RESEARCH ARTICLE

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Homocysteine as a predictor of cognitive decline in Alzheimer's disease

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Objective: Moderately elevated levels of plasma total homocysteine are associated with an increased risk of developing Alzheimer's disease. We have tested whether baseline concentrations of homocysteine relate to the subsequent rate of cognitive decline in patients with established Alzheimer's disease (AD).

Methods: In 97 patients with AD, 73 pathologically-confirmed, we analysed the decline of global cognitive test scores (CAMCOG) over time from the first assessment for at least three 6-monthly visits up to a maximum of 9.5 years (in total 689 assessments). Non-linear mixed-effects statistical models were used.

Results: Baseline homocysteine levels showed a concentration-response relationship with the subsequent rate of decline in CAMCOG scores: the higher the homocysteine, the faster the decline. The relationship was significant in patients aged < 75 years who had not suffered a prior stroke. For example, in patients aged 65 years with a baseline homocysteine of 14 μmol/L, the decline from a CAMCOG score of 88 to a score of 44 occurred 19.2 (95% CI 6.8, 31.6) months earlier than in patients with a baseline homocysteine of 10 μmol/L.

Conclusions: Raised homocysteine concentrations within the normal range among the elderly strongly relate to the rate of global cognitive decline in patients with Alzheimer disease. Plasma homocysteine can readily be lowered by B-vitamin treatment and trials should be carried out to see if such treatments can slow the rate of cognitive decline in relatively young patients with Alzheimer disease.

Key words: homocysteine; Alzheimer's disease; cognitive decline; non-linear model
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ABBREVIATIONS — AD Alzheimer's disease CAMCOG cognitive component of the Cambridge Examination for Mental Disorders of the Elderly tHcy plasma total homocysteine

Introduction

Progressive decline in cognitive abilities is a defining characteristic of Alzheimer's disease (AD). The rate of cognitive decline is highly variable from patient to patient (Teri *et al.*, 1995) and a number of factors have been proposed that might account in part for this variability. These include: gender, age at onset of symptoms, (Jacobs *et al.*, 1994; Teri *et al.*, 1995) baseline cognitive status, (Burns *et al.*, 1991; Morris *et al.*, 1993) period of education, (Rasmusson *et al.*, 1996; Stern *et al.*, 1999; Teri *et al.*, 1995) behavioural problems, (Mortimer *et al.*, 1992) psychosis, (Chui *et al.*, 1994; Scarmeas *et al.*, 2005) extrapyramidal signs (Chui *et al.*, 1994; Scarmeas *et al.*, 2005) and plasma levels of amyloid β-protein and C-reactive protein (Locascio *et al.*, 2008).

The progression of cognitive decline is not only associated with decline in functional abilities in the

patient, leading to institutionalization and early death, (Wolfson *et al.*, 2001) but also leads to increased costs to society (Ernst *et al.*, 1997; Wolstenholme *et al.*, 2002; Zhu *et al.*, 2006). Thus, finding ways to slow the rate of cognitive decline would have important clinical and public health implications. Furthermore, the identification of biological factors that influence the rate of cognitive decline could improve our scientific understanding of the underlying mechanisms.

Different alleles of the gene for apolipoprotein E (*APOE*) are associated with different rates of cognitive decline in AD (Martins *et al.*, 2005). Genes, and most of the factors listed above, are not readily modifiable. In this report, we describe evidence that the blood level of a modifiable factor, plasma total homocysteine (tHcy), is related to the rate of cognitive decline.

Moderately raised levels of tHcy have been identified as a candidate risk factor for the development of AD (Clarke *et al.*, 1998; McCaddon *et al.*, 1998; McCaddon, 2006; Seshadri, 2006; Smith, 2008). We here examine whether tHcy levels measured at initial diagnosis of AD are also related to the rate of subsequent cognitive decline over a period of several years. We have applied non-linear mixed-effects statistical models, since previous studies have shown that cognitive decline in AD better fits a non-linear model (inverse 'S' curve) than a linear model (Martins *et al.*, 2005; Stern *et al.*, 1996).

