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The impact of homocysteine and B vitamins on Alzheimer's disease, cognitive performance and structural brain changes

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To my family

ABSTRACT English

The overall aim of this thesis was to investigate the relation of homocysteine (tHcy), vitamin B12, and folate with Alzheimer's disease (AD), cognitive performance, and structural brain changes in population-based studies of Finnish and Swedish elderly individuals.

Study I. Serum levels of tHcy, holotranscobalamin (holoTC, the active fraction of vitamin B12), and folate were assessed in 274 individuals aged 65-79 years and without dementia from the Cardiovascular Risk Factors, Aging, and Dementia (CAIDE) study. Participants were followed-up for 7 years to detect incident AD. The odds ratios (OR) (95% confidence interval [CI]) for AD were 1.16 (1.04-1.31) for each increase of 1 μmol/L tHcy and 0.980 (0.965-9.995) for each increase of 1pmol/L holoTC. While adjusting for holoTC attenuated the tHcy-AD relation, the holoTC-AD link was less influence by controlling for tHcy. The protective effects of holoTC were more pronounced with increasing age.

Study II. In the CAIDE study, performance in several cognitive domains was assessed on two occasions. Higher tHcy values among 65-79 years old persons were associated with worse performance on global cognition, episodic memory, executive functions and verbal expression 7 years later. Elevated holoTC was related to better performance on global cognition, executive functions, and psychomotor speed. After excluding participants with incident dementia, tHcy and holoTC remained associated with several cognitive domains, and folate became associated with global cognition and verbal expression. The protective effects of holoTC were present over the whole normal range of holoTC.

Study III. The associations of baseline plasma tHcy with neuropathological and post-mortem magnetic resonance imaging (MRI) findings up to 10 years later were investigated in the Vantaa 85+ study including individuals aged >85 years. tHcy levels in the highest quartile were related to about 2.5-fold increased OR for higher neurofibrillary tangles burden. This association was present particularly in subjects with dementia, cerebral infarcts, and with longer follow-up duration. tHcy tended to relate to amyloid -β accumulation in people with longer follow-up time. Higher tHcy levels were also associated with more severe medial temporal lobe atrophy and periventricular white matter hyperintensities on post-mortem MRI.

Study IV. Plasma B12 and red blood cell folate were examined in relation to brain volumes in a Swedish population-based study (The Swedish National Study of Aging and Care in Kungsholmen (SNAC-K)), including dementia-free individuals aged >60 years with MRI scans at 2-3 occasions over 6 years. Higher baseline plasma B12 concentrations were associated with decreased rate of total brain tissue and grey matter volume loss, even in elderly who did not develop dementia. The protective effects of vitamin B12 were present over the whole distribution of vitamin B12 levels.

Conclusions: Results of this project indicate that lower B12, elevated tHcy, and lower folate levels are involved in late-life cognitive impairment. Assessments of B12 and folate status (including functional indicators such as tHcy or holoTC) are recommendable in elderly at risk of dementia. Adequately timed and powered randomized controlled trials are needed to determine the impact of B-vitamin supplementation on preventing cognitive decline and dementia-related pathology.

Keywords: Alzheimer's disease, Alzheimer pathology, cerebrovascular pathology, cognition, elderly, dementia, folate, holotranscobalamin, homocysteine, population-based study, vitamin B12

SAMMANFATTNING Svenska

Det övergripande syftet med denna avhandling var att undersöka hur homocystein (tHcy), vitamin B12 och folat är relaterade till Alzheimer's sjukdom (AD), kognition och strukturella hjärnförändringar genom populationsbaserade studier på finska och svenska äldre personer.

Studie I. Serumnivåer av tHcy, holotranscobalamin (holoTC, den aktiva delen av vitamin B12) och folat mättes hos 274 individer mellan 65-79 år gamla och utan demens som ingick i Cardiovascular Risk Factors, Aging and Dementia (CAIDE) studien. Deltagarna följdes upp under 7 år för att upptäcka de som drabbades av AD. Oddskvoten (OR) (95% konfidensintervall (CI)) att drabbas av AD var 1.16 (1.04-1.31) per 1pmol/L ökning av holoTC. Sambandet mellan tHcy och AD dämpades när man tog hänsyn till holoTC. Kopplingen mellan holoTC och AD påverkades däremot i lägre grad av tHcy. HoloTC hade en mer uttalad skyddande effekt med stigande ålder.

Studie II. I CAIDE-studien bedömdes den kognitiva prestationen vid två tillfällen. Högre tHcy värden bland 65-79 år gamla personer var förknippade med sämre prestation inom global kognition, episodiskt minne, exekutiva funktioner och verbala förmåga 7 år senare. Förhöjda holoTC-värden var kopplade till bättre prestation inom global kognition, exekutiva funktioner och psykomotorisk hastighet. Efter exkludering av deltagare som drabbats av demens var fortfarande nivåerna av tHcy och holoTC kopplade till prestationen inom flera kognitiva domäner och dessutom visade folat ett samband med global kognition och verbal förmåga. HoloTC:s skyddande effekter visade sig genom hela det normala intervallet för holoTC.

Studie III. I Vantaa 85+ studien undersöktes hur plasmanivåer av tHcy hos individer över 85 år var kopplade till patologiska hjärnförändringar vid obduktion upp till 10 år senare. tHcy-nivåer i den översta kvartilen var relaterade till en förhöjd oddskvot på ca 2.5 för fler neurofibrillära nystan. Denna koppling var speciellt uttalad för personer med demens, hjärninfarkter och med en längre uppföljningstid. tHcy tenderade att vara kopplat till en högre ansamling av beta-amyloid hos personer med en längre uppföljningstid. Högre tHcy-nivåer var kopplade till mer uttalad atrofi i den mediala temporalloben och till periventrikulära vitsubstansskador på magnetisk resonanstomografi (MRT).

