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Plasma homocysteine, Alzheimer and cerebrovascular pathology: a population-based autopsy study

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Elevated plasma total homocysteine is associated with increased risk of dementia/Alzheimer's disease, but underlying pathophysiological mechanisms are not fully understood. This study investigated possible links between baseline homocysteine, and post-mortem neuropathological and magnetic resonance imaging findings up to 10 years later in the Vantaa 85+ population including people aged ⩾85 years. Two hundred and sixty-five individuals had homocysteine and autopsy data, of which 103 had post-mortem brain magnetic resonance imaging scans. Methenamine silver staining was used for amyloid-β and modified Bielschowsky method for neurofibrillary tangles and neuritic plaques. Macroscopic infarcts were identified from cerebral hemispheres, brainstem and cerebellum slices. Standardized methods were used to determine microscopic infarcts, cerebral amyloid angiopathy, and α-synuclein pathology. Magnetic resonance imaging was used for visual ratings of the degree of medial temporal lobe atrophy, and periventricular and deep white matter hyperintensities. Elevated baseline homocysteine was associated with increased neurofibrillary tangles count at the time of death: for the highest homocysteine quartile, odds ratio (95% confidence interval) was 2.60 (1.28–5.28). The association was observed particularly in people with dementia, in the presence of cerebral infarcts, and with longer time between the baseline homocysteine assessment and death. Also, elevated homocysteine tended to relate to amyloid-β accumulation, but this was seen only with longer baseline-death interval: odds ratio (95% confidence interval) was 2.52 (0.88–7.19) for the highest homocysteine quartile. On post-mortem magnetic resonance imaging, for the highest homocysteine quartile odds ratio (95% confidence interval) was 3.78 (1.12–12.79) for more severe medial temporal atrophy and 4.69 (1.14–19.33) for more severe periventricular white matter hyperintensities. All associations were

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independent of several potential confounders, including common vascular risk factors. No relationships between homocysteine and cerebral macro- or microinfarcts, cerebral amyloid angiopathy or α-synuclein pathology were detected. These results suggest that elevated homocysteine in adults aged ⩾85 years may contribute to increased Alzheimer-type pathology, particularly neurofibrillary tangles burden. This effect seems to be more pronounced in the presence of cerebrovascular pathology. Randomized controlled trials are needed to determine the impact of homocysteine-lowering treatments on dementia-related pathology.

Keywords: homocysteine; Alzheimer's disease; Alzheimer pathology; cerebrovascular pathology; elderly

Abbreviation: CERAD = The Consortium to Establish a Registry for Alzheimer's Disease

Introduction

Plasma total homocysteine concentration is a sensitive marker of folate and B12 status and its increased levels are associated with a variety of disorders including cardiovascular, cerebrovascular and peripheral vascular conditions (Refsum *et al.*, 2004; Smith, 2008). Due to the known effects of folate and B12 deficiencies, and abnormal homocysteine metabolism on development and maintenance of the nervous system (Harris *et al.*, 2008), several plausible mechanisms through which high homocysteine might also increase the risk of dementia have been postulated, e.g. the impact on cerebrovascular pathology, direct neurotoxic effects or the influence on amyloid-β peptide generation and tau hyperphosphorylation; but none of these has been proven unequivocally (Obeid and Herrmann, 2006; Seshadri, 2006; Sontag *et al.*, 2007; Smith, 2008; Coppieters and Dragunow, 2011). Population-based longitudinal studies on the associations between homocysteine and dementia/Alzheimer's disease, rate of cognitive decline, or structural brain changes have shown conflicting results (Seshadri *et al.*, 2002, 2008; Vermeer *et al.*, 2002; den Heijer *et al.*, 2003; Smith, 2008; Hooshmand *et al.*, 2010, 2012; Rastas *et al.*, 2010; Jochemsen *et al.*, 2013). A few cross-sectional studies have reported associations between homocysteine and plasma amyloid-β levels (Flicker *et al.*, 2004; Irizarry *et al.*, 2005; Luchsinger *et al.*, 2007), and between metabolites of the homocysteine cycle and CSF phospho-tau (Obeid *et al.*, 2007; Popp *et al.*, 2009), but contradictory results also exist (Alexopoulos *et al.*, 2009; Smach *et al.*, 2011).

The present study investigated possible links between plasma homocysteine levels, and post-mortem neuropathological and MRI findings in the Vantaa 85+ population including people aged ⩾85 years. A previous study of this population found no significant association between homocysteine and dementia (Rastas *et al.*, 2010). Although clinical diagnoses of dementia were shown to correlate well with brain autopsy findings in the Vantaa 85+ study (Barkhof *et al.*, 2007; Oinas *et al.*, 2009; Ahtiluoto *et al.*, 2010; Polvikoski *et al.*, 2010; Tanskanen *et al.*, 2012*b*), the association between brain pathologies and dementia is known to be more complex in older compared with younger elderly (Savva *et al.*, 2009). It is important to determine if homocysteine has any impact on the actual neuropathological findings because elevated homocysteine is a modifiable factor and thus a potential target for preventive interventions.

Materials and methods

Study population

The Vantaa 85+ study has previously been described in detail (Polvikoski *et al.*, 1995; Ahtiluoto *et al.*, 2010). In brief, the study included 553 participants who were clinically examined at baseline, and represented 92% of the 601 individuals aged ⩾85 years and living in Vantaa, Finland in 1991. In 291 of the participants who died during the 10-year follow-up, consented post-mortem examination was conducted. Post-mortem brain MRI scans were also done in 119 of the 291 participants. The Vantaa 85+ study was approved by the Ethics Committee of the Health Centre of the city of Vantaa. Participants gave informed consent before enrolment in the study. Permission for the autopsy was obtained from the closest relative in all cases. The National Authority for Medicolegal Affairs (VALVIRA) approved the collection of tissue samples for research.

