (a Population-Attributable Risk of 16% has, for instance, been estimated in the Framingham Study) (6). Larger and longer trials will be needed (*i*) to determine the optimal tHcy threshold warranting B-vitamin supplementation and (*ii*) to monitor the treatment effect on the crucial outcome, the incidence of dementia.

Methods

This study was approved by the Oxfordshire National Health Service research ethics committee A on January 6, 2006 (Central Office for Research Ethics Committees, COREC 04/Q1604/100). 156 volunteers who fulfilled the Petersen criteria (18) for MCI underwent the same longitudinal imaging protocol with structural, high-resolution T1-weighted images acquired at baseline and after 2 y on a 1.5T Sonata MRI system (1-mm^3 isotropic resolution; Siemens Medical Systems). An optimized FSL-VBM analysis was

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carried out (fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLVBM) (20, 21), with all images from both time points processed following the same protocol to avoid the known bias of using one of the two time points as reference. To achieve accurate inference, including FWE correction for multiple comparisons across space, we used permutation-based nonparametric inference within the framework of the general linear model (5,000 permutations) (49). We tested for GM changes between baseline and follow-up scan in placebo and B-vitamin groups, and for differences in GM changes between the two populations. The B-vitamin and placebo groups were further divided according to their tHcy levels at baseline (≤11.06 μmol/L or >11.06 μmol/L, the median value). Results were considered significant at $P < 0.05$, FWE-corrected for multiple comparisons across space using Threshold-Free Cluster Enhancement (50). All other statistical tests were carried out in R. Network analysis (51) was performed using Tetrad (www.phil.cmu.edu/projects/tetrad/). Goodness of fit was determined with a χ^2 test. Using a subsampling procedure, we further verified the stability of the optimized network. More details of the method are provided in *SI Methods*.

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Supporting Information

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SI Methods

Ethics. The study was carried out according to the principles expressed in the Declaration of Helsinki and was approved by the Oxfordshire National Health Service research ethics committee A on January 6, 2006 (Central Office for Research Ethics Committees, COREC 04/Q1604/100). Each subject gave written consent for their participation.

Subjects. Details of recruitment into the VITACOG trial, the cognitive testing, biochemical measurements, randomization, B-vitamin treatment, and ethical approval have been described previously (1, 2). Briefly, inclusion criteria included age ≥70 y, study partner available as informant, and diagnosis of amnestic or nonamnestic mild cognitive impairment (MCI) according to Petersen criteria (3). The diagnosis of MCI included a subjective concern about memory and a cognitive screening test using word-recall (TICS-M) with a score >1.5 SD below the norm that did not interfere with activities of daily living. The cutoff scores were based on locally established norms from subjects of the same age in the same city. Exclusion criteria included a diagnosis of dementia or being treated with antidementia drugs; active cancer; major stroke within the past 3 mo; treatment with methotrexate, anticancer, or antiepileptic drugs; taking folic acid >300 μg/d, pyridoxine >3 mg/d or vitamin B12 >1.5 μg/d by mouth or any dose by injection. Participants received a tablet containing 0.8 mg folic acid, 0.5 mg cyanocobalamin and 20 mg pyridoxine HCl, or a placebo tablet.

Baseline demographics characteristics, plasma biochemistry and cognitive test scores of these subjects are given in Table 1. In particular, the plasma total homocysteine (tHcy) concentrations at baseline were 11.4 ± 3.1 μmol/L in the placebo group and 11.8 ± 3.6 μmol/L in the B-vitamin group and, after 2 y, 12.6 ± 4.1 μmol/L in the placebo group and 8.9 ± 2.5 μmol/L in the B-vitamin group ($P < 0.001$). Thus, tHcy level was 29.4% lower in the B-vitamin group than in the placebo group at the end of the study.

Image Acquisition. From the 223 subjects who had been followed up for the 2 y, 187 had volunteered (84%) for two MRI scans and were eligible to be scanned (exclusion criteria included claustrophobia, severe back ache, presence of metallic implants, pacemaker and intraocular metallic foreign bodies). Seven subjects withdrew before the second scan and, of the 180 remaining, 12 T1-weighted scans were found to be technically unsatisfactory, leaving a total of 168 subjects who were included in our previous, whole-brain SIENA analysis (2). All subjects underwent the same imaging protocol with whole-brain T1-weighted scans acquired at baseline and after 2 y using the same 1.5-T Sonata MR imager (Siemens Medical Systems) with a standard quadrature head coil and maximum 40 $mT.m^{-1}$ gradient capability. Subjects were scanned with a 3D T1-weighted fast low-angle shot (FLASH) sequence using the following parameters: coronal orientation, matrix 256 × 256, 208 slices, 1 × 1 mm^2 in-plane resolution, slice thickness 1 mm, echo/repetition times 5.65/12 ms, flip angle 19°. To increase signal-to-noise ratio, scanning was repeated three times and averaged after acquisition and cross-repeat alignment.