Methods

Subjects

The study population comprised Caucasian patients in the longitudinal OPTIMA cohort. We included patients age 50 or older with a clinical diagnosis of dementia at baseline who either then or at follow-up had a diagnosis of AD (see below) and for whom CAMCOG scores were available from three or more assessments at least 5 months apart. Only patients with CAMCOG score greater than 35 at baseline were included. This limited the study population to 97 AD patients. The OPTIMA protocols (Clarke *et al.*, 1998) have been approved by the Central Oxford Ethics Committee (No 1656). All participants gave informed consent.

Data collection

Research nurses administered the Cambridge Examination for Mental Disorders of the Elderly (CAMDEX) (Roth *et al.*, 1988) to all participants at their first assessment and at approximately 6-month intervals. The CAMDEX includes a cognitive assessment (CAMCOG) with a maximum score of 107. Follow-up with the CAMCOG continued until patients were too impaired to complete the first few questions on the CAMDEX (name, birth date, address). The final diagnosis was from the most recent medical assessment or based on postmortem assessment, if available. The current study includes only patients with clinical diagnoses of possible ($n = 6$) or probable AD ($n = 18$) using established operational criteria (McKhann *et al.*, 1984) or those with histopathologically confirmed AD ($n = 73$) using CERAD 'Probable' or 'Definite' criteria. None of the patients had familial AD. Patients were categorized as ever or never smokers. Raised cardiovascular risk ($n = 48$) was defined as one or more of the following prior to, or during, the study period: myocardial infarction ($n = 7$); stroke ($n = 28$); diabetes or use of antidiabetic drugs ($n = 7$); use of antihypertensive drugs ($n = 26$). Treatment with centrally acting drugs ($n = 22$) that might modify cognitive function or disease progression included donepezil ($n = 15$), galantamine ($n = 3$), rivastigmine ($n = 2$), dothiepin ($n = 2$), risperidone ($n = 2$) and memantine ($n = 1$), with 3 patients taking two of these drugs.

Blood sampling and analysis

Blood drawn from non-fasting subjects into tubes containing EDTA was stored at -80°C until biochemical analyses were performed. Plasma total homocysteine (tHcy) was determined with a fluorescence polarization immunoassay using the Abbott IMx® analyzer (Shipchandler and Moore, 1995). The concentration of plasma folate was measured by a Lactobacillus casei microbiological assay and plasma vitamin B_{12} concentration by a Lactobacillus leichmannii microbiological assay. Our laboratory CVs were 3% for tHcy, and 7% each for both folate and vitamin B12. Apolipoprotein E (APOE) genotypes were determined by a one-stage PCR assay.

Statistical methods

Simple comparisons were made using Student's t test or the χ^2 test. Otherwise, the CAMCOG data were analysed using non-linear mixed-effects models (Davidian and Giltinan, 1995; Vonesh and Chinchilli, 1997). The non-linear analysis uses a logistic (S-shaped) curve (Pinheiro and Bates, 2000) to model the expected

CAMCOG score *j* months relative to the first visit. We used a three-parameter logistic function:

$$\text{CAMCOG} = \frac{\text{Asymptote}}{1 + 0.025 \exp\left[-3.65\left(\frac{\text{Onset-time}-j}{\text{Half-time}}\right)\right]} + \text{Residual}$$

where *j* is the number of months relative (before or after) to the first visit. The three parameters can be interpreted as follows:

Asymptote: The expected maximal CAMCOG score just before the cognitive function starts to decline.
Onset-time: The time (in months) relative to the first visit when the patient reached 97.5% of the asymptotic value of their CAMCOG score. We selected this time because it is likely to be shortly before cognitive function started to decline steeply, but while cognitive function still remained fairly intact. The age at onset of symptoms may then be defined as the Onset-time + Age at first visit (i.e. age at baseline).
Half-time: The duration of time (in months) from the Onset-time to the time when the CAMCOG score reaches 50% of its initial maximal value (i.e. half of the asymptote). It is an alternative measure to the rate-of-decline: in patients with a fast rate of decline, the 'Half-time' will be short. In this study, we are assessing the effect of tHcy, in comparison with other factors, on *Half-time*. In this non-linear mixed effects model, the advantage of 'Half-time' compared with the rate-of decline is that the former can be modelled as a direct linear function of tHcy and other covariates, while the latter is the derivative of the CAMCOG score over time and, hence, will vary according to which part of the curve is assessed.

These parameters, and the curve created with the model, are graphically depicted in Figure 1.