Subjects in the present study were selected based on the availability of baseline homocysteine measurements (265 individuals with autopsy data, of which 103 with post-mortem brain MRI scans). The autopsy population had slightly lower homocysteine values [20.5 (8.0) versus 22.3 (8.5), $P = 0.014$] and included more females (83.4 % versus 75.9%, $P = 0.024$) compared with the rest of the study population. There was no difference with regard to baseline age, *APOE4* status, living in institutions, consumption of vitamins, Mini-Mental State Examination, and history of cardiovascular diseases.

Clinical assessment

Evaluation included an interview by a trained nurse using a questionnaire (Rastas *et al.*, 2010) concerning health, health-related behaviour, and medications, and a clinical examination by a physician. Information on medical history and medications for each participant was also verified from primary health care records. Dementia was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, third edition, revised (DSM-III-R) criteria, as described previously (Ahtiluoto *et al.*, 2010; Rastas *et al.*, 2010).

Plasma homocysteine measurements

Non-fasting blood samples taken in 1991 were stored at −20°C for 8 years, and homocysteine levels were analysed at the Department of Clinical Chemistry at the Helsinki University Central Hospital by fluorescence polarization immunoassay using Abbott AxSYM analyser (Abbott Laboratories Inc).

APOE genotyping (blood samples)

DNA mini-sequencing and DNA amplification with PCR followed by restriction enzyme digestion with HhaI, were independently used in two laboratories (Department of Medicine, University of Helsinki, and Mayo Clinic, Jackonsville, FL) with identical results (Polvikoski *et al.*, 1995; Myllykangas *et al.*, 2002; Ahtiluoto *et al.*, 2010).

Neuropathology

Brains were fixed in phosphate-buffered 4% formaldehyde solution for at least 2 weeks. Examinations were conducted independently of all clinical data. The same dissection, examination and sampling protocol was used for each brain.

Alzheimer-related pathology

Specimens were obtained from the middle frontal, superior temporal, and middle temporal gyri and inferior parietal lobule, according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) protocol (Mirra *et al.*, 1991). Paraffin sections were cut at a thickness of 8 μm for staining with methenamine silver for amyloid-β (Yamaguchi *et al.*, 1990) and 10 μm for staining with modified Bielschowsky method (Yamamoto and Hirano, 1986) for neuritic plaques and neurofibrillary tangles. Methenamine silver staining has been shown to be as sensitive as amyloid-β immunostaining for detecting senile plaques, including diffuse plaques (Allsop *et al.*, 1989; Yamaguchi *et al.*, 1990).

To estimate the amount of amyloid-β deposited in the cerebral cortex, the fraction of cortical area covered by methenamine silver-stained plaques in sections cut from the four specimens was measured as described previously (Polvikoski *et al.*, 1995). Briefly, the contiguous cortical microscopic fields, extending from the pial surface to the white matter, were examined, using a ×10 objective under ×10 ocular field with a square microscopic graticule, 1.25 mm in width, along a line perpendicular to the pial surface. The graticule consisted of 10 horizontal and 10 vertical lines with 100 intersections. All intersections that overlay a methenamine silver-positive plaque were counted. In every specimen at least seven (maximum 10) cortical columns (width 1.25 mm) were examined from the pial surface to the white matter. The average area fraction of cortex covered by methenamine silver-positive plaques was calculated for all four specimens from each individual to minimize the effect of variation in extent of amyloid-β deposition in different brain regions. The final value (percentage) provided an estimate of the extent of amyloid-β deposition in the cerebral cortex.

After Bielschowsky silver staining, neurofibrillary tangles in sections cut from the four specimens were counted as described previously (Myllykangas *et al.*, 1999). In each specimen, the neurofibrillary tangles were counted in five random columns (width 0.5 mm) extending from the pial surface to the white matter, using a grid of 0.5 × 0.5 mm and a × 20 objective under ×10 ocular field. The average neurofibrillary tangle number was determined by dividing the total neurofibrillary tangle number in all four cortical sections by four. The CERAD scores and Braak stages were defined as originally described (Braak and Braak, 1991; Mirra *et al.*, 1991).

Macroscopic infarcts

Cavitary lesions or solid cerebral infarcts visible to the naked eye were identified and measured by examining the intact brain, 1-cm thick coronal slices of the cerebral hemispheres, 5-mm thick transverse slices of the brainstem, and sagittal slices of the cerebellum. All lesions were subsequently histologically ascertained to be infarcts (Polvikoski *et al.*, 2010).

Cortical microinfarcts

Microinfarcts were analysed in the haematoxylin and eosin stained tissue sections in six brain regions (frontal, parietal, temporal and occipital lobes, hippocampus and cerebellum), and were defined as focal lesions <2 mm, invisible to the naked eye, with neuronal loss, glia cell and macrophage reaction and/or cystic tissue necrosis (Tanskanen *et al.*, 2012*b*). Lesions located close to larger infarcts were considered to represent the border zone of the larger lesion, and were not counted as true microinfarcts.

Cerebral amyloid angiopathy

Cerebral amyloid angiopathy was analysed in six brain regions (see above) as previously described (Tanskanen *et al.*, 2012*a*). Cerebral amyloid angiopathy diagnosis was based on Congo red staining and confirmed using immunohistochemistry against amyloid-β peptide (clone 4G8, detecting amino acids 17–24). The percentage of blood vessels with cerebral amyloid angiopathy was analysed separately for parenchymal and leptomeningeal vessels in each tissue section of the six regions. Parenchymal and leptomeningeal percentage values were combined, and total percentage value for each subject was calculated as the sum of combined percentages for the six brain regions divided by six.