Studie IV. B12 i plasma och folat i röda blodceller undersöktes med avseende på deras relation till hjärnvolym i en svensk populationsbaserad studie (The Swedish National Study of Aging and Care in Kungsholmen (SNAC-K)). SNAC-K inkluderade personer över 60 år, utan demens och med MRT-skanningar vid 2-3 tillfällen under 6 år. Högre koncentrationer av B12 i serum vid baseline var kopplade till en lägre takt i förlusten av hjärnvävnad, både avseende total hjärnvävnad och grå substans, även hos personer utan demens. B12:s skyddande effekter visade sig genom hela fördelningen av B12-värden.

Slutsatser: Resultaten från detta projekt tyder på att lägre nivåer av B12, förhöjda tHcy-värden och lägre folatnivåer hänger ihop med nedsatt kognition sent i livet. Mätning av B12 och folat-status (inklusive funktionella indikatorer som tHcy eller holoTC) kan rekommenderas hos äldre som löper risk att drabbas av demens. Randomiserade kliniska prövningar av god kvalité behövs för att fastställa hur kosttillägg av B-vitamin kan förebygga kognitiv försämring och demensrelaterade patologier.

Nyckelord: Alzheimers sjukdom, Alzheimerpatologi, blodkärlsrelaterad hjärnpatologi, kognition, äldre, demens, folat, holotranscobalamin, homocystein, populationsbaserad studie, vitamin B12.

ZUSAMMENFASSUNG Deutsch

Das übergreifende Ziel dieser Arbeit ist es, den Zusammenhang von Homocystein (tHcy), Vitamin B12 und Folat mit der Alzheimer Erkrankung (AD), kognitiver Fähigkeit und strukturellen Veränderungen des Gehirns zu erforschen und zu verstehen, basierend auf Bevölkerungsstudien von Finnen und Schweden im fortgeschrittenen Alter.

Studie I. Serum Spiegel von tHcy, von Holo-Transcobalamin (holoTC), die aktive Form von Vitamin B12, sowie von Folat wurden zu Studienbeginn bei 274 demenzfreien Testpersonen im Alter von 65-79 Jahren durch die Cardiovascular Risk Factors, Aging, and Dementia (CAIDE)-Studie erfasst. Die Testpersonen wurden nach 7 Jahren erneut untersucht, um Neuerkrankungen von AD zu identifizieren. Die Odds Ratio (OR) (95% Konfidenzintervall [CI]) für AD war 1.16 (1.04-1.31) für jede Steigerung von 1 μmol/L tHcy und 0,980 (0.965-9.995) für jede Steigerung von 1 μmol/L holoTC zu Studienbeginn. Korrektur für HoloTC verminderte den tHcy-AD Zusammenhang, wohingegen der holoTC-AD Zusammenhang durch Anpassung von holoTC weniger beeinflusst wurde. Die protektiven Effekte von holoTC wurden deutlicher sichtbar mit steigendem Alter.

Studie II. In der CAIDE Studie wurden Fähigkeiten in mehreren kognitiven Bereichen erfasst, sowohl zu Beginn der Studie als beim Follow-Up 7 Jahre später. Höhere tHcy Werte zu Beginn der Studie standen in Zusammenhang mit verschlechterter Fähigkeit in globaler Kognition, episodischem Gedächtnis, Exekutivfunktionen und verbale Ausdruck. Erhöhtes holoTC war mit einer verbesserten Fähigkeit in globaler Kognition, Exekutivfunktionen und psychomotorischer Geschwindigkeit assoziiert. tHcy und holoTC standen weiterhin in Zusammenhang mit mehreren kognitiven Bereichen nach Ausschluss von Individuen mit Neuerkrankung an Demenz. Zudem führte dies zur Assoziation von Folat mit globaler Kognition und verbalem Ausdruck. Dieser protektive Effekt von holoTC war über das gesamte normale Intervall von holoTC zu sehen.

Studie III. Die Assoziation von Plasma tHcy zu Studienbeginn mit Ergebnissen von Post Mortem neuropathologischen Untersuchungen und magnetischer Resonanzthomographie (MRI) bis zu 10 Jahre später wurde in der Vantaa 85+ Studie mit Testpersonen im Alter von >85 Jahren zu Studienbeginn untersucht. tHcy Level in der höchsten Quartile war assoziiert mit einer bis zu 2.5-fachen Steigerung der OR für erhöhte Belastung mit neurofibrillary tangles. Diese Assoziation war besonders in Individuen mit Demenz zu sehen, bei Vorhandensein von zerebralen Infarkten und bei längerer Follow-Up Dauer. Homocystein neigte dazu, mit amyloid- β- Akkumulation zu korrelieren bei Leuten mit längerer Follow-Up Dauer. Höhere Homocystein Level waren auch mit ernsthafterer medial temporal lobe -Atrophie und periventrikularer weiße Substanz Hyperintensitäten assoziiert.

Studie IV. Plasma B12 und Folat aus Erythrozyten wurden in Zusammenhang auf zerebrales Volumen mit MRI Scans zu 2-3 Gelegenheiten innerhalb von 6 Jahren in einer Schwedischen populationsbasierten Studie (The Swedish National Study of Aging and Care in Kungsholmen (SNAC-K)) untersucht, die demenzfreie Testpersonen im Alter von >60 Jahren umfasst. Höhere Plasma B12 Konzentrationen zu Studienbeginn waren mit geringerer Gesamt- und Grauer Substanz-Gerhirnvolumenreduktion assoziiert, sogar in nicht demenzkranken älteren Personen. Die schützenden Eigenschaften von Vitamin B12 zeigten sich über die gesamte Verteilung von Vitamin B12.

