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Homocysteine concentrations in the cognitive progression of Alzheimer's disease

RUNNING HEADER: Homocysteine and cognition in Alzheimer's disease

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Abstract:

Objectives: Hyperhomocysteinemia in Alzheimer's disease (AD) is widely reported and appears to worsen as the disease progresses. While active dietary intervention with vitamins B12 and folate decreases homocysteine blood levels, with promising clinical outcomes in Mild Cognitive Impairment (MCI), this so far has not been replicated in established AD populations. The aim of the study is to explore the relationship between hyperhomocysteinemia and relevant vitamins as the disease progresses.

Methods: In this longitudinal cohort study, 38 participants with mild to moderate AD were followed for an average period of 13 months. Plasma folate, vitamin B12, and homocysteine concentrations were measured at baseline and at follow-up. Dietary intake of B vitamins was also measured. Spearman's correlations were conducted by homocysteine and B vitamin status.

Results: As expected, cognitive status significantly declined over the follow-up period and this was paralleled by a significant increase in homocysteine concentrations ($p=0.006$). However, during this follow-up period there was no significant decline in neither dietary intake, nor the corresponding blood concentrations of vitamin B12/folate, with both remaining within normal values. Changes in blood concentrations of B vitamins were not associated with changes in homocysteine levels ($p>0.05$).

Conclusion: In this study, the increase in homocysteine observed in AD patients as the disease progresses cannot be solely explained by dietary and blood levels of folate and vitamin B12. Other dietary and non-dietary factors may contribute to hyperhomocysteinemia and its toxic effect in AD, which needs to be explored to optimise timely intervention strategies.

Keywords: homocysteine, B vitamins, Alzheimer's disease, cognition

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1. Introduction

Dementia is a common disorder with its prevalence set to rise worldwide. In the absence of a cure there is a growing need to identify treatments that can slow the cognitive progression of the disease, thus enabling those with dementia to retain appropriate functional abilities for longer. Currently, there are only a small number of symptomatic medications licensed for the treatment of Alzheimer's disease (AD), the most prevalent type of dementia. Importantly, the effectiveness of these licensed medications are often considered limited (Casey et al., 2010).

Abnormal elevation of homocysteine levels has been implicated as a marker for AD.

Hyperhomocysteinemia is associated with increased cognitive decline in healthy older adults with higher risk of cognitive impairment (e.g. (Morris, 2012; Smith et al., 2010; Vogel et al., 2009). Apart from a few notable exceptions, most studies report mild to moderate hyperhomocysteinemia in dementia populations compared to healthy controls (for a review see (Zhuo et al., 2011). However, research into the specific role of homocysteine on cognitive decline in a population with established AD is limited. Although not consistently reported (e.g. (Huang et al., 2010), studies have found that homocysteine levels in AD can predict rates of cognitive decline (Huang et al., 2013; Oulhaj et al., 2010).

It has therefore been of interest to establish whether reducing homocysteine levels can have a beneficial effect on the slowing down of AD progression. A key avenue of research is the supplementation with vitamin B12 and folate. These vitamins act as cofactors for the methylation of homocysteine to methionine, and therefore in the absence of these dietary nutrients homocysteine concentrations increase. It has been estimated that 65% of the hyperhomocysteinemia cases among the Framingham elderly population-based cohort could be accounted for by inadequate folate, or, to a lesser extent, inadequate vitamin B12 or