Image Preprocessing. We wanted to investigate voxelwise, localized gray matter (GM) volume changes over the 2-y period between placebo and vitamin B-treated groups across the whole brain. As it was important that the use of nonlinear deformations to register native scans into a common space was carried out with appropriate accuracy, we conducted an "optimized" voxel-based morphometry (VBM) protocol (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLVBM) (4, 5) using FMRIB Software Library (FSL) tools (6), in which a symmetric study-specific GM template was built from the MCI participants' images. For this template, brain-extracted GM-segmented images from all subjects at both baseline time point (t0) and second time point 2 y later (t1) were nonlinearly registered to the Montreal Neurological Institute MNI152 GM template and averaged together with their respective mirror images to create the left/right symmetric study-specific GM template. All the native GM volume images were then nonlinearly normalized onto this template. Then, the optimized FSL-VBM protocol introduces a compensation (or "modulation") for the contraction/enlargement caused by the nonlinear component of the transformation: each voxel of each registered GM image was multiplied by the Jacobian of the warp field. Finally, every modulated normalized GM volume image at time point t1 was subtracted from its respective modulated normalized GM volume image at baseline t0. All "t0-t1" GM volume images were then concatenated and smoothed with an isotropic Gaussian kernel with a sigma of 3 mm (∼7 mm full width at half maximum). The percentage change in total GM volume was derived from the average GM partial volume values for each subject inside the entire GM mask at t0 and t1.

In reviewing the scans while still blind to the group membership of each participant, we had to exclude from the FSL-VBM analysis an additional 12 subjects for the following reasons: subcortical lacunar infarct, megadolicobasilar artery, meningioma, very focal atrophy (lateral fissure and intraparietal sulcus), insufficient gray/white matter contrast, and two instances for which "t0-t1" differences exceeded 3σ from the average of all subjects. Thus, scans from 156 subjects remained for analysis (87% of the 180 subjects scanned at the two time points).

Upon visual inspection, white matter hypointensities in T1-weighted images did not affect the nonlinear registration of the segmented images. Furthermore, they did not impact results, as they were outside the GM mask used for the statistical analysis (GM mask automatically generated following the FSL-VBM protocol and masked by the brain mask of the MNI152 template). On average, subjects with low and high tHcy levels had similar volume of WM "lesions" (∼1.3 × 1.3 × 1.3 cm^3, as measured crudely by assessing the volume in the registered segmented images represented by the voxels above a partial volume value of 0.8 outside of the GM mask).

Statistical Analysis. Based on a priori criteria, a median split for tHcy (11.06 μmol/L) was used to ensure sufficient sample size in the two subgroups. We thus considered four groups in our statistical design: subjects in the placebo group with low level of tHcy at baseline (P_h1), subjects in the placebo group with high tHcy level (P_h2), subjects in the B-vitamin group with low tHcy level (B_h1), and subjects in the B-vitamin group with high tHcy level (B_h2).

We first looked for:

- The regions that showed significant changes over the 2 y separating the two scanning time points for the placebo group (*Results* and Fig. 1*A*) and the B-vitamin group (*Results* and Fig. 1*B*): (P_h1 + P_h2) and (B_h1 + B_h2),
- The regions where the "t0-t1" changes between the two time points in the placebo group were significantly different from the changes in the B-vitamin group (*Results*, Fig. 2, and Fig.

S1): (P_h1 + P_h2) − (B_h1 + B_h2) = (P_h1 − B_h_1) + (P_h2 − B_h2).

Then, examining the impact of baseline level of tHcy on the results, we looked for:

- The regions where, looking at the placebo group and at the B-vitamin group separately, the "t0-t1" changes in the subjects with high tHcy level were significantly different from the changes in the subjects with low tHcy (main text and Fig. S2): (P_h2 − P_h1) and (B_h2 − B_h1),
- The regions where, looking at the subjects with low tHcy level and with high tHcy separately, the t0 − t1 changes in the placebo group were significantly different from the changes in the B-vitamin group (Fig. 3, main text and Fig. S3): (P_h1 − B_h1) and (P_h2 − B_h2); and
- The regions where there was a formal interaction in the t0 − t1 changes between baseline tHcy level and treatment status (main text and Fig. S4): (P_h2 − P_h1) − (B_h2 − B_h1) = (P_h2 − B_h2) − (P_h1 − B_h1).