Ergebnis: Die Resultate dieser Arbeit deuten an, dass sowohl niedriges B12 und Folat als auch erhöhtes Homocystein eine Rolle in der Entwicklung von Kognitiv Störung. Funktionale Indikatoren von B12 und Folat, wie z.B. tHcy und holoTC, sollten berücksichtigt werden wenn der Zusammenhang zwischen B12 und Folat mit kognitiver Verschlechterung und strukturellen Veränderungen im Gehirn untersucht wird. Es bedarf sorgfältig geplanter randomisierter kontrollierter Studien mit ausreichender statistischer Power und adäquater Länge, um den Einfluss von B-Vitamin Supplementation zur Verhinderung von kognitiven Verschlechterungen und Demenz bedingten Pathologien zu erfassen.

Stichwörter: Alzheimer Erkrankung, Alzheimer Pathologie, zerebrovaskuläre Pathologie, Kognition, ältere Menschen, Demenz, Folat, Holotranscobalamin, Homocystein, populationsbasierte Studie, Vitamin B12.

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
APOE	Apolipoprotein E
APP	Amyloid beta precursor protein
ATC	Anatomical Therapeutic Chemical Classification System
Aβ	β-amyloid
BMI	Body mass index
CAA	Cerebral amyloid angiopathy
CAIDE	The Finnish Cardiovascular Risk Factors, Aging, and Dementia Study
CBS	Cystathionine β-synthase
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CH2THF	5,10-methylenetetrahydrofolate
CH3THF	5-methyltetrahydrofolate
CI	Confidence interval
CIND	Cognitive Impairment No Dementia
CMIA	Chemiluminescent Microparticle Immunoassay
CSF	Cerebrospinal fluid
CT	Computer tomography
CV	Coefficient of variation
DBP	Diastolic blood pressure
DLB	Dementia with Lewy bodies
DSM-III-R, (DSM-IV)	Diagnostic and Statistical Manual of Mental Disorders, revised third edition, (Fourth edition)
FDG-PET	fluorodeoxyglucose uptake – Positron emission tomography
FFE	Fast field echo
FINMONICA	Finnish part of Monitoring trends and determinants of Cardiovascular disease study
FINGER	The Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability
GM	Grey Matter
Hb	Hemoglobin
Hcy	Homocysteine
HoloTC	Holotranscobalamin
ICD	International Classification of Diseases
MEIA	Microparticle Enzyme Immunoassay
MMSE	Mini-Mental State Examination
MRI	Magnetic resonance imaging
NFT	Neurofibrillary tangle
MTA	Medial temporal lobe atrophy

NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association
NINCDS-AIREN	National Institute of Neurological and Communicative Disorders and Stroke-Association Internationale pour la Recherché et l'Enseignement en Neurosciences
OR	Odds ratio
PCR	Polymerase chain reaction
PDD	Parkinson's disease dementia
PUFA	polyunsaturated fatty acid
RBC	Red Blood Cell
RCT	Randomized controlled trial
RD	Relative difference
Q	Quartile
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
SES	Socioeconomic status
SNAC-K	The Swedish National Study on Aging and Care in Kungsholmen
SP	Senile plaques
SPM	Statistical Parametric Mapping
tHcy	Total serum/plasma homocysteine
SPSS	Statistical Package for the Social Sciences
TBT	Total brain tissue volume
VaD	Vascular Dementia
VALVIRA	The National Authority for Medicolegal Affairs
WHICAP	The Washington-Heights Inwood Columbia Aging Project
WM	White matter
WMH	White matter hyperintensities

LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following original papers, referred to in the text by their Roman numerals:

I. **Hooshmand B,** Solomon A, Kåreholt I, Leiviskä J, Rusanen M, Ahtiluoto S, Winblad B, Laatikainen T, Soininen H, Kivipelto M. *Neurology*. 2010; 19;75(16):1408-14.

II. **Hooshmand B**, Solomon A, Kåreholt I, Rusanen M, Hänninen T, Leiviskä J, Winblad B, Laatikainen T, Soininen H, Kivipelto M. Classification and prediction of clinical Alzheimer's diagnosis based on MRI and plasma vitamin E measures. Associations between serum homocysteine, holotranscobalamin, folate and cognition in the elderly: a longitudinal study. *J Intern Med*. 2012;271(2):204-12.

III. **Hooshmand B**, Polvikoski T, Kivipelto M, Tanskanen M, Myllykangas L, Erkinjuntti T, Mäkelä M, Oinas M, Paetau A, Scheltens P, van Straaten E.C.W., Sulkava R, Solomon A. Plasma homocysteine, Alzheimer and cerebrovascular pathology: a population-based autopsy study. *Brain.* 2013;136;2707-2716.

IV. **Hooshmand B,** Mangialasche F, Kalpouzos G, Jonsson Laukka E, Bäckman L, Fratiglioni L, Kivipelto M. Vitamin B12 and folate in relation to rate of brain atrophy: a longitudinal population based study.
Submitted.

OTHER PUBLICATIONS BY THE AUTHOR NOT INCLUDED IN THE THESIS

1. Miralbell J, Spulber G, **Hooshmand B**, Besga A, Mataró M, Cedazo-Minguez A, Kivipelto M, Wahlund LO. Grey matter and cognitive patterns in cognitive impaired subjects using CSF biomarker cut-offs. J Alzheimers Dis. 2012; 29(4):741-9

2. Besga A, Cedazo-Minguez A, Kåreholt I, Solomon A, Björkhem I, Winblad B, Leoni V, **Hooshmand B**, Spulber G, Gonzalez-Pinto A, Kivipelto M, Wahlund LO. Differences in brain cholesterol metabolism and insulin in two subgroups of patients with different CSF biomarkers but similar white matter lesions suggest different pathogenic mechanisms. Neurosci Lett. 2012; 510(2):121-6.