vitamin B6 status and intake (Selhub et al., 1993). It has even been shown that B vitamins taken at the earliest stages of the disease (i.e. Mild Cognitive Impairment (MCI)) are able to slow down the rate of brain atrophy (Smith et al., 2010) as well as the cognitive decline (de Jager et al., 2012), and may reduce conversion rates into dementia (Blasko et al., 2013). Despite this, there is little evidence that increasing the intake of these vitamins are able to combat cognitive decline later in the disease course, i.e. among participants with established AD. Serum levels of vitamin B12 have been found to have no association with cognitive progression in AD populations (Huang et al., 2010; Oulhaj et al., 2010; Small et al., 1997a, 1997b; Tu et al., 2010). Randomised controlled trials (RCTs) in AD populations have found that relevant vitamin treatments are unable to attenuate cognitive progression in the samples as a whole (Aisen et al., 2008; Kwok et al., 2011; Sun et al., 2007). Interrogation of these findings suggest that B vitamin supplementation may still have a role in attenuating cognitive decline in AD only in the presence of particularly elevated homocysteine levels. For example, Kwok and colleagues found that participants who received B vitamin treatment with elevated levels of homocysteine (13 $\mu\text{mol/L}$ or greater) had significantly smaller decline in the construction domain of Mattis Dementia Rating Scale (MDRS) than those receiving placebo (Kwok et al., 2011). Interestingly, participants in another study that had more modest levels of homocysteine (mean = 9.2 $\mu\text{mol/L}$, SD = 3.2) at the onset of the trial did not report any positive effects on cognition, while a subgroup with mild AD showed some benefit of the treatment (Aisen et al., 2008).

Discerning the exact role of homocysteine and B vitamins on cognitive decline is complicated, as hyperhomocysteinemia is only one of many contributing markers in the disease progression. The neurotoxicity of homocysteine and its direct effect on brain atrophy has been established (Madsen et al., 2015). Unlike the early indications from MCI, lowering homocysteine levels in established AD may not produce the same clinical benefits as research

so far has shown. Other non-dietary factors may also contribute to hyperhomocysteinemia and moderate its toxicity. Longitudinal studies assessing the relationship between homocysteine concentrations and cognitive decline in established AD, and its possible association with both dietary and blood vitamin B12 and folate levels, are limited (Clarke et al., 1998; Oulhaj et al., 2010).

The aim of this study was to establish whether the extensively reported hyperhomocysteinemia in AD as the disease progresses is associated with dietary and blood levels of B vitamins. In line with current knowledge, we hypothesised that blood homocysteine levels would significantly increase over the follow-up period. In addition, we hypothesised that this increase in homocysteine is explained by an associated decrease in vitamin B levels.

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2. Materials and Methods

2.1 Participants

All participants were recruited in Sussex (UK) memory assessment clinics and had mild to moderate AD (MMSE>12). The mean age of AD participants was 81.3 years (SD=6.0). The inclusion and exclusion criteria for this study has previously been reported elsewhere (Farina et al., 2016). In brief, eligible participants had previously been clinically assessed using the International Statistical Classification of Diseases, 10th revision (ICD-10; World Health Organisation, 1992), and received a diagnosis of probable dementia of Alzheimer's type. AD patients all had a personal consultee (relative or friend), and were either clinically or self-referred. For inclusion in the study described here, participants were also required to provide a blood sample on two occasions so that homocysteine concentrations could be measured. Ethical approval was obtained from a National Research Ethics Service Committee.

2.2 Neuropsychological testing and dietary data

Demographic data including age, gender and ethnicity were recorded. As previously reported (see Farina, Tabet, & Rusted, 2016), a battery of neuropsychological tests and the Food Frequency Questionnaire (FFQ) were completed by participants and lasted approximately 2-3 hours. The Addenbrooke's Cognitive Examination Revised (ACE-R; Mioshi et al., 2006) was used to provide a standardised measure of dementia severity. The ACE-R total score was also used as a comprehensive measure of global cognitive status. The Cornell Scale of Depression in Dementia (CSDD; Alexopoulos et al., 1988) was used to screen for the potential presence of major depression. IQ was estimated using the National Adult Reading Test (NART; Nelson, 1982).

Dietary intake of nutrients were estimated using the EPIC FFQ (Bingham et al., 2007). The questionnaire was completed by the carer of the participant, who described the average dietary intake of the participant over the past year. Once collected, data was analysed by the Department of Nutritional Sciences, University of Surrey, UK. Nutritional values for individual foods were estimated and summed. Nutrient intakes were expressed as percentages of the age and gender appropriate Recommended Nutritional Intakes (RNIs).