α-Synuclein pathology

Sections of substantia nigra stained with the haematoxylin and eosin method and sections of substantia nigra and hippocampus stained with antibodies against α-synuclein were used to screen for Lewy-related pathology (Oinas *et al.*, 2009). If any Lewy-related pathology was detected in screened areas, the immunohistochemistry for α-synuclein was performed on cortical samples, as recommended by the first dementia with Lewy bodies consensus guidelines (McKeith *et al.*, 1996). The type of α-synuclein pathology was determined for every subject (Oinas *et al.*, 2009). Of the 265 subjects in this study, 80 had brainstem, limbic or diffuse neocortical α-synuclein pathology, and 10 had α-synuclein pathology confined to the hippocampal region only (samples from the amygdala were not examined). These 10 were excluded from synuclein analyses because McKeith categories for dementia with Lewy bodies do not recognize this entity (McKeith *et al.*, 1996).

Post-mortem magnetic resonance imaging procedures

Formalin-fixed brains were prepared for MRI scans by removing the pons, cerebellum, and medulla oblongata. The brains were marked with plastic pieces to synchronize the planes used in MRI scanning and pathological sections. The markers were attached superior to the temporal lobe to form a plane with the mamillary bodies, which were marked with a plastic ring. Orientation of the coronal magnetic resonance slices was defined parallel to the markers. Transverse images were obtained perpendicular to the coronal plane (Barkhof *et al.*, 2007; Polvikoski *et al.*, 2010). Before scanning, specimens were rinsed in for at least 15 min and subsequently placed in 0.1 mM $MnCl_2$ solution and imaged with a 1.5 T Vision system (Siemens). The degree of medial temporal lobe atrophy was determined using a five step (score 0–4) well-established visual rating scale (Scheltens *et al.*, 1992), by a highly trained observer, blinded to clinical and neuropathological findings. The Scheltens scale

(Scheltens *et al.*, 1993) was used to visually rate the severity of white matter hyperintensities (Polvikoski *et al.*, 2010).

Statistical analyses

Population characteristics

Individuals were compared according to homocysteine levels below or above the proposed cut-off of 20 μmol/l in elderly >65 years without folic acid supplementation (Refsum *et al.*, 2004). An additional comparison was made between autopsied participants with and without post-mortem MRI scans. We used χ^2 or Fisher exact test for the proportions and student *t*-test or Mann-Whitney U test for continuous variables, when appropriate. Dichotomous variables were created to indicate presence/absence of cardiovascular risk factors or conditions (i.e. hypertension, myocardial infarct, heart failure, atrial fibrillation), *APOE4* status, living in institutions, and use of B-vitamin supplements (no mandatory folic acid fortification is done in Finland). Spearman's rank-order correlations were assessed between dementia, and neuropathological and MRI variables.

Homocysteine and neuropathology

Homocysteine values were categorized into quartiles (calculated for the 265 subjects in the autopsy population) and the anticipated low-risk category [i.e. the lowest homocysteine quartile (Q)] was set as reference: Q1 ⩽ 15.5 μmol/l, Q2 = 15.6–18.4 μmol/l, Q3 = 18.5–23.45 μmol/l, Q4 ⩾ 23.5 μmol/l. The tangle count, amyloid-β load and cerebral amyloid angiopathy were not normally distributed, and were categorized in three groups: (i) no neurofibrillary tangle, amyloid-β load or cerebral amyloid angiopathy (reference group); (ii) tangle count, amyloid-β load or cerebral amyloid angiopathy below median value (calculated for participants who had these pathologies); and (iii) tangle count, amyloid-β load or cerebral amyloid angiopathy above the median. Because the tangle count, amyloid-β load, cerebral amyloid angiopathy, Braak and CERAD categories are ordered according to severity, ordinal logistic regressions were used to assess the associations of homocysteine with these pathologies.

For brain infarcts, dichotomous variables were created: without/with macroinfarcts; without/with microinfarcts; without/with either macro- or microinfarcts. For macroinfarcts, several other categorical variables were created according to number (more/fewer than three), location (cortical/white matter/ basal ganglia, thalamus, cerebellum, and brain stem) or size (smaller/larger than 4 mm; smaller/larger than 15 mm; smaller/larger than 30 mm diameter) (Polvikoski *et al.*, 2010). The presence of α-synuclein pathology was treated as a dichotomous outcome (yes/no). Associations between homocysteine, cerebral infarcts and α-synuclein pathology were assessed with logistic regressions as appropriate.

Additional analyses were done to investigate the effects of dementia, cerebral infarcts or time (shorter/longer than median follow-up interval) on relations between homocysteine and Alzheimer-related neuropathological outcomes. Interaction terms were entered in the models in order to investigate possible interactions between homocysteine, age, gender and *APOE* in relation to the outcomes.

Homocysteine, white matter hyperintensities and medial temporal atrophy on post-mortem magnetic resonance imaging

Homocysteine values were categorized into quartiles (calculated for the 103 subjects in the post-mortem MRI population) and the anticipated low-risk category (i.e. the lowest homocysteine quartile) was set as reference: Q1 ⩽ 16.1 μmol/l, Q2 = 16.2–19.3 μmol/l, Q3 = 19.4–25.4 μmol/l, Q4 ⩾ 25.5 μmol/l. Because for some of the white matter hyperintensities or medial temporal atrophy scores the number of subjects was small, recategorization was done to create categories of comparable size for each MRI outcome variable. Periventricular white matter hyperintensities (sum for caps and bands) were categorized as mild (score 0–3, $n = 48$ subjects), and more severe (score 4–6, $n = 55$). Deep white matter hyperintensities (sum for frontal, parietal, temporal and occipital regions) were categorized as mild (score 0–3, $n = 35$ subjects), moderate (score 4–8, $n = 38$) and severe (score 9–24, $n = 30$). Medial temporal atrophy (highest of the left or right medial temporal atrophy score) was categorized as mild (score 0–1, $n = 34$ subjects), moderate (score 2, $n = 35$) and severe (score 3–4, $n = 34$). Binary and ordinal logistic regressions were used to investigate relations between white matter hyperintensities, medial temporal atrophy and homocysteine.