CONTENTS

توانا بود هر که دانا بود

Knowledge is power

Ferdowsi, *Shahname*, Preface

1 INTRODUCTION

1.1 Dementia and Alzheimer's disease

According to the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria, the essential feature of dementia is the development of multiple cognitive deficits that include memory impairment and at least one of the following cognitive disturbances: aphasia, apraxia, agnosia, or disturbance in executive functioning. To fulfill the criteria for dementia diagnosis, the cognitive decline must be sufficiently severe to cause significant impairment in social or occupational functioning, and it must present a decline from a previous level of functioning[1].

The most common causes of dementia are Alzheimer's disease (AD) and cerebrovascular disease. Other types of dementia include frontotemporal lobar degeneration (FTLD), dementia with Lewy bodies (DLB), Parkinson's disease dementia (PDD), alcohol-related dementia, HIV-related dementia and Creutzfeldt-Jakob disease. Increasing evidence from population-based neuropathological and neuroimaging studies suggests that mixed brain pathologies (particularly neurodegenerative and vascular) account for a large number of dementia cases, especially at older ages[2].

Advanced age is a well-established risk factor for dementia and AD. Dementia prevalence is very low in individuals younger than 60 years, and increases exponentially with increasing age. The age-specific prevalence rates of dementia are estimated to be approximately 1% in people aged 60-64 years, 1.5% between 65-69 years, 3% between 70-74 years, 6% between 75-79 years, 13% between 80-84 years, 24% between 85-89 years, 34% between 90-94 years, and 45% in the oldest age group of 95+ years[3].

Dementia has become a focus of great public health interest during the past decades, as the world population is aging. The worldwide increase in the number of older adults, which is particularly pronounced in the 80+ age group, explains the epidemic proportions assumed by dementia. According to the World Alzheimer Report, there were 35.6 million people living with dementia worldwide in 2010, a number that will increase to 65.7 million by 2030 and 115.4 million by 2050 unless effective means of reducing disease incidence are found[4].

Dementia and AD are recognized as a major cause of disability, institutionalization, and mortality in the elderly, as well as a cause of immense distress among family members and caregivers. The high number of people affected by dementia places enormous pressure on

society and health care systems. The worldwide societal costs of dementia have increased by 34% in between 2005 and 2009[5], and the total estimated costs of dementia were around 604 billion USD in 2010, including the costs of informal care (unpaid care provided by family and others), direct costs of social care (provided by community care professionals, and in residential home settings), and direct costs of medical care (costs of treating dementia and related conditions in primary and secondary care)[4]. Consequently, finding effective preventive strategies for dementia should be one of the top priorities in public health policies worldwide.

1.2 Neuropathology of Alzheimer's disease

The pathophysiology of AD is not yet fully understood. AD neuropathology is characterized by the formation of extracellular amyloid plaques and intraneuronal neurofibrillary tangles (NFT)[6, 7], leading to neuronal dysfunction and death. The amyloid plaques are products of sequential proteolytic processing of the amyloid precursor protein (APP), an integral membrane protein found in many tissues and concentrated in the synapse of neurons. The proteases involved in the proteolytic processing of APP are the α-, β-, and γ-secretases. The cleavage by β- and γ-secretases results in the formation of 39-43 amino-acid β-amyloid (Aβ) peptides. While $A\beta_{1-40}$ constitutes the most frequent form of Aβ, $A\beta_{1-42}$ (representing only about 10% of all Aβ species in the brain) displays an increased tendency to aggregate and accumulate as extracellular amyloid deposits[8]. The two major types of amyloid plaques in the AD brain are neuritic plaques (NP) and diffuse plaques. NPs contain dense bundles of amyoid fibrils and are surrounded by dystrophic neurites, astrocytes, and microgelia. Diffuse plaques contain unstructured amyloid and are not surrounded by dystrophic neurites. Aβ aggregates are also found in the walls of cerebral blood vessels causing cerebral amyloid angiopathy (CAA).

The amyloid hypothesis has been the most well-defined and studied conceptual framework for AD since the early 1990s. It has undergone several modifications over time, mainly concerning the type of Aβ causing AD: amyloid plaques were initially pointed out, later protofibrils, followed by increased concentrations of $A\beta_{1–42}$, then increased $A\beta_{1–42}$:$A\beta_{1–40}$ ratio, and currently oligomeric Aβ[9]. However, AD involves more than just amyloid-related pathology, and amyloid also has physiological functions that are only beginning to be described[10].

The other neuropathological hallmark of AD is the formation of NFTs inside neurons. NFTs are the result of pathological phosphorylation of the microtubule-associated tau protein. Tau

hyperphosphorilation leads to oligomerization and microtubule destabilization within the cell, and ultimately to neuronal apoptosis[11]. Braak and Braak proposed a model for AD development based on the presentation of NFTs, with a hierarchical progression of the changes[6, 7]. During the preclinical phase of the disease, NFTs appear in the entorhinal region of the medial temporal lobe, spreading to the hippocampus and finally to the neocortex during the later stages of AD. As a result, there is marked neuronal loss and brain atrophy observed especially in the temporal lobe structures. Later during the disease course, cortical and central atrophy is also evident[12]. This pattern is subject to only minor inter-individual variation, and allows the identification of 6 stages in the evolution of lesions: the clinically silent transentorhinal stages I and II; the limbic stages III and IV (when the onset of clinical symptoms usually occurs); and the neocortical stages V and VI, corresponding to fully developed AD[6, 13].

Other changes found in AD include synaptic loss and proliferation of reactive astrocytes in the entorhinal cortex, hippocampus, amygdala and association areas of frontal, temporal, parietal, and occipital cortex[14, 15]. In addition, oxidative stress, inflammation, and cerebrovascular lesions seem to be important in AD[16].