2.3 Biochemical assays

Plasma homocysteine, vitamin B12, and folate were analysed at the Department of Pharmacology, University of Oxford. Plasma concentrations of total Cobalamin (B12) and folate were measured by automated (Perkin-Elmer MultiProbe 11 liquid handling system, Perkin-Elmer Life and Analytical Sciences) microbiological assays using *Lactobacillus leichmanii* and *L. casei*, respectively (Kelleher and Broin, 1991; Molloy and Scott, 1997). Between-day coefficients of variation for B12 were 7.1%, and 7.4% for folate. Total plasma homocysteine was analysed by liquid-chromatography according to a modified protocol described previously (Refsum et al., 2004), using a QTRAP 5500 (AB Sciex, Framingham, Massachusetts, US) coupled to a Prominence LC-20AD_{XR} binary pump (Shimadzu, Kyoto, Japan). In short, 10 µL of plasma was added to an equal volume of an internal standard mix containing isotopically labelled homocysteine. Samples were neutralised using an ammonia solution before reduction with dithioerythriol at room temperature for 15 min. Plasma proteins were precipitated with perchloric acid (4% v/v) and cleared by centrifugation. Supernatant was diluted 1:10 in water/sodium 1-heptane sulfonate [1M]/ perchloric acid [20%, 3.3 M] 5/3/1 v/v/v, and injected onto a Kinetex C18 column (30 x 4.6 mm, 2.6 µm, Phenomenex, Torrance, CA, US) at a flow rate of 0.8 mL/min. Gradient elution was

employed starting at 100% mobile phase A (water + 0.05% formic acid), with a final composition of 40% A and 60% mobile phase B (methanol + 0.05% formic acid). Data acquisition and analysis were performed with Analyst 1.6.1 (AB Sciex, Framingham, Massachusetts, US). Quantitation was based on the ratio of analyte peak area/internal standard peak area against a linear calibration curve with a 1/x weighting. The coefficient of variation for was 4.0%.

2.4 Statistical Analysis

Descriptive statistics (e.g. means and frequencies) were reported for the participant demographics. All findings reported were of participants that were able to provide a sample of blood at baseline and at follow-up so that plasma homocysteine concentrations could be analysed. A series of Spearman's Rho correlations were conducted between baseline measures of B vitamin status and plasma homocysteine levels to determine the relationship between them. Additional Spearman's Rho correlations were run between B vitamin status and homocysteine change scores. No adjustments were made for multiple comparisons. Vitamin B12 and folate levels, plasma homocysteine and global cognitive status were compared between time points. Plasma homocysteine scores were logarithmically transformed. For non-parametric data, a Wilcoxon Signed Rank test was used, and for parametric data, a Paired-sample t-test was used.

The statistical significance threshold was set at $p < 0.05$. All statistical analyses were performed using SPSS (IBM SPSS statistics V.21; SPSS Inc, Chicago, Illinois, USA).

3. Results

3.1. Demographics

Thirty-eight participants with a diagnosis of AD met the inclusion criteria and were tested at baseline and on average 389.4 days later (SD= 26.2). Participants mean age was 81.3 years (SD=6.0), had a mild to moderate diagnosis at baseline, and were all free from major depression (CSDD \leq 10). See Table 1 for full participant demographics.

-----Table 1 Here -----

3.2. Validation of dietary intake vs plasma levels of B vitamins

Dietary intake of vitamin B12 did not significantly correlate with plasma vitamin B12 concentrations at baseline ($r_s = .12$, $p = .46$) or at follow-up ($r_s = -.18$, $p = .29$). Dietary intake of folate did however significantly correlate with plasma folate at baseline ($r_s = .43$, $p < .01$) but not at follow-up ($r_s = .06$, $p = .72$).

3.2. The relationship between plasma homocysteine concentrations and vitamin B12 and folate status

Baseline plasma homocysteine concentrations significantly correlated with plasma folate ($r_s = -.58$, $p < .001$) and vitamin B12 concentrations ($r_s = -.42$, $p < .01$). Baseline plasma homocysteine concentrations also significantly correlated with dietary intake of folate ($r_s = -.34$, $p = .04$), but not with dietary intake of vitamin B12 ($r_s = -.20$, $p = .24$). See Table 2.