All analyses in the autopsy and post-mortem MRI populations were adjusted for age at death, gender, *APOE4* status and follow-up duration (Model 1), and additionally for cardiovascular conditions, living in institutions, and use of B-vitamin supplements (Model 2). Results are shown as odds ratios (OR) with 95% confidence intervals (CI). We analysed the data using Stata software version 12, and the level of significance was <0.05.

Results

Population characteristics

Socio-demographic and clinical characteristics according to homocysteine levels are shown in Table 1 for the autopsy population (265 subjects) and Table 2 for the post-mortem MRI population (103 of the 265 subjects). The follow-up duration was somewhat shorter in subjects with homocysteine >20 μmol/l, but there were no other differences according to homocysteine levels. Because brain MRI scans were done in the first participants who died and were autopsied, the post-mortem MRI population was younger at the time of death [mean (standard deviation, SD) age 90.8 (3.4) versus 93.8 (3.2), $P < 0.001$], and had slightly higher homocysteine levels (median 19.3 versus 18.0, $P = 0.061$). It also included more subjects with dementia at baseline (54.4% versus 35.2%, $P = 0.002$), cardiovascular conditions (87.4% versus 72.2%, $P = 0.004$), or living in institutions (58.8% versus 25.3%, $P < 0.001$) compared to the rest of the autopsy population. Neuropathological characteristics were not significantly different between autopsied subjects with and without MRI scans.

Dementia was correlated with neurofibrillary tangle count (Spearman rho 0.316; $P < 0.001$), Braak stage (0.271; $P < 0.001$), amyloid-β load (0.348; $P < 0.001$), CERAD score (0.272; $P < 0.001$), cerebral macroinfarcts (0.180; $P = 0.004$), cerebral amyloid angiopathy (0.234; $P < 0.001$), α-synuclein pathology (0.177; $P = 0.004$), medial temporal atrophy score (0.317; $P = 0.001$), but not with periventricular (−0.084; $P = 0.400$) or deep (0.099; $P = 0.322$) white matter hyperintensities. Relations between clinical dementia syndromes, neuropathological and MRI findings in the Vantaa 85+ population have been previously described in detail (Barkhof *et al.*, 2007; Oinas *et al.*, 2009; Ahtiluoto *et al.*, 2010; Polvikoski *et al.*, 2010; Tanskanen *et al.*, 2012*b*).

Table 1 Characteristics of the autopsy population according to homocysteine values

Characteristics	Homocysteine ⩽20 μmol/l *n* = 154	Homocysteine >20 μmol/l *n* = 111	*P*-value	No.
Age at baseline, years	88.4 (2.8)	88.9 (3.2)	0.15	265
Age at death, years	92.6 (3.3)	92.6 (3.9)	0.93	265
Duration of follow-up, years*	3.7 (2.3–6.0)	3.3 (1.6–5.5)	*0.06*	265
Females, *n* (%)	130 (84.4)	91 (82.0)	0.60	265
Baseline cardiovascular conditions, *n* (%)	117 (76.0)	90 (81.1)	0.32	265
APOE4 allele, *n* (%)	49 (31.8)	33 (29.7)	0.72	265
Living in institution at baseline, *n* (%)	53 (34.6)	48 (43.2)	0.16	264
Mini-Mental State Examination score at baseline*	20.5 (9.3–25.0)	18 (11–24)	0.39	260
Dementia at baseline, *n* (%)	63 (40.9)	50 (45.0)	0.50	265
Dementia at death, *n* (%)	99 (64.3)	70 (63.1)	0.84	265
Use of vitamins, *n* (%)	8 (5.2)	5 (4.5)	0.80	263
Amyloid-β load, *n* (%)			0.86	265
None	24 (15.6)	20 (18.0)		
<Median of those with amyloid-β	66 (42.9)	45 (40.5)		
>Median of those with amyloid-β	64 (41.6)	46 (41.4)		
CERAD score, *n* (%)			0.68	265
None or sparse	50 (32.5)	41 (36.9)		
Moderate	85 (55.2)	59 (53.2)		
Frequent	19 (12.3)	11 (9.9)		
CAA, *n* (%)			0.52	261
None	47 (30.9)	33 (30.3)		
<Median of those with CAA	49 (32.2)	42 (38.5)		
>Median of those with CAA	56 (36.8)	34 (31.2)		
Tangle count, *n* (%)			0.56	265
None	57 (37.0)	34 (30.6)		
<Median of those with tangles	50 (32.5)	39 (35.1)		
>Median of those with tangles	47 (30.5)	38 (34.2)		
Braak stage, *n* (%)			0.33	265
0–2	46 (29.9)	27 (24.3)		
3–4	69 (44.8)	60 (54.1)		
5–6	39 (25.3)	24 (21.6)		
α-Synuclein pathology, *n* (%)	47 (32.4)	33 (30.0)	0.68	255
Cerebral macroinfarcts, *n* (%)	85 (55.2)	58 (52.3)	0.64	265
Cerebral microinfarcts, *n* (%)	30 (19.7)	14 (12.8)	0.14	261
All cerebral infarcts, *n* (%)	96 (62.3)	65 (58.6)	0.53	265

Values are mean (standard deviation) or *n* (%) unless otherwise stated.
*Median (interquartile range), Mann-Whitney U test was used.
The 20 μmol/l cut-off for homocysteine was chosen according to proposed upper reference limit in elderly >65 years without folate supplementation (Refsum *et al*., 2004).
CAA = cerebral amyloid angiopathy.