The clinical relevance of these brain changes is not entirely clear. AD-related changes can often be found in persons with milder cognitive impairment, or even with normal cognition[17, 18]. Not all individuals with Alzheimer-type brain changes develop cognitive impairment later on. Also, little is known about the time course from the accumulation of AD changes until the onset of symptoms. In addition, as AD usually occurs in old age, it is frequently accompanied by other common late-life pathologies, especially cerebrovascular disease and Lewy body pathology. Simultaneously occurring pathologies can lower the threshold for clinical manifestations, increasing the likelihood of cognitive impairment diagnosed as AD[19-21].

1.3. Proposed revisions of diagnostic criteria for Alzheimer's disease

The clinical onset of AD is insidious and often characterized by memory impairment. The disease evolves gradually, leading to global cognitive impairment[22]. Currently, the diagnosis of AD is based mainly on clinical findings. There are several sets of similar (but not identical) diagnostic criteria, including the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)[23], the DSM-IV [1], and the International Classification of Diseases, 10th

revision (ICD-10) criteria[24]. The NINCDS-ADRDA criteria have been widely used in research[25]. According to these criteria, the patient has probable AD when the presence of dementia has been established by clinical examination, and confirmed by neuropsychological tests (i.e. deficits in two or more areas of cognition, progressive worsening of memory and other cognitive functions). The onset is between 40-90 years, typically after the age of 65 years. No disturbance of consciousness should be present, and other disorders that could cause dementia should be absent. In addition, the criteria refer to aphasia, apraxia, and agnosia, impaired activities of daily living, family history of AD, and normal laboratory assessments. The diagnosis of definite AD requires that the patient has met the clinical criteria for probable AD, and has histopathological evidence of AD at autopsy.

Diagnostic criteria for AD and other dementia-related disorders are currently being updated. In the recently published DSM V[26], dementia has been replaced by Major Neurocognitive Disorder, and a diagnosis of Mild Neurocognitive Disorder has also been added. Mild and Major Neurocognitive Disorder no longer specify memory as the compulsory central domain of cognitive impairment, as criteria for dementia do in ICD-10 and DSM-IV. There is additionally a diagnosis of Major or Mild Neurocognitive Disorder due to multiple etiologies, acknowledging the role of mixed brain pathologies in cognitive impairment at older ages. AD diagnosis is focused on memory impairment, but non-memory features are also mentioned. Epidemiological studies will be needed to establish what types of pathologies are actually diagnosed as Minor or Major Neurocognitive Disorder, their risk factors and prognosis. The updated 11th edition of ICD is expected to be published during 2015.

In addition to DSM V criteria, two sets of research criteria for diagnosing AD have been proposed[27, 28]. These new recommendations still have clinical criteria at the core of the diagnosis, but incorporate even biomarkers (genetic, biochemical, neuroimaging) that can be detected in vivo and are believed to reflect AD pathology. This is expected to enhance the specificity of AD diagnosis. Biomarkers of Aβ accumulation are abnormal tracer retention on amyloid positron emission tomography (PET) imaging, and low CSF $A\beta_{1\text{-}42.}$ Biomarkers of neuronal degeneration or injury are also included, i.e. elevated CSF tau (both total and phosphorylated tau), decreased fluorodeoxyglucose uptake on PET (FDG-PET) in a specific topographic pattern involving temporoparietal cortex; and atrophy on MRI, again in a specific topographic pattern involving medial, basal, and lateral temporal lobes and medial and lateral parietal cortices.

The choice of these biomarkers is based on the assumption that amyloid plaques and NFTs define AD neuropathology. The diagnostic criteria also postulate the presence of a temporal order of biomarker abnormalities, and assign a key, early role to Aβ in AD pathophysiology. Validation and standardization of the proposed biomarkers is needed before they can be more widely implemented in clinical practice.

1.4 Risk and protective factors for AD

Dementia and AD have been extensively studied in the past decades with the aim of identifying efficacious prevention and treatment strategies. It has become clear that dementia/AD is a complex multifactorial condition involving several interrelated mechanisms in which the interactions of genetic and environmental factors play a major role. The main proposed risk and protective factors for dementia/AD are summarized in table 1. It is important to note that many of these factors are also related to cardiovascular or cerebrovascular conditions.

Epidemiological findings support the possibility to prevent or delay dementia/AD onset by addressing modifiable risk/protective factors. It has been estimated that half of AD cases worldwide are potentially attributable to modifiable risk factors; a 10-25% reduction in these factors could potentially prevent three million AD cases worldwide, with a reduction in all risk factors having the greatest impact on dementia prevalence[29].

Due to the long preclinical phase of AD, a life-course perspective is essential for formulating effective preventive strategies and identifying optimal time windows for applying them. Dementia risk is the result of exposure to multiple factors during the life span, and the role of different factors can vary with age. Cumulative exposures may have multiplicative or additive effects over the life course[30].

Table 1. Main proposed risk and protective factors for late-onset dementia and AD

Risk factors	Protective factors
Age **Genetic** • Familial aggregation • *APOE*ε4 • Different genes (i.e. *CR1*, *PICALM*, *CLU*, *TREM2*, *TOMM40*) have been proposed (www.alzgene.org) **Vascular and metabolic** • Cerebrovascular lesions • Cardiovascular diseases • Diabetes mellitus and pre-diabetes *Midlife positive association but late-life negative association* • Hypertension • High BMI (overweight and obesity) • High serum cholesterol **Lifestyle** • Smoking • High alcohol intake **Diet** • Saturated fats • Homocysteine **Others** • Depression • Traumatic brain injury • Occupational exposure (heavy metals, ELF-EMFs) • Infective agents (Herpes Simplex Virus Type I, Spirochetes)	**Genetic** • Different genes (e.g. *APP*, *APOE*ε2) have been proposed (www.alzgene.org) **Psychosocial factors** • High education and socioeconomic status • High work complexity • Rich social network and social engagement • Mentally stimulating activities **Lifestyle** • Physical activity • Moderate alcohol intake **Diet** • Mediterranean diet • Polyunsaturated (PUFA) and fish-related fats • Vitamins B12, folate, B6 • Antioxidant vitamins (A, C, E) • Vitamin D **Drugs** • Antihypertensive drugs • Statins • Hormone replacement therapy (HRT) • Non steroid anti-inflammatory drugs (NSAIDs)