We also compared baseline GM volume between placebo and B-vitamin groups and found no significant difference [minimum familywise error (FWE), P = 0.33]. In a further test, we investigated the possible influence of GM volume at baseline on the second time point GM volume by regressing out the former from the latter voxelwise. We found extremely similar results between this approach and subtracting GM volume at baseline from the second timepoint as in our main analysis.

Baseline GM volume differences between low and high tHcy level groups were also investigated. There was no significant difference between low and high tHcy level groups after correction for multiple comparisons, although a trend ($P < 0.01$, uncorrected) could be seen with lower GM volume with higher tHcy level essentially in the left amygdalohippocampal complex.

Furthermore, similarly to our assessment of the impact of baseline tHcy levels on GM atrophy, we looked for the impact of carrying apolipoprotein E (ApoE) ε4 on GM atrophy. No results reached significance after correction for multiple comparisons. There was, however, a trend for higher atrophy in ApoE ε4 carriers in the placebo group, especially in the medial temporal lobe, fusiform and piriform cortex, which did not exist in the treatment group ($P < 0.01$, uncorrected), although a formal interaction between treatment and ApoE ε4 status was not significant.

Finally, for all subjects, we correlated "t0-t1" GM volume changes with the changes in global cognition and memory function using the clinical dementia rating–sum of boxes (CDR-SOB), Mini-Mental State Examination (MMSE), the Hopkins Verbal Learning Test (HVLT) delayed recall and the category fluency (animals).

To achieve accurate inference, including FWE correction for multiple comparisons across space, we used permutation-based nonparametric inference within the framework of the general linear model (5,000 permutations) (7). Results were considered significant for $P < 0.05$, FWE-corrected for multiple comparisons across space using a Threshold-Free Cluster Enhancement (TFCE) approach, which avoids the use of an arbitrary threshold for the initial cluster formation (8).

All results were identified using a combination of three complementary atlases: the Harvard–Oxford structural cortical probability maps based on MRI T1-weighted images, Jülich cytoarchitectonic probabilistic maps based on postmortem brains, and Talairach Daemon labels corresponding approximately to Brodmann areas (9, 10).

Directed Acyclic Graph Analysis in Subjects with High tHcy Level. We modeled nine different variables as a directed acyclic graph: (*i*) treatment category and changes ("delta") over the 2-y period in: (*ii*) plasma folate, (*iii*) plasma vitamin B12 and (*iv*) plasma tHcy, (*v*) GM volume, (*vi*) CDR-SOB, (*vii*) MMSE, (*viii*) HVLT delayed recall, and (*ix*) category fluency. Indeed, as changes in vitamin B6 were shown to have no impact on tHcy over time (P = 0.19), we considered changes only in folate and vitamin B12 concentrations in this analysis. To ensure that there was no bias in the GM measure, we considered the regions where all participants with high tHcy lost GM volume, regardless of their treatment status. We looked for the optimal Bayesian network explaining these variables using Greedy Equivalence Search (GES) (11) (www.phil.cmu.edu/projects/tetrad/), which allowed us to establish statistical conditional dependencies (edges) between these variables—primarily to estimate direct causal connections between variables. The Linear, Non-Gaussian, Acyclic causal Models (LiNGAM) (12) approach was then used to determine further the direction of the edges, i.e. the causality between the variables. We estimated the goodness of fit of the model using a χ^2 test, whereby the null hypothesis was that our model of interest is equivalent to a saturated model with all possible connections between all variables. For this χ^2 test, a large, nonsignificant P value is evidence of good fit, in that the fitted model is indistinguishable from a saturated model with many more parameters. Moreover, we used a subsampling procedure to evaluate the stability of the direction of the edges in our optimized network. Using five random splits of the data (into two groups of 38 and 39 subjects), we created 10 reduced versions of the data. Holding fixed the undirected edges found in the complete dataset by GES, we found highly consistent directionality determined by PC-LiNGAM. Only one edge had less than 80% stability (directionality the same in eight out of 10 subsamples): "delta_b12" → "delta_tHcy" was identified five out of 10 times as "delta_tHcy" → "delta_b12".