Homocysteine and neuropathology

Associations between baseline homocysteine and neuropathological measurements are shown in Table 3. For individuals in the highest homocysteine quartile, OR (95% CI) was 2.60 (1.28–5.28) for having a higher neurofibrillary tangle count, even after all adjustments (Model 2). After adjusting for age at death, gender, follow-up duration and *APOE4* status (Model 1), OR (95% CI) for a more severe Braak stage was 1.96 (1.05–3.68) for the highest homocysteine quartile. This association became borderline significant (OR 1.80, $P = 0.07$) with additional adjustment for history of cardiovascular conditions, use of vitamins, and living in institutions (Table 3, Model 2). The pattern of association for the highest homocysteine quartile remained even after excluding subjects with dementia at baseline: OR (95% CI) was 3.44 (1.22–9.68) for higher neurofibrillary tangle count, and 2.32 (0.91–5.91) for more severe Braak stage.

In stratified analyses investigating the impact of dementia status at death, cerebral infarcts and follow-up time (Table 4), the highest homocysteine quartile was related to higher neurofibrillary tangle count particularly among participants with dementia: OR (95% CI) was 3.46 (1.43–8.38); participants with cerebral micro- or macroinfarcts: 3.98 (1.56–10.15); and participants with longer time between the baseline homocysteine measurement and death: 3.25 (1.19–8.90).

Higher homocysteine was not significantly related to increased amyloid-β burden or CERAD score (Table 3), and this was not influenced by dementia status at death or cerebral infarcts (Table 5). However, participants with longer time between the baseline homocysteine measurement and death tended to have

Table 2 Characteristics of the post-mortem MRI population according to homocysteine values

Characteristics	Homocysteine ⩽20 μmol/l *n* = 55	Homocysteine >20 μmol/l *n* = 48	*P*-value	No.
Age at baseline, years	89.1 (2.9)	88.9 (3.7)	0.76	103
Age at death, years	91.1 (3.0)	90.5 (3.7)	0.40	103
Duration of follow-up, years*	2.0 (1.2–2.9)	1.3 (0.7–2.8)	**0.03**	103
Females, *n* (%)	49 (89.1)	42 (87.5)	0.80	103
Baseline cardiovascular conditions, *n* (%)	47 (85.5)	43 (89.6)	0.53	103
APOE4 allele, *n* (%)	19 (34.5)	16 (33.3)	0.90	103
Living in institution at baseline, *n* (%)	28 (51.9)	32 (66.7)	0.13	102
Mini-Mental State Examination score at baseline*	14 (0–24)	15 (8–22)	0.89	99
Dementia at baseline, *n* (%)	27 (49.1)	29 (60.4)	0.25	103
Dementia at death, *n* (%)	32 (58.2)	30 (62.5)	0.66	103
Use of vitamins, *n* (%)	4 (7.3)	4 (8.3)	0.99	103
MTA score*	2 (1–3)	2 (2–3)	0.10	103
Total WMH score*	10 (6–16)	10 (7–14.8)	0.93	103
Periventricular WMH score*	4 (3–5)	4 (3–5)	0.93	103
Deep WMH score*	5 (1–12)	5 (2.3–9)	0.88	103

Values are *n* (%) unless otherwise stated.
*Median (interquartile range), Mann-Whitney U test was used.
The 20 μmol/l cut-off for homocysteine was chosen according to proposed upper reference limit in elderly >65 years without folate supplementation (Refsum *et al.*, 2004).
MTA = medial temporal atrophy; WMH = white matter hyperintensitiy.

a higher amyloid-β burden ($P = 0.085$): for the highest homocysteine quartile, OR (95% CI) was 2.52 (0.88–7.19) (Table 5). No significant relations between homocysteine and cerebral amyloid angiopathy, cerebral infarcts, or α-synuclein pathology were found (Table 3).

Homocysteine, white matter hyperintensities and medial temporal atrophy on post-mortem magnetic resonance imaging

After adjusting for age at death, gender, follow-up time, *APOE4* status, history of cardiovascular conditions, use of vitamins, and living in institutions, higher homocysteine levels were associated with higher medial temporal atrophy and periventricular white matter hyperintensity scores (Table 3). For the highest homocysteine quartile, OR (95% CI) were 3.78 (1.12–12.79) for more severe medial temporal atrophy, and 4.69 (1.14–19.33) for more severe periventricular white matter hyperintensities. No significant association was found between homocysteine and deep white matter hyperintensities scores.

Discussion

Our results show that elevated baseline homocysteine was associated with increased neurofibrillary tangle burden at the time of death (up to 10 years later) in the elderly aged over 85 years. The association was observed particularly in people with dementia, in the presence of cerebral infarcts, and with longer time between the baseline homocysteine assessment and death. There was no significant association between homocysteine and amyloid-β accumulation, although elevated homocysteine tended to relate to amyloid-β accumulation in people with longer follow-up time. On post-mortem MRI, higher homocysteine was associated with more severe medial temporal atrophy and periventricular white matter hyperintensities. All observed associations were independent of several potential confounders, including common vascular risk factors.