APP: amyloid precursor protein; *APOE:* Apolipoprotein E; BMI: body mass index; CLU: clusterin; CR1: complement component receptor 1; ELF-EMFs: extremely-low-frequency electromagnetic fields; PICALM: phosphatidylinositol binding clathrin assembly protein; PUFA: polyunsaturated fatty acid; SES: socioeconomic status; TOMM40: translocase of outer mitochondrial membrane 40 homolog; TREM2: triggering receptor expressed on myeloid cells 2.

Age-dependent associations with dementia/AD have been suggested for several factors. For example, elevated blood pressure, body mass index (BMI), and total cholesterol levels at midlife have been associated with an increased dementia/AD risk, while at older ages these relations are less straightforward[31-35]. A pattern of decline in blood pressure, BMI or cholesterol from midlife to late-life has been reported in individuals who develop dementia/AD later on[30, 31, 36, 37]. This pattern cannot be entirely explained by use of medication or intentional lifestyle changes. Reverse causality is one possible explanation, i.e. a midlife factor increases AD risk, but once the disease begins it affects the same factor that has contributed to it. Because the temporality of exposure in relation to outcome is more difficult

to establish at older ages when many individuals can have preclinical AD, studies with shorter follow-up and older populations can sometimes report opposite results compared to midlife, long-term follow-up studies.

Diabetes mellitus has also been associated with an increased risk of dementia/AD, but the association is stronger when diabetes occurs in midlife than in late-life[38]. Smoking is another risk factor for AD[39], and about 14% of all AD cases have been estimated to be potentially attributable to smoking[29]. A J-shaped relation between alcohol consumption and dementia/AD risk has been described, i.e. heavy drinking increases the risk, while moderate alcohol intake may be protective[37]. This protective effect may however be due to confounding (i.e. other healthy lifestyle factors that tend to accompany moderate alcohol consumption). Although it is not entirely clear whether depression is a risk factor for or a preclinical symptom of dementia, studies with long-term follow-up support the risk factor hypothesis[40].

Several protective factors for AD have also been identified, including high education and socioeconomic status (SES) in early life, as well as a number of factors in adult life: high work complexity, rich social network, social engagement, mentally-stimulating activities, and regular physical exercise[37, 41, 42]. Living with a partner during midlife has been associated with reduced risk of cognitive impairment and dementia later in life, suggesting that being in a relationship entails cognitive and social challenges that can increase cognitive reserve[43]. Active engagement in mental, physical, and social activities even at older ages may postpone the onset of dementia, possibly by increasing cognitive reserve[44]. In addition, several follow-up studies reported a decreased risk of dementia/AD associated with healthy dietary patterns and nutritional factors, such as high adherence to a Mediterranean diet, vitamin E, and ω-3 polyunsaturated fatty acids (PUFA), which is often measured as fish consumption[45-48].

Genetic-environmental interactions can also influence the risk of dementia. Population-based studies suggest an effect modification for the *APOE* ε4 allele, the most important genetic risk factor for sporadic AD. *APOE* ε4 carriers seem more vulnerable to risk factors like alcohol drinking, smoking, physical inactivity, and high intake of saturate fat, indicating that people with genetic susceptibility may be able to reduce their initial AD risk by lifestyle interventions[37, 49].

In addition to research on individual risk factors, integrated risk assessment tools have also started to be formulated for dementia/AD. Based on results from the Cardiovascular Risk Factors, Aging and Dementia (CAIDE) study in Finland, a midlife risk score for predicting

dementia risk 20 years later was proposed[50]. The CAIDE Dementia Risk Score includes factors such as age, sex, lower education, high systolic blood pressure, BMI, cholesterol and low level of leisure-time physical activity. The risk of dementia increases as the score increases in a dose-response trend, making it possible to identify individuals who may benefit from preventive interventions[50]. The CAIDE Dementia Risk Score has been used to select participants in the ongoing Finnish Geriatric intervention (FINGER) study to prevent cognitive impairment and disability[51].

Risk assessment tools for dementia in older populations present some differences compared to midlife risk profiles. In the Swedish Kungsholmen Project, the cumulative effect of vascular risk factors and vascular diseases on dementia/AD risk was investigated in people aged 75+ years. These factors were aggregated according to two pathophysiological hypotheses: the brain hypoperfusion profile, defined by chronic heart failure, low pulse pressure, and low diastolic pressure, and the atherosclerosis profile, which included high systolic pressure, diabetes mellitus or prediabetes, and stroke[52]. A Late-life Dementia Risk Index was developed based on the American Cardiovascular Health Cognition Study in people aged 65+ years, including age, presence of the *APOE*ε4 allele, BMI<18.5, lack of alcohol consumption, comorbid vascular conditions (internal carotid artery thickening, angina, coronary artery by-pass surgery, stroke, peripheral artery disease), evidence of brain abnormalities showed by magnetic resonance imaging (MRI) (white matter diseases or enlarged ventricles), cognitive test scores and physical performance[53, 54].

There is still a lack of detailed knowledge concerning risk/protective factors and the most effective ways to target them in order to prevent dementia/AD[55]. Randomized controlled trials (RCTs) are needed to confirm the effect of risk reduction strategies targeting multiple risk factors simultaneously. Multidomain interventional RCT are already ongoing, and will provide new insights into prevention of cognitive impairment and dementia/AD (www.edpi.org).