Because individual observed CAMCOG curves differ between patients, we included random components in the model to control for unobserved predictors that may have a significant effect on the cognitive decline, and to account for the correlation structure that may exist between consecutive CAMCOG scores within the same patient (Davidian and Giltinan, 1995; Pinheiro and Bates, 2000; Vonesh and Chinchilli, 1997). Thus, the two parameters *Onset-time* and *Half-time* are decomposed within the model into fixed and random effects so that the fixed effects of the model provide a statistical representation of the 'average' decline in CAMCOG, while the two random effects describe how patients deviate from the average pattern. For further details of the method, see the supporting information on the web.

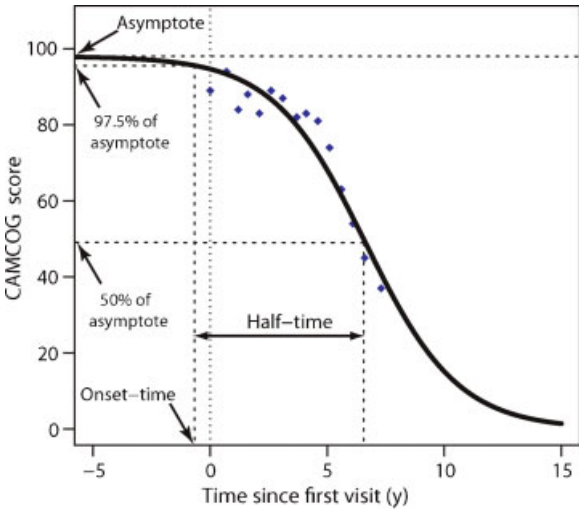

Figure 1 Illustration of the non-linear mixed model in an AD patient The data are from a male patient, aged 59.8 years, followed for 7.3 years, with 15 CAMCOG assessments and recruited at an early stage of disease. By using the non-linear mixed modelling, we estimated the three relevant parameters, i.e. the '*asymptote*', corresponding to the expected maximal CAMCOG score before cognitive function started to decline, the '*Onset-time*', corresponding to the time at 97.5% of the maximal CAMCOG score and the '*Half-time*', i.e. the duration of time from the Onset-time until the half-maximal CAMCOG score.

We fitted the model to the data set using the 'nlme' procedure in R (R-Development-Core-Team, 2004). The non-linear analysis modelled the effect of tHcy on *Half-time*, controlling for all other covariates, including age at baseline (continuous), gender, *APOE* genotype (binary or three categories), years of education (continuous), use of centrally-acting drugs (binary), systolic blood pressure at baseline (continuous), CVD risk category (binary) as well as factors known to influence tHcy levels: serum folate, vitamin B12, creatinine (continuous) and smoking (binary). We used both the likelihood ratio and Wald tests to test for non-significant sets of parameters and consequently for model building. The Akaike information criterion was sometimes used for comparing non-hierarchical models. An effect with a *p*-value (two-sided) < 0.05 was considered significant.

Results

Characteristics of the study population

We examined 97 patients, with a diagnosis of AD, every 6 months over periods ranging from 1.5 to 9.5 years. The average number (SD) of visits per patient was 7.1 (3.7); in total, the study included 689 visits. Some

Table 1 Characteristics of the study population

	Study population ($n = 97$)
Age in years, mean (SD)	71.9 (8.2)
Men	43 (44.3%)
CAMCOG score at baseline, mean (SD)	68.3 (15.9)
Age left at school in years, mean (SD)	15.5 (1.8)
Systolic blood pressure in mm Hg, mean (SD)	149.5 (21.9)
Diastolic blood pressure in mm Hg, mean (SD)	85.3 (10.5)
Ever smoker	26 (26.8%)
Plasma tHcy in μmol/L, mean (SD)	14.2 (4.8)
Serum creatinine in μmol/L, mean (SD)	95.7 (24.6)
Serum vitamin B12 in pmol/L, mean (SD)	326 (184)
Serum folate in nmol/L, mean (SD)	9.2 (4.8)
*APOE*4 negatives	28 (28.9%)
*APOE*4 heterozygotes	52 (53.6%)
*APOE*4 homozygotes	17 (17.5%)
Centrally acting drugs	22 (22.7%)
History of stroke	28 (28.9%)
Diabetes	7 (7.2%)
Antihypertensive drugs	26 (26.8%)
History of myocardial infarction	7 (7.2%)
High CVD category*	48 (49.5%)
Age of onset in years, mean (SD)**	69.0 (8.5)
Time since onset in years, mean (SD)**	3.1 (2.4)
Duration in study in years, mean (SD)	3.4 (1.9)
Number of visits	7.1 (3.7)