Vitamin B12 and folate are essential factors for the remethylation of homocysteine to methionine and the subsequent S-adenosyl methionine formation, the primary methyl donor for many biochemical reactions involved in normal brain functions such as production of cell membrane phospholipids, myelin, monoaminergic neurotransmitters and nucleic acid. Subsequently, S-adenosyl methionine is converted into S-adenosyl homocysteine, a potent competitive inhibitor of several methyl transferases, after donating its methyl group to other cellular components. S-adenosyl homocysteine is further hydrolyzed into homocysteine in a reversible reaction that favours the S-adenosyl homocysteine formation when homocysteine concentrations increase (Refsum *et al.*, 2004; Obeid *et al.*, 2007). Experimental studies have suggested that low concentrations of S-adenosyl methionine or low S-adenosyl methionine:S-adenosyl homocysteine ratio are associated with insufficient methylation and decreased activity of protein phosphatase 2 A (PP2A), an enzyme involved in the dephosphorylation of phospho-tau. The insufficient dephosphorylation may lead to an increase in the hyperphosphorylated form of tau, which has decreased affinity for microtubules. As a result, the unbound hyperphosphorylated tau pool becomes available to self-assembly and tangle formation (Obeid and Herrmann, 2006; Obeid *et al.*, 2007; Sontag *et al.*, 2007; Smith, 2008; Zhang *et al.*, 2008; Popp *et al.*, 2009; Coppieters and Dragunow, 2011; Chai *et al.*, 2013).

In our study, homocysteine was associated with both neurofibrillary tangle burden and more severe medial temporal atrophy on post-mortem MRI. Associations between homocysteine and medial

Table 3 The associations of homocysteine (quartiles) with neuropathology and post-mortem MRI measurements (OR, 95% CI)

	Q1	Q2	Q3	Q4
Tangle count[a]				
Model 1 ($n = 265$)	Ref	*1.78 (0.91–3.51)*	*1.84 (0.94–3.63)*	**2.87 (1.43–5.79)**
Model 2 ($n = 262$)	Ref	*1.79 (0.90–3.56)*	*1.85 (0.93–3.67)*	**2.60 (1.28–5.28)**
Braak stage[a]				
Model 1 ($n = 265$)	Ref	1.12 (0.60–2.06)	1.67 (0.89–3.12)	**1.96 (1.05–3.68)**
Model 2 ($n = 262$)	Ref	1.10 (0.59–2.07)	1.66 (0.89–3.12)	*1.80 (0.95–3.40)*
Amyloid-β load[a]				
Model 1 ($n = 265$)	Ref	1.53 (0.76–3.09)	1.17 (0.58–2.35)	1.73 (0.83–3.57)
Model 2 ($n = 262$)	Ref	1.60 (0.78–3.29)	1.16 (0.57–2.34)	1.55 (0.74–3.24)
CERAD score[a]				
Model 1 ($n = 265$)	Ref	0.97 (0.49–1.93)	0.86 (0.43–1.74)	1.21 (0.60–2.45)
Model 2 ($n = 262$)	Ref	0.99 (0.50–2.00)	0.87 (0.43–1.75)	1.12 (0.55–2.29)
CAA[a]				
Model 1 ($n = 261$)	Ref	1.08 (0.52–1.97)	0.88 (0.44–1.73)	1.30 (0.66–2.57)
Model 2 ($n = 258$)	Ref	1.07 (0.54–2.11)	0.90 (0.46–1.78)	1.31 (0.66–2.61)
Cerebral macroinfarcts[a]				
Model 1 ($n = 265$)	Ref	1.18 (0.59–2.36)	0.89 (0.44–1.81)	0.78 (0.39–1.57)
Model 2 ($n = 262$)	Ref	0.96 (0.47–1.98)	0.81 (0.39–1.67)	0.65 (0.31–1.35)
Cerebral microinfarcts[a]				
Model 1 ($n = 261$)	Ref	1.88 (0.78–4.56)	0.77 (0.27–2.19)	1.02 (0.39–2.68)
Model 2 ($n = 258$)	Ref	1.59 (0.64–3.95)	0.70 (0.24–2.02)	1.03 (0.39–2.73)
All cerebral infarcts[a]				
Model 1 ($n = 265$)	Ref	1.34 (0.65–2.74)	0.96 (0.47–1.97)	0.87 (0.43–1.77)
Model 2 ($n = 262$)	Ref	1.10 (0.52–2.32)	0.87 (0.42–1.81)	0.74 (0.35–1.54)
α-Synuclein pathology[a]				
Model 1 ($n = 255$)	Ref	1.29 (0.60–2.79)	1.09 (0.49–2.44)	1.07 (0.49–2.33)
Model 2 ($n = 252$)	Ref	0.98 (0.44–2.19)	1.01 (0.44–2.30)	0.90 (0.40–2.04)
MTA score[b]				
Model 1 ($n = 103$)	Ref	0.85 (0.29–2.46)	1.28 (0.45–3.67)	**3.94 (1.19–13.07)**
Model 2 ($n = 102$)	Ref	0.96 (0.32–2.84)	1.26 (0.43–3.69)	**3.78 (1.12–12.79)**
Periventricular WMH[b]				
Model 1 ($n = 103$)	Ref	1.82 (0.55–5.97)	1.02 (0.30–3.47)	**4.91 (1.21–19.95)**
Model 2 ($n = 102$)	Ref	1.95 (0.59–6.50)	1.08 (0.31–3.75)	**4.69 (1.14–19.33)**
Deep WMH[b]				
Model 1 ($n = 103$)	Ref	0.71 (0.26–1.99)	0.56 (0.20–1.52)	1.31 (0.43–3.98)
Model 2 ($n = 102$)	Ref	0.89 (0.29–2.31)	0.60 (0.21–1.68)	1.19 (0.38–3.69)