1.5 Homocysteine (Hcy)

Hcy, a sulfur-containing non-essential amino-acid, was first described in 1932 by the Nobel laureate Vincent du Vigneaud and his group as an intermediary sulfur metabolite in the methionine cycle, by treating methionine with sulfuric acid[56].

The potential role of Hcy in pathological processes was described 30 years later, when an inborn error of Hcy metabolism in children with intellectual disabilities was first demonstrated[57, 58]. 7 years later, Kilmer McCuly suggested that Hcy had deleterious effects on the vascular system, and could possibly be a marker of atherosclerosis[59]. In 1976, Wilcken found elevated Hcy levels in 7 out of 25 patients with coronary artery disease after methionine loading, and concluded that patients with premature-onset coronary artery disease had reduced ability to metabolize Hcy[60]. Later on, Brattström reported an association between moderate hyperhomocysteinemia and ischemic stroke[61].

Hcy is metabolized through two pathways: remethylation to the essential amino-acid methionine (thus completing the methionine cycle) or transsulfuration to the sulfur amino acid cysteine via cystathionine. Figure 1 illustrates the simplified Hcy metabolism through the methionine cycle. In the methionine cycle, methionine is first metabolized to s-adenosylmethionine (SAM), the primary methyl donor for most neuronal methylation reactions, including the synthesis of catecholamine neurotransmitters, proteins, nucleic acids, phospholipids and myelin[62-64]. After donating its methyl group, SAM is subsequently converted to s-adenosyl homocysteine (SAH). SAH is further hydrolyzed to Hcy in a reversible reaction. Hcy would need to be remethylated in order to complete the methionine cycle. This step requires two water-soluble vitamins: vitamin B12 as a co-factor and 5-methyltetrahydrofolate (CH3THF) as a methyl group donor substrate for the enzyme methionine synthase (MS). CH3THF is itself the product of 5,10-methylenetetrahydrofolate (CH2THF) reduction by the enzyme methylenetetrahydrofolate reductase in the folate cycle. Therefore, functional lack of B12 or folate would impair the remethylation of Hcy, resulting in increased total homocysteine levels[65, 66].

Hcy can also enter the catabolic transsulfuration pathway by first converting to cysthationine, a reaction which requires a B6-dependent enzyme, cystathionine β-synthase (CBS). However, the transsulfuration pathway has somewhat limited tissue distribution and may be limited in mammalian brain which possesses CBS but little γ-cystathionase [67-70].

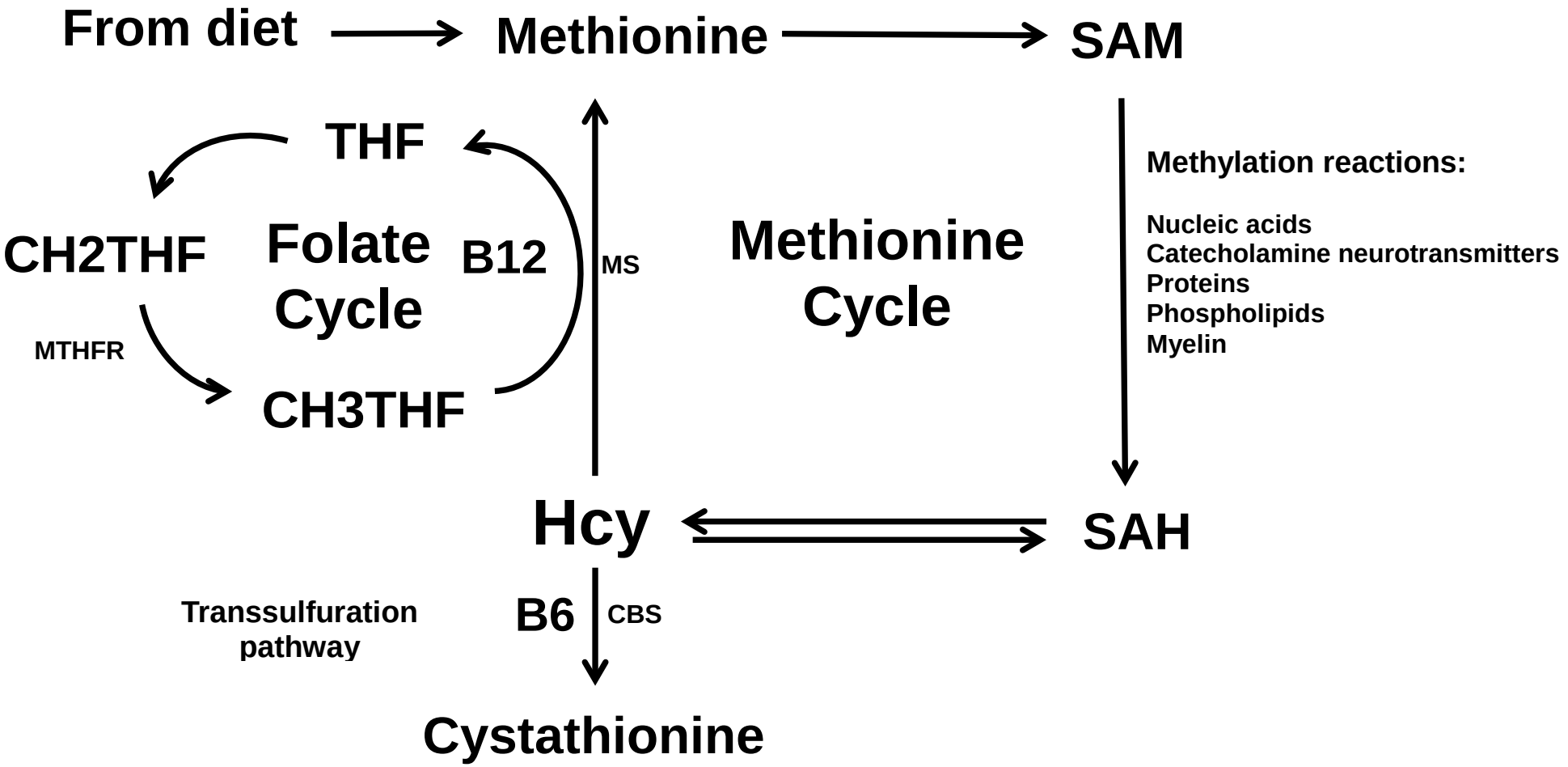

Figure 1. Simplified homocysteine metabolism cycle.