Data are number (%) unless otherwise specified.
*Defined as one or more of the following prior to or during the study period: Myocardial infarct; stroke, diabetes or use of antihypertensive drugs.
**Based on information from a family member.

descriptive statistics about the patients at their first visit to OPTIMA are given in Table 1. Typical examples of the patterns of cognitive decline in different patients are shown in Figure 2. It is apparent that despite similar starting points, the individual patterns vary considerably, with the rate of decline differing from patient to patient. Furthermore, the change in CAMCOG score seems not to be linear with time.

Effect of homocysteine and other covariates on *Half-time*

We fitted a non-linear mixed effects model, as described in Methods, to the data using both the likelihood ratio and Wald tests to test for non-significant sets of parameters with all the relevant covariates. The final model contained the main effects of tHcy, age at baseline, stroke, education, treatment with centrally acting drugs and two-way interaction terms between tHcy and age, tHcy and stroke, age and stroke, and a three-way interaction between tHcy, age and stroke. The other covariates did not contribute, and were therefore not included. In later analyses, we have used this model unless otherwise stated.

Table 2 lists the covariates that were associated with *Half-time* from the fitted model. From this final model, the asymptote or the maximal CAMCOG score was estimated at 87.1 (SD 0.8). On average, from the model, patients entered the study 25.82 (3.32) months after the clinical onset of the disease. In a group of patients aged 70 years, baseline tHcy of 14 μmol/L, with average schooling and without stroke and not on treatment with centrally acting drugs, the average *Half-time* was 43.3 (4.2) months. A longer period at school had a negative influence, i.e. *Half-time* became shorter, while centrally-acting drugs and stroke appeared to have a positive influence, i.e. *Half-time* became longer. The effect of tHcy on *Half-time* was significant, but it significantly interacted with age and also with stroke. Applying this model to the individual patients whose cognitive trajectories are shown in Figure 2 gave the results shown in Figure 3. It is clear that the model provides a reasonably good fit to the observations.

Using this final model, we can then estimate the change in *Half-time* associated with any set of our covariates. In Figure 4, we show the estimated *Half-time* according to tHcy at baseline in four different age groups in patients who left school at age of 16 years, who had not suffered from a stroke and who did not receive treatment with centrally acting drugs. The corresponding predicted rates-of-decline (CAMCOG points per year) are also shown, estimated from the period of maximal and nearly linear rate of decline from 75 to 25% of the CAMCOG score (see Methods). Notably, at age < 75 years and in patients without stroke, there was a significant association with tHcy, so that the higher the concentration of tHcy, the shorter the *Half-time*, i.e. the more rapid rate of decline in the CAMCOG score. At age > 75 years or in patients with stroke (independent of age), we did not find a significant association between tHcy and *Half-time*. Thus elevated tHcy is a strong risk factor for rapid cognitive decline in relatively young AD patients but becomes progressively less important with increasing age.

The potential effect of homocysteine-lowering therapy on the course of CAMCOG

The model can be used to predict the effects of lowering tHcy levels on subsequent changes in CAMCOG scores. The predicted effect of lowering baseline tHcy on the course of CAMCOG over time in 4 different age groups is shown in Figure 5, while Table 3 shows the