Significant results ($P < 0.05$) are in bold, trends ($P < 0.10$) are in italics.
Model 1: adjusted for age at death, duration of follow-up, gender, and *APOE4* allele.
Model 2: additionally adjusted for cardiovascular conditions, living in institutions, and use of vitamins.
[a]Homocysteine quartiles in the autopsy population were: Q1 ⩽ 15.5 μmol/l, Q2 = 15.6–18.4 μmol/l, Q3 = 18.5–23.45 μmol/l, Q4 ⩾ 23.5 μmol/l.
[b]Homocysteine quartiles in the post-mortem MRI population were: Q1 ⩽ 16.1 μmol/l, Q2 = 16.2–19.3 μmol/l, Q3 = 19.4–25.4 μmol/l, Q4 ⩾ 25.5 μmol/l.
CAA = cerebral amyloid angiopathy; MTA = medial temporal atrophy; WMH = white matter hyperintensity.

Table 4 The associations of homocysteine (quartiles) with neurofibrillary tangle count according to dementia status, presence of infarcts, and time between baseline homocysteine assessment and death (OR, 95% CI)

	Q1	Q2	Q3	Q4
No dementia ($n = 94$)	Ref	1.08 (0.30–3.86)	2.23 (0.68–7.26)	1.82 (0.48–6.92)
Dementia ($n = 168$)	Ref	**2.40 (1.01–5.67)**	2.01 (0.83–4.85)	**3.46 (1.43–8.38)**
No cerebral infarcts ($n = 103$)	Ref	2.20 (0.62–7.83)	2.10 (0.65–6.83)	1.73 (0.52–5.72)
Cerebral infarcts ($n = 159$)	Ref	1.95 (0.84–4.55)	*2.16 (0.88–5.28)*	**3.98 (1.56–10.15)**
Follow-up time ⩽ 3.5 y ($n = 132$)	Ref	1.41 (0.51–3.92)	1.37 (0.50–3.76)	1.66 (0.58–4.76)
Follow-up time > 3.5 y ($n = 130$)	Ref	*2.45 (0.92–6.53)*	**2.88 (1.07–7.74)**	**3.25 (1.19–8.90)**

Significant results ($P < 0.05$) are in bold, trends ($P < 0.10$) are in italics. Analyses are adjusted for age at death, duration of follow-up, gender, *APOE4* allele, cardiovascular conditions, living in institutions and use of vitamins. Cerebral infarcts refer to the presence of either macro- or microinfarcts. The 3.5 years cut-off for follow-up time in stratified analyses represents the median value for the duration of follow-up in the autopsy population. Homocysteine quartiles in the autopsy population were: Q1 ⩽ 15.5 μmol/l, Q2 = 15.6–18.4 μmol/l, Q3 = 18.5–23.45 μmol/l, Q4 ⩾ 23.5 μmol/l.

Table 5 The associations of homocysteine (quartiles) with amyloid-β load according to dementia status, presence of infarcts and time between baseline homocysteine assessment and death (OR, 95% CI)

	Q1	Q2	Q3	Q4
No dementia ($n = 94$)	Ref	1.06 (0.31–3.64)	0.72 (0.23–2.25)	0.75 (0.21–2.68)
With dementia ($n = 168$)	Ref	*2.23 (0.86–5.76)*	1.78 (0.68–4.62)	2.06 (0.78–5.42)
No cerebral infarcts ($n = 103$)	Ref	2.55 (0.75–8.68)	1.20 (0.39–3.73)	2.20 (0.64–6.83)
Cerebral infarcts ($n = 159$)	Ref	1.30 (0.51–3.28)	1.07 (0.41–2.79)	1.10 (0.41–2.92)
Follow-up $\leqslant 3.5$ y ($n = 132$)	Ref	1.35 (0.34–4.24)	1.15 (0.38–3.47)	0.76 (0.24–2.37)
Follow-up > 3.5 y ($n = 130$)	Ref	2.26 (0.85–5.98)	1.48 (0.56–3.90)	*2.52 (0.88–7.19)*

Significant results ($P < 0.05$) are in bold, trends ($P < 0.10$) are in italics. Analyses are adjusted for age at death, duration of follow-up, gender, *APOE4* allele, cardiovascular conditions, living in institutions, and use of vitamins. Cerebral infarcts refer to the presence of either macro- or microinfarcts. The 3.5 year cut-off for follow-up time in stratified analyses represents the median value for the duration of follow-up in the autopsy population. Homocysteine quartiles in the autopsy population were: Q1 $\leqslant$ 15.5 μmol/l, Q2 = 15.6–18.4 μmol/l, Q3 = 18.5–23.45 μmol/l, Q4 $\geqslant$ 23.5 μmol/l.

temporal atrophy or hippocampal atrophy have been reported in some (Clarke *et al.*, 1998; den Heijer *et al.*, 2003) but not all (Seshadri *et al.*, 2008) previous studies using *in vivo* MRI. Medial temporal atrophy is indicative of primary neurodegenerative pathology in the medial temporal lobe (Barkhof *et al.*, 2007), and is pathologically characterized by a high neurofibrillary tangle burden according to a previous study in the Vantaa 85+ population (Polvikoski *et al.*, 2010). Homocysteine may thus be directly involved in the pathology of brain areas affected by Alzheimer's disease. The association of homocysteine with neurofibrillary tangle count in the Vantaa 85+ population was stronger compared to Braak staging. Such a discrepancy between individual neurofibrillary tangle counts and the Braak stages is not entirely surprising, because occasional neocortical tangle counts can be seen in any Braak stages less than stage V (Polvikoski *et al.*, 2010).