-----Table 2 Here -----

3.3. Longitudinal changes in dietary intake and plasma levels

Dietary and plasma levels of vitamin B12 and folate declined between time-points. However, we only observed borderline significant decrease in plasma B12 concentrations ($p = .05$). Interestingly, plasma homocysteine concentrations significantly increased between time-points ($p = .001$) as presented in Table 3.

-----Table 3 Here -----

3.4. Relationship between B vitamins and change in homocysteine

No baseline indices of vitamin B status was significantly associated with longitudinal changes of plasma homocysteine ($p > 0.05$) (See Table 2). However, there was a trend toward significance between change in homocysteine compared to change in plasma B12 levels ($r_s = -.30$, $p = .07$). This was not observed when compared to changes in plasma folate levels ($r_s = -.10$, $p = .54$).

4. Discussion

The primary aim of this research was to assess whether the longitudinal changes in homocysteine levels were related to B vitamin (vitamin B12 and folate) concentrations in a cohort with established AD. This study found that homocysteine levels did increase over the study period, even in a cohort with high homocysteine levels at baseline. However, we were unable to show that the continued increase in homocysteine levels were driven by changes in B vitamin status.

It is important to highlight that participants in this study reported consuming well above the Reference Nutritional Intake (RNI; (Department of Health, 1991) for folate (mean = 169.6 %) and vitamin B12 (mean = 529.3%) at baseline. Whilst FFQs do tend to overestimate intake, there is an indication in the present sample that patients during the early stages of AD consume nutrients sufficient to meet the requirements of 97.5% of their non-dementia counterparts. In addition, the corresponding plasma concentrations of folate and vitamin B12 were deemed as being well above deficiency levels (Smith and Refsum, 2012; World Health Organization, 2012). There was also no significant decline in B vitamin intake and plasma concentrations over the study period. In any case, all values were still safely well above recommended levels at the end of the study. In contrast, plasma homocysteine concentrations significantly increased between time-points. Therefore, it can be inferred that maintaining an adequate day-to-day intake of B vitamins, even in the relatively high homocysteine levels reported in this sample, is insufficient to maintain baseline homocysteine levels. Correlation analysis between change in homocysteine scores and indices of B vitamin status revealed that there was no significant association, though a trend was found between change in B12 levels and homocysteine levels ($p=0.07$). This raises the question whether other factors may be contributing to increasing homocysteine levels within AD and indirectly may explain that why clinical trials with established AD has not so far shown clinical benefit. It has previously been recommended that higher B vitamin target concentrations are needed to control homocysteine concentrations in certain populations (Smith and Refsum, 2012) and whilst the present study does not provide direct evidence to support this recommendation, it certainly could explain some of our current findings.

More favourable data has been presented in a MCI cohort where vitamin supplementation not only lowered homocysteine levels but protected against brain atrophy (Smith et al., 2010), and cognitive and clinical decline in those with elevated homocysteine at baseline (de Jager et al.,

2012). It is still worth acknowledging that the role of homocysteine in the progression of AD is likely to be more complex, and may interact with other factors. For example, B vitamin supplementation only slowed brain atrophy rates and cognitive decline in participants with sufficiently high plasma levels of omega-3 fatty acids in a MCI population (Jernerén et al., 2015; Oulhaj et al., 2016). Omega-3 fatty acids were not measured in the present study, and therefore we are unable to comment on the role of omega-3 in the context of the current findings.

This study has several limitations which need to be acknowledged. A key limitation is that the relatively small sample size increases the likelihood of type II error. Dietary intake estimates calculated from the FFQ should not be considered as absolute values but rather estimates for the comparison within and between participants at different time-points. The participants recruited for this study were recruited for the purposes to identify the role of lifestyle factors on the cognitive progression of dementia (see Farina et al., 2016), and as a result no control group was recruited. This prevents us from discussing how these vitamins and homocysteine concentrations in our sample differ to a cognitively healthy population, and whether the increase in homocysteine over time reported here differs significantly from what we expect in age-matched controls (Frick et al., 2004; Refsum et al., 2004). However, it is well established that homocysteine levels are elevated in AD compared to healthy controls (for review see (Zhuo et al., 2011). The mean homocysteine concentration at baseline reported in the present study, 16.0 $\mu\text{mol/L}$, is considered elevated and is not dissimilar to that reported in a similar AD cohort (e.g. mean age = 82.8 years, average total homocysteine = 18.3 $\mu\text{mol/L}$) (Joosten et al., 1997). Another potential limitation is the choice of measurement for B vitamin status in the bloods, which may have affected accuracy. For example, holotranscobalamin (holoTC) has been argued as being more reliable marker of B12 status than total vitamin B12 alone

(Harrington, 2017), particularly in those who are vitamin B12 deficient (Nexo and Hoffmann-Lücke, 2011).