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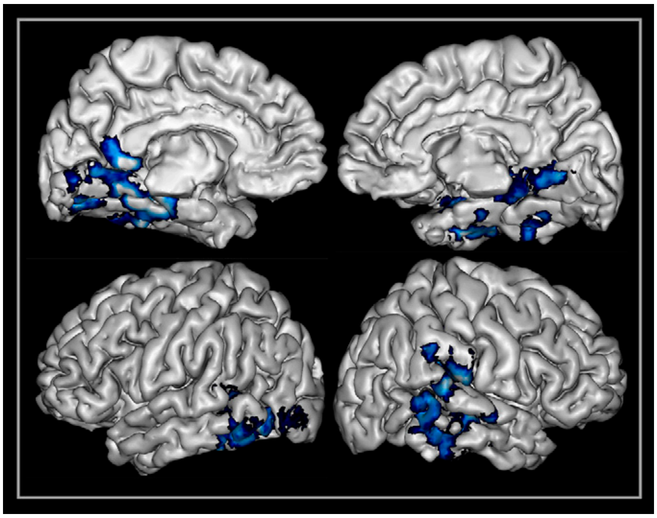

Fig. S1. B-vitamin treatment significantly reduces regional loss of GM. 3D rendering of brain regions in blue–white where B-vitamin treatment significantly reduces GM loss over the 2-y period (P < 0.05 FWE-corrected). All blue/white areas correspond to regions of significant loss in placebo and known to be vulnerable in Alzheimer's disease (Fig. 1).

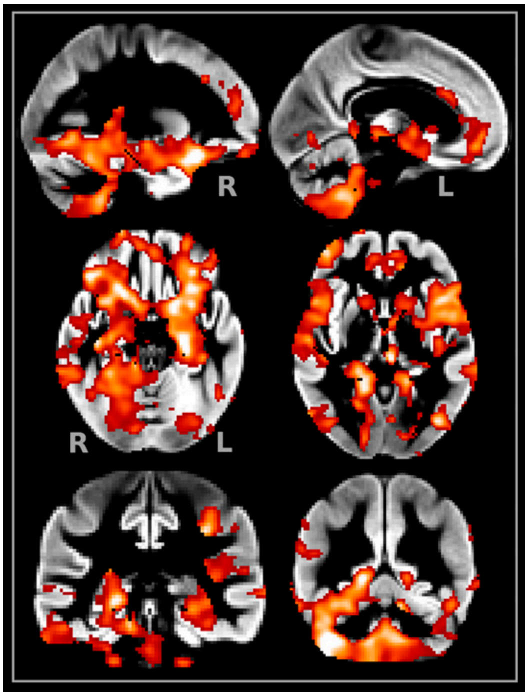

Fig. S2. Increased GM loss over the 2-y period in participants in the placebo group with higher tHcy compared with lower tHcy. The 76 participants in the placebo group were split into two categories depending on whether they had higher or lower tHcy levels than the overall median value (11.06 μmol/L). Participants with higher baseline tHcy levels (n = 35) had greater GM atrophy (red, P < 0.05 FWE-corrected) over the 2-y period compared with those with lower tHcy levels. In contrast, there was no difference in atrophy between participants with high and low baseline tHcy levels in the B-vitamin group.

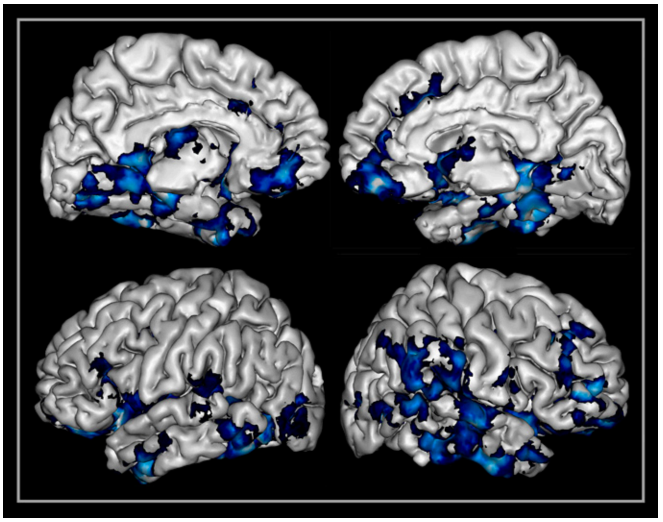

Fig. S3. B-vitamin treatment is effective only in participants with higher tHcy levels. 3D rendering of brain regions in blue–white where B-vitamin treatment significantly reduces GM loss in participants with high tHcy levels (>11.06 μmol/L) at baseline ($P < 0.05$ FWE-corrected). No significant effect of B-vitamin treatment was found in the participants with low baseline tHcy levels.