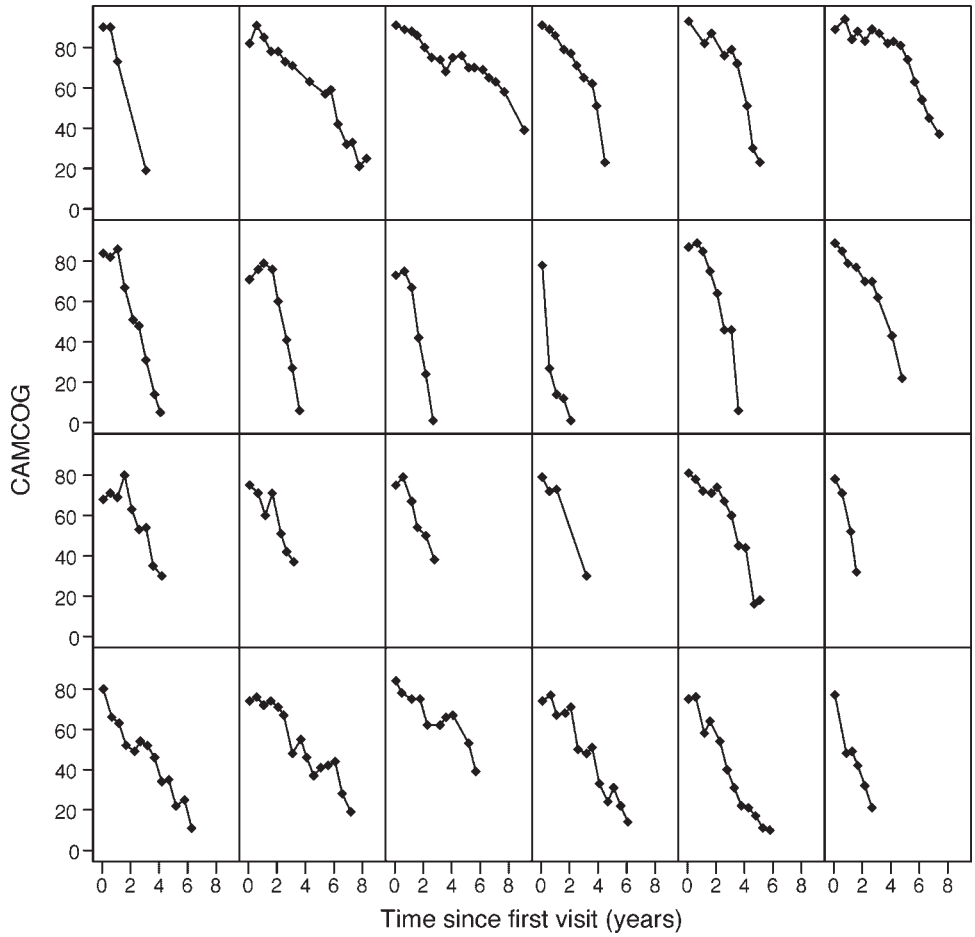

Figure 2 Observed longitudinal CAMCOG scores in a selection of the AD patients with CAMCOG scores greater than 75 points at baseline and less than 40 points at last visit ($n = 24$).

Table 2 The parameter estimates in the total population of 97 patients with clinical dementia using a non-linear mixed-effect model*

	Parameter estimates (SD) months	*p*-value
Average Onset time	−25.82 (3.32)	<0.001
Average *Half-time*	43.32 (4.23)	<0.001
Effect of 1 μmol/L increase in tHcy	−2.72 (1.15)	0.019
Effect of age at baseline per year	0.94 (0.43)	0.029
Effect of stroke	21.18 (7.64)	0.006
Effect of schooling per year	−4.67 (1.51)	0.002
Effect of centrally acting drugs	17.72 (7.48)	0.018
tHcy * Age	0.42 (0.12)	<0.001
tHcy * Stroke	2.72 (1.15)	0.018
Age * Stroke	−2.81 (0.97)	0.004
tHcy * Age * Stroke	−0.42 (0.12)	0.001

*In a model where the covariates are centred as follows: tHcy at baseline, 14 μmol/L; Age left school, 16 years; Age at baseline, 70 years. The asymptote for the CAMCOG score was 87.07 (SD 0.82), $p < 0.001$.

predicted effect of lowering tHcy levels by 4 μmol/L on the *Half-time* in patients with different ages at baseline. It is apparent that there is a marked difference in *Half-time*, and thus, the rate of decline, according to age and tHcy levels.