In summary, AD patients in the present sample showed stable and adequate levels of folate and vitamin B12 for the duration of the study, based on commonly used recommendations and cut-off values. Despite this, homocysteine levels were elevated at baseline and increased further as the disease progressed. Whilst there is an established relationship between elevated homocysteine and dietary intake of B vitamins in older adults (Selhub et al., 1993), this study could indicate that the recommended guidelines in terms of plasma levels of B vitamins might have to be reconsidered, as suggested by others (Smith and Refsum, 2012). However, we should not neglect the potential of other factors, with evidence from a MCI population identifying omega-3 may contribute to hyperhomocysteinemia (Jernerén et al., 2015; Oulhaj et al., 2016). Overall this might explain why so far dietary vitamin B intervention has not showed direct clinical benefit in AD. Ultimately, understanding the underlying mechanism of hyperhomocysteinemia in AD beyond vitamin B cofactors might help better optimise treatment strategies, which may result in direct clinical benefit to patients.

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Table 1. Baseline demographic data of AD participants (n=38)

	%	Mean	SD
Age (years)		81.3	6.0
Gender			
Male	53		
Female	47		
Diagnosis			
AD	71		
Mixed	29		
Race			
Caucasian	100		
Time Since Diagnosis (years)		1.1	1.5
Premorbid IQ		115.0	8.9
Age leaving full-time education (years)		16.1	2.4
Pulse Pressure (Systolic Pressure – Diastolic Pressure)		75.4	16.8
Creatinine ($\mu\text{mol/l}$)		91.9	31.5
Cornel Scale of Depression in Dementia		2.5	2.7
Body Mass Index		26.2	3.8

Table 2. Spearman's correlation between ACE-R change scores, baseline dietary B vitamins, baseline plasma B vitamins and baseline plasma homocysteine concentrations.

	Baseline Dietary Vitamin B12		Baseline Plasma Folate		Baseline Plasma Vitamin B12		Baseline Plasma Homocysteine		Plasma Homocysteine Change scores	
	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
Baseline Dietary Folate	.44	.006	.43	.008	-.11	.531	-.34	.039	.01	.972
Baseline Dietary Vitamin B12			.27	.100	.12	.457	-.20	.236	.11	.508
Baseline Plasma Folate					.26	.116	-.58	< .001	-.21	.201
Baseline Plasma Vitamin B12							-.42	.009	0.08	.643

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Table 3. The means (and standard deviations) of blood and dietary markers of B vitamins, plasma homocysteine and global cognition at each time-point for participants that completed both time points (n=38).

	Baseline		Follow-up		Diff (95% CI)	p
	Mean	SD	Mean	SD		
Plasma						
Homocysteine (µmol/l)	16.0	5.3	17.4	6.3	1.3 (0.6, 2.1)	.001
B12 (pmol/l)	347.0	171.0	311.4	207.9	-35.6 (-91.3, 20.1)	.053
Folate (nmol/l)	25.9	23.3	21.8	19.5	-4.2 (-10.9, 2.6)	.175
Diet						
B12 (% RNI)	529.3	286.3	498.6	206.6	-30.7 (-124.1, 62.6)	1.00
Folate (% RNI)	169.6	58.3	168.8	101.1	-0.7 (-25.3, 23.8)	.085
Global Cognition						
ACE-R	69.6	10.0	65.6	13.3	-4.0 (-6.8, -1.2)	.006

RNI: Recommended Nutritional Intake, ACE-R: Addenbrooks Cognitive Examination-Revised, CI: Confidence Intervals