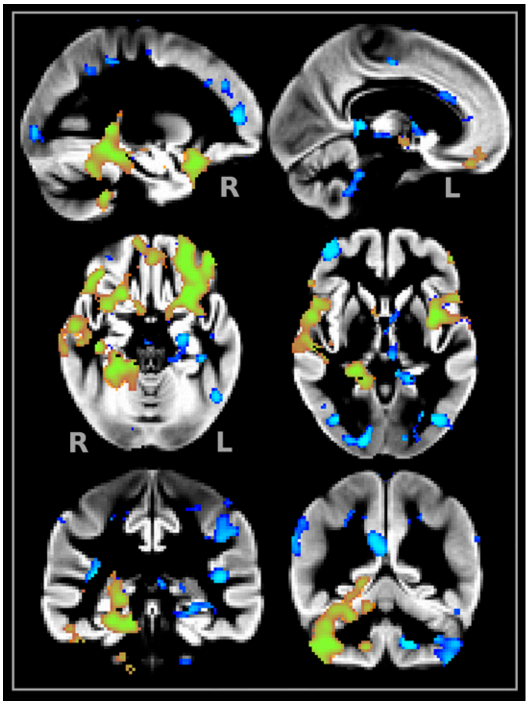

Fig. S4. Interaction between tHcy levels and treatment status in GM loss. The interaction between high and low tHcy levels and placebo and B-vitamin groups showed significant regions corresponding to where GM loss was reduced with treatment in participants with high tHcy levels (in red–yellow $P < 0.05$ FWE-corrected; in blue $P < 0.05$ uncorrected; overlap in green).

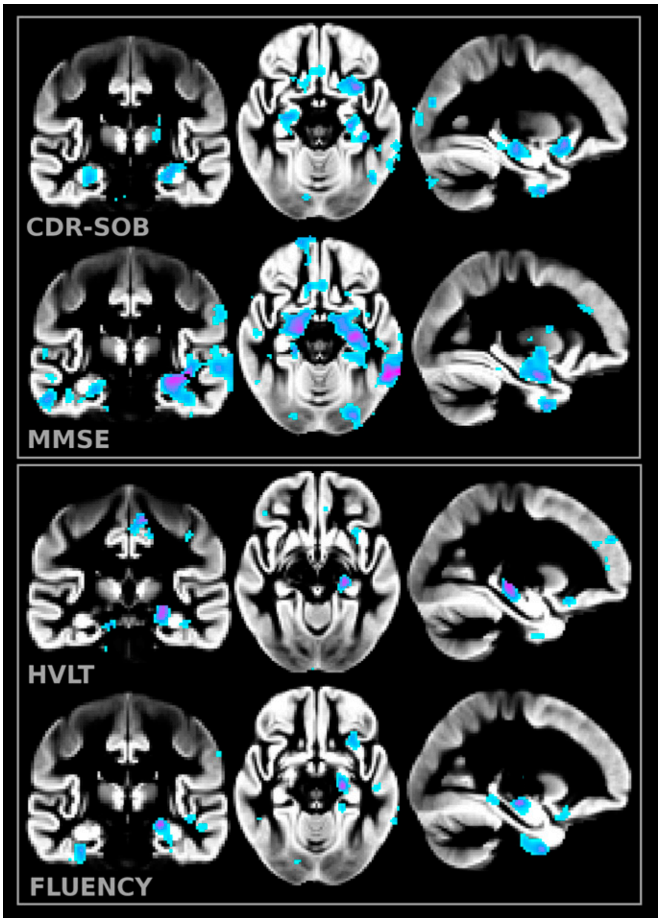

Fig. S5. Correlation between GM loss and cognitive decline. Significant correlation was found between GM loss over 2 y and global function mainly in the medial temporal areas, entorhinal and piriform cortex (CDR-SOB and MMSE, $P < 0.05$ FWE-corrected) and, at an uncorrected threshold, with memory function mainly in the left medial temporal areas (HVLT-R delayed recall and category fluency, $P < 0.001$).

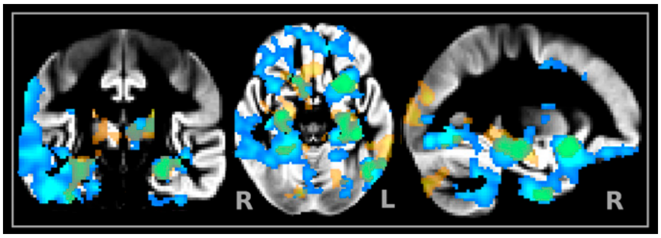

Fig. S6. Regions related to the global cognitive decline overlap with those benefiting from B-vitamin treatment. Correlation between GM loss and worsening of CDR-SOB scores (yellow) is overlaid onto the significant effect of B-vitamin treatment on atrophy in those with higher tHcy level (in blue, adapted from Fig. 3). Overlap is shown in green.