Effect of homocysteine and other covariates on *Half-time* when age is categorized as a binary variable

Since the non-linear mixed effects model indicated that the effect of tHcy becomes statistically significant below an age of approximately 75 years, we used an alternative approach to see if this could be confirmed. We divided the data set into two groups: the first group included 56 patients aged ≤ 75 years among which 41 (i.e. 73.2%) had no history of stroke. The second group included 41 patients aged > 75 years among which 28 (i.e. 68.3%) had no history of stroke (supporting information: Table S1). We then fitted the model to all 97 patients using age at baseline as a binary covariate,

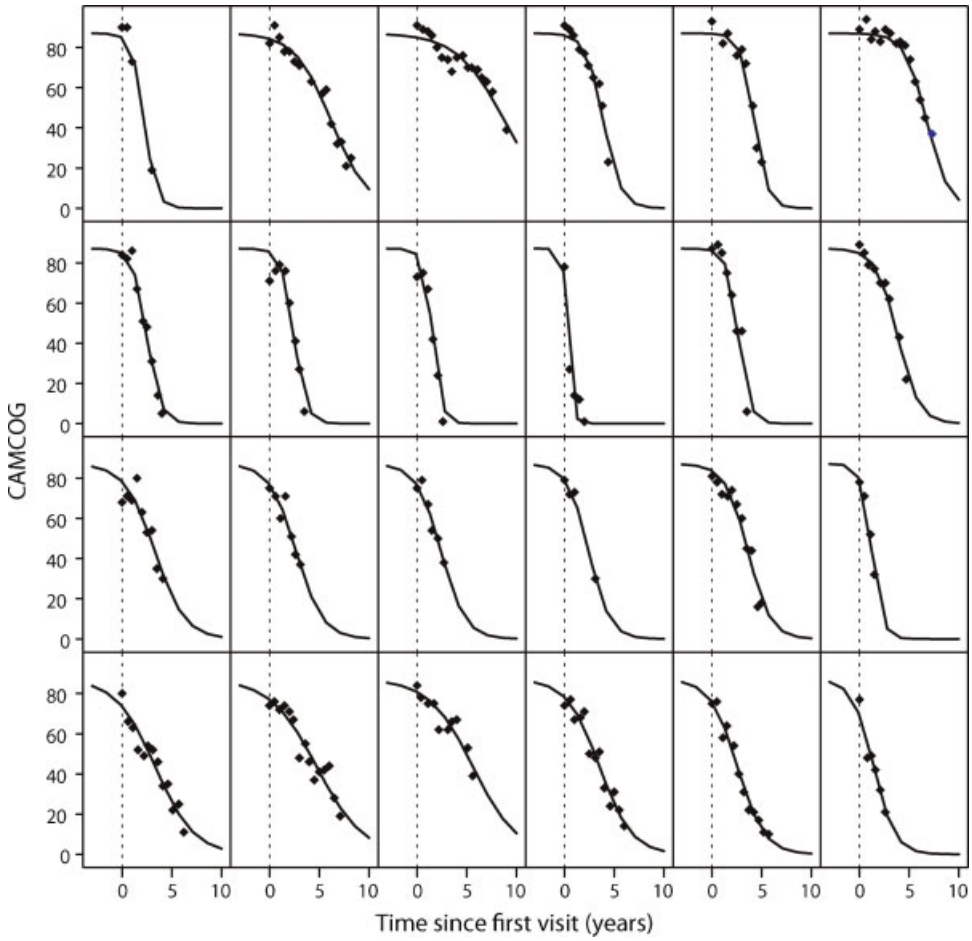

Figure 3 The predicted CAMCOG decline curves in a selection of AD patients using non-linear mixed modelling The final model was fitted to all 97 subjects and the figure shows how this model (curves) fits with the observed CAMCOG data (points) in each of the 24 patients of Figure 2. The model takes into account the covariates and interaction terms listed in Table 2.

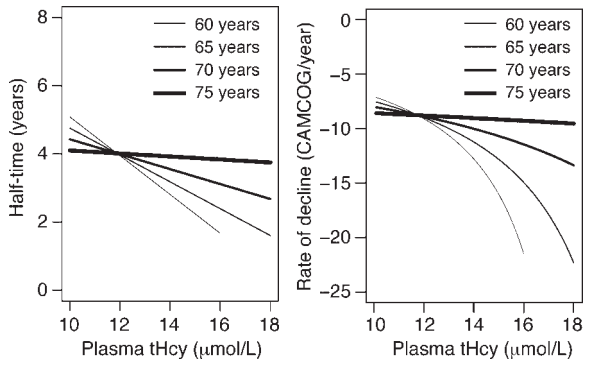

Figure 4 The effect of tHcy on *Half-time* and rate of cognitive decline in four age-groups of AD patients. Using the non-linear mixed final model and data from all the patients ($n = 97$), the expected *Half-time* (left panel) and Rate-of-decline (right panel) were estimated in four age-groups and at three different tHcy levels. The final models included the covariates and interaction terms listed in Table 2, and curves shown are based on a population who left school aged 16 years, without stroke and who did not received treatment with centrally acting drugs. Rate-of-decline was estimated from between 75 and 25% of the asymptote.