The association between elevated homocysteine, neurofibrillary tangle burden and even amyloid-β load was observed particularly with longer time between the baseline homocysteine assessment and death, suggesting that the impact of homocysteine on neurofibrillary tangle formation and possibly also amyloid-β accumulation is a long-term process. Elevated homocysteine may influence amyloid-β generation and its neurotoxicity through several mechanisms. Raised homocysteine may stimulate the endoplasmic reticulum stress response in endothelial cells, neurons and glia, activating presenilin-induced amyloid-β generation (Outinen *et al.*, 1999); deficient methylation upregulates presenilin gene function and amyloid-β generation (Scarpa *et al.*, 2003); homocysteine may convert to homocysteic acid, a highly potent neurotoxic metabolite and an *N*-methyl-D-aspartate receptor agonist, which may further promote amyloid-β generation in the brain (Hasegawa *et al.*, 2005).

Although an association between homocysteine and vascular disease has long been recognized, no associations between homocysteine and cerebral macro- or microinfarcts were found in our study. On the other hand, as elevated homocysteine is related to increased mortality (Refsum *et al.*, 2004), the lack of association may be related to selective survival; the individuals with higher homocysteine or more severe cerebrovascular lesions may have died before the beginning of the Vantaa 85+ study. However, homocysteine was related to neurofibrillary tangle burden particularly in individuals with cerebral infarcts, suggesting that blood perfusion of the brain may modulate the impact of homocysteine on tau pathology.

In the Vantaa 85+ population, homocysteine was associated with periventricular but not with deep white matter hyperintensities on post-mortem MRI. A stronger relation between homocysteine and periventricular rather than deep white matter hyperintensities on *in vivo* MRI has been previously reported (Vermeer *et al.*, 2002), although other *in vivo* MRI studies did not support this pattern (Hogervorst *et al.*, 2002; Sachdev *et al.*, 2004). Although deep white matter hyperintensities are considered markers for cerebral small vessel disease and related problems in white matter perfusion and ischaemia, different pathogenetic mechanisms (i.e. blood–brain barrier dysfunction, disturbances in CSF production) may lead to periventricular white matter hyperintensities (Schmidt *et al.*, 2011). It has been suggested that periventricular white matter hyperintensities may be an epiphenomenon of brain atrophy and may not be independently related to Alzheimer's disease (Fazekas *et al.*, 1996; Barber *et al.*, 2000). However, based on the rather small number of Vantaa 85+ participants with homocysteine measurements and post-mortem MRI scans, an association of homocysteine with deep white matter hyperintensities cannot be completely ruled out.

Consistent with the proposed aetiological differences between the deep and periventricular white matter hyperintensities, presence of multiple cerebral infarcts was associated with the deep white matter hyperintensities, but not with periventricular white matter hyperintensities in this study population (Polvikoski *et al.*, 2010). In contrast, periventricular white matter hyperintensities were associated with neurofibrillary tangle burden (Polvikoski *et al.*, 2010), which is in agreement with our results relating homocysteine with both periventricular white matter hyperintensities and neurofibrillary tangle burden.

Based on these results, it seems less likely that the association between the homocysteine and neurofibrillary pathology might come directly via homocysteine-associated cerebrovascular disease, because no significant association between the homocysteine and cerebral infarcts or deep white matter hyperintensities was found. These findings support a direct association between homocysteine and neurofibrillary formation, and the association between homocysteine and periventricular white matter changes might be an indirect consequence of the Alzheimer-related brain atrophy, subsequent ventricular dilatation and ruptures of the

ependymal lining with increased leakage of CSF into the surrounding periventricular white matter. Alternatively, the periventricular white matter changes might result directly from hippocampal and neocortical neurodegeneration and subsequent loss of axons in fibre tracts, which run near the lateral ventricles.

The major strength of this study is the prospective population-based design with a high autopsy rate over 10 years, and the inclusion of participants aged $\geqslant$85 years. Although few studies have investigated the impact of homocysteine on Alzheimer-type neuropathology, none had a longitudinal design. However, selective survival may have contributed to an underestimation of the relations between homocysteine and neuropathological or MRI findings. The availability of homocysteine measurements at only one time point may have also underestimated these relations due to regression dilution (Hooshmand *et al.*, 2010). Furthermore, we could not assess the role of main homocysteine determinants (such as B12 and folate) in relation to neuropathology or MRI measurements. Quantitative, systematic methods were used in our study to identify neuropathological changes, but due to the use of traditional silver staining methods, there may be differences compared with studies using immunohistochemistry for Alzheimer-related pathology (Bennett *et al.*, 2012).

In conclusion, our results suggest that elevated homocysteine in adults aged $\geqslant$85 years contribute to increased Alzheimer pathology, particularly neurofibrillary tangle burden. This association was observed particularly when study participants were followed-up for a longer time. Interestingly, this effect seems to be more pronounced in the presence of cerebrovascular pathology, despite the lack of direct association between cerebrovascular pathology and homocysteine. Future studies will need to investigate in more detail possible underlying mechanisms, i.e. oxidative stress and endothelial activation. High homocysteine and low B12 and folate levels are surprisingly common conditions in the elderly (Smith, 2008; Allen, 2009). However, few randomized controlled trials have so far investigated the usefulness of B12 and folate supplementation in preventing cognitive impairment or dementia, with mixed results (Smith, 2008; Doets *et al.*, 2013; Ford and Almeida, 2012). Limitations of statistical power, choice of target population, study duration and autopsy confirmation makes such studies difficult to interpret. The neurofibrillary tangle and amyloid-β accumulation is a long-term process and adequately timed and powered randomized controlled trials are needed to determine efficient treatment guidelines (e.g. dose, treatment start and duration, target population).

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