starting with a global model containing all potential covariates and building the final model using both the Wald test and the Likelihood ratio test as described in Methods. The final model shows that the only group in which the effect of tHcy is statistically significant is the one with patients aged ≤ 75 years and without history of stroke. If tHcy in those ≤ 75 years was increased by 1 μmol/L, *Half-time* became significantly shorter ($p = 0.02$) by 3.77 (SD 1.61) months. Supporting material Figure 1S shows *Half-time* as well as the predicted course of CAMCOG for three levels of tHcy. These results are in full concordance with what we found earlier using age at baseline as a continuous covariate.

Discussion

In this study, and in a previous report, (Martins *et al.*, 2005) we have shown that a non-linear model provides

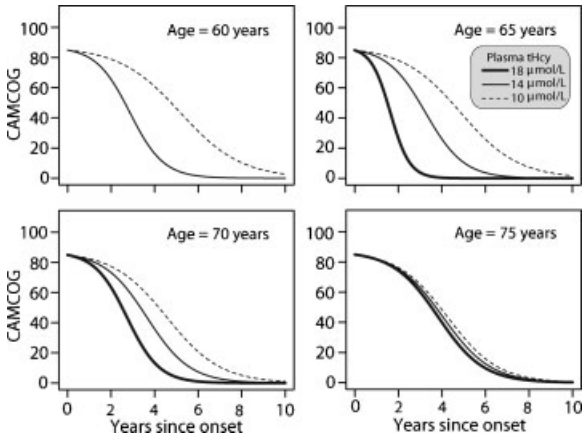

Figure 5 Potential effect of tHcy-reducing therapy on the course of CAMCOG in four age groups of AD patients. Using the final non-linear mixed model and data from all patients ($n = 97$), the expected CAMCOG course was estimated in four age-groups and at three different tHcy levels. (The plot for tHcy of 18 μMol/L in those aged 60 is not shown because none of the patients of this age had such a high tHcy level.) The final model included the covariates and interaction terms listed in Table 2, and the curves shown are based on a population who left school aged 16 years, without stroke and who did not received treatment with centrally acting drugs.

a good fit to the observed changes in cognition (as assessed by the CAMCOG score) over time in patients with AD. Parameters in a non-linear model have a natural biological interpretation. Non-linear models also provide more reliable predictions for the response variable outside the range of the data than linear models, as shown earlier for AD (Stern *et al.*, 1996).

The model we have used should prove useful in the search for factors that influence the rate of cognitive decline (as revealed by the parameter *Half-time*), but the relatively small size of our cohort means that in this study we could only detect factors with a strong effect. Thus, we did not have sufficient power to show the effect of *APOE* genotype that we described before in a larger cohort from OPTIMA (Martins *et al.*, 2005). Our final model shows that education is important:

Table 3 The predicted effect (with 95% confidence intervals) of lowering tHcy levels by 4 μmol/L on the *Half-time* in patients with different age at baseline and without stroke

	Increase in *Half-time* (months)	
	Estimate	95% Confidence intervals
Age = 60 years	27.5	11, 43.9
Age = 65 years	19.2	6.8, 31.6
Age = 70 years	10.9	1.9, 19.9

those with longer period of schooling have a faster rate of decline, consistent with previous reports (Rasmusson *et al.*, 1996; Stern *et al.*, 1999; Teri *et al.*, 1995). Age is a significant predictor, with younger patients showing a slightly shorter *Half-time* of almost 1 month for each year they are younger. In contrast, those patients treated with centrally-acting drugs (mainly cholinesterase inhibitors) or who had a history of stroke showed a slower rate of decline. The number of subjects treated with an individual drug was too small for us to confirm or otherwise the report (Ellul *et al.*, 2007) that certain drugs are associated with slower decline and others with more rapid decline. The protective effect of a prior history of stroke on the rate of cognitive decline is surprising in relation to the additive effect of vascular disease and AD pathology found in other studies (Esiri *et al.*, 1999; Nagy *et al.*, 1997; Snowdon *et al.*, 1997) and contrasts with the increased rate of decline found after incident stroke in patients with AD (Regan *et al.*, 2006). One possibility is that the stroke group in general represent a different aetiology, and hence, overall experience a lower rate of decline. Furthermore, the apparent protective effect of stroke may be related to the drug treatments, e.g. anti-hypertensive drugs, (Mielke *et al.*, 2007) that many of these patients received, since in developing the model we found that anti-hypertensive treatment was protective but it was replaced by stroke in the final model.

The novel finding of our study is that higher tHcy levels within the normal range for the elderly are associated with a more rapid rate of cognitive decline in patients with AD. In this cohort, the effect was only significant in patients without stroke and who were < 75 years old. In an earlier study on patients with AD, we reported that levels of tHcy above 11.1 μmol/L were associated with more rapid disease progression as assessed by the decrease in size of the medial temporal lobe (Clarke *et al.*, 1998). Two other reports found no effect of tHcy levels on cognitive decline in AD (Locascio *et al.*, 2008; Regan *et al.*, 2006), but in one study the average age of the patients was close to 80 years (Regan *et al.*, 2006) and in the other study an effect of tHcy on the decline in activities of daily living was observed (Locascio *et al.*, 2008). There is a more extensive literature on the possible association of tHcy with cognitive decline in the elderly (McCaddon, 2006; Smith, 2008), and two recent community studies are relevant. In a study in the UK, where the mean baseline tHcy was 14.5 μmol/L, raised tHcy and markers of low vitamin B12 status were associated with an increased risk of subsequent cognitive decline (Clarke *et al.*, 2007), while in a study in the USA, where the mean baseline tHcy was 11.5 μmol/L, tHcy was not associated

with cognitive decline, but markers of low vitamin B12 status were (Tangney *et al.*, 2009).

There are several potential clinical implications of our findings. First, irrespective of whether or not the association of plasma tHcy levels with cognitive decline is causal, tHcy appears to be a good prognostic marker for the rate of cognitive decline. For patients of the same age, those with high-normal tHcy are likely to decline more rapidly than those with low-normal tHcy. Second, if the association turns out to be causal, then lowering tHcy levels should delay cognitive decline in patients < 75 years old. Reductions of 3–4 μmol/L in tHcy levels can readily be achieved by treatment with folic acid and vitamin B-12 (Homocysteine-Lowering-Trialist-Collaboration, 2005). We have shown that the estimated effect of a 4 μmol/L fall in tHcy on the *Half-time* ranges from a lengthening of 10.9–27.5 months, depending on age (Table 3), which is highly significant clinically. Clinical trials should be carried out to see if this prediction can be born out in practice. The results of one such trial have recently been reported and overall they were negative (Aisen *et al.*, 2008), although it is noteworthy that a slowing of cognitive decline was found for those patients with mild AD at the start of the trial. The trial was carried out in USA, where grain products are fortified with folic acid, and the pretreatment mean baseline tHcy level was below 10 μmol/L, a level lower than the levels where we have found an association of tHcy with the rate of cognitive decline. Our results suggest that a *post hoc* analysis of this trial should further stratify patients for age and for a history of stroke. Third, in setting up new homocysteine-reducing trials, our data suggest that the design should favour younger patients without vascular disease. Finally, both the design and the analysis of clinical trials involving dementia patients will be enhanced by the use of non-linear modelling.

Key Points

- Plasma total homocysteine levels measured at baseline predict the rate of cognitive decline in patients with AD.
- The association was most marked in patients under 75 years old who had not had a stroke.
- If the effect is causal, it is predicted that lowering the levels of plasma homocysteine by B-vitamin treatment could markedly slow cognitive decline.
- Non-linear statistical analysis is a powerful method for studying the rate of cognitive decline.

Conflict of interest

ADS declares a financial conflict of interest since he was named as co-inventor on a patent filed in 1996 on the use of folic acid in the treatment of AD. The patent is held by the University of Oxford but, under the rules of the University, he stands to gain financially if the patent is exploited. None of the other authors have competing interests.

Ackowledgements

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Supporting information

Supporting information may be found in the online version of this article.

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