CBS: Cystathionine β-synthase, CH2THF: 5, 10-methylenetetrahydrofolate, CH3THF: 5-methyltetrahydrofolate, Hcy: homocysteine, MS: methionine synthase, MTHFR: methylenetetrahydrofolate reductase, SAH: S-adenosyl homocysteine, SAM: S-adenosyl methionine.

Determinants of Hcy concentration

Several genetic, physiologic, and life-style factors, as well as various diseases and medications can affect blood Hcy levels. For example, Hcy concentration tends to increase with age. After puberty, males have higher mean Hcy values than females, but gender differences become less evident with increasing age[71]. Other conditions affecting Hcy include dietary B-vitamin deficiencies, renal impairment, certain genetic polymorphisms (i.e. MTHFR C677T), smoking, excessive coffee consumption, physical activity, alcohol intake, diabetes, thyroid function, anti-folate medications (such as drugs used in rheumatologic or infectious diseases), vitamin B12-antagonists (such as metformin or anti-acid medications), and vitamin B6-antagonists (such as isoniazid or theophyllin)[66]. Most of these factors influence Hcy by altering the functions or blood concentrations of B-vitamins (especially B12 and folate), or by influencing renal functions[66]. Therefore, vitamin B12 and folate, together with renal function, are considered as the main determinants of Hcy status particularly among elderly.

Effects of Hcy on vascular and Alzheimer-related pathology

Hyperhomocysteinemia is a known risk factor for atherosclerosis, coronary artery disease, and stroke. Long-standing hyperhomocysteinemia may lead to intima thickening and disturbance of endothelial functions in cerebrovascular arterioles and capillaries[63, 64]. In experimental studies, excess Hcy has been shown to affect blood vessels through a variety of mechanisms. Hcy promotes inflammatory processes, thus exposing several sensitive receptors on endothelial cells to oxidizing agents and increasing oxidative stress; it also promotes uptake of low-density lipoprotein cholesterol by macrophages[72, 73]. Hcy exposes certain ligands (receptors) for platelets on the endothelial surface and activates platelets, thus promoting platelet clotting[74]; it also affects smooth muscle cells, another vital blood vessel component. Furthermore, Hcy generates procoagulant activity in the tissues and decreases anti-clotting factor antithrombin III activity[75]. It is positively related to asymmetric dimethylarginine which also has adverse effects on vascular tissue such as inhibiting the nitric oxide synthesis, the main vascular dilating factor[76].

Cardiovascular conditions and dementia/AD share several risk factors, and vascular changes have been described in the brains of patients with AD[2, 64]. Elevated Hcy can thus be hypothesized to associate with AD through vascular-related mechanisms. In addition, it has been suggested that elevated Hcy may accelerate the production of Aβ, the accumulation of Aβ in plaques, and increased tau phosphorylation[63, 77].

1.6 Folate

Folate, the naturally-occurring form of folic acid (occasionally denoted as vitamin B9), was first discovered in 1931 by the British pathologist Lucy Wills[78]. Folic acid was isolated from spinach in 1941 (folium is the Latin name for leaf). From 1946, folic acid was available in its crystalline form. Around 1950 it was found that folate supplementation worsened prognosis in children with acute lymphatic leukemia. This observation initiated the development of anti-folates. As mentioned earlier, anti-folates like methotrexate and trimethoprim can cause a rise in plasma Hcy. Methotrexate is a potent cytostatic agent and it also possesses anti-inflammatory and immunosuppressive properties.

Folate is found in foods from both plant and animal sources, such as leafy green vegetables, beans, whole grains, potatoes, liver and beef. However, absorption of folic acid from supplements is much more effective than absorption of folate from food.

Folate plays a crucial role in purine synthesis, in monoamine synthesis, and as methyl group donor in methylation of DNA in gene regulating reactions. Folate deficiency can occur at all ages and is usually a result of low intake due to poor diet, malabsorption, alcoholism, infections, certain genetic polymorphisms, pregnancy and lactation, and use of certain medications such as methotrexate, anti-epileptics or trimethoprim[79]. It is particularly important to ensure good folate and B12 status during pregnancy (due to the increased risk of neural tube defects associated with folate deficiency), and during infancy.

Folate in blood is contained in erythrocytes (red blood cells (RBC) folate) and plasma. RBC-folate levels indicate a person's folate consumption during recent months, while plasma or serum folate levels reflect the few days prior to blood sampling.

1.7 Vitamin B12 (Cobalamin)

A fatal type of anemia associated with stomach degeneration was described already in the early 19th century. The condition was later on named *pernicious anemia.* It was found that patients with this type of anemia had giant peripheral blood cells, which were called megaloblasts. Pernicious anemia remained incurable until 1926, when George Minot and William Murphy reported a set of experiments in which a special raw liver diet had been used to treat patients. Together with George H. Whipple, they were awarded the Nobel Prize in

Physiology or Medicine in 1934 “for their discoveries concerning liver therapy in cases of anemia”. In 1956, Dorothy Hodgkin succeeded in elucidating the complex crystalline structure of B12; due to its ruby color, B12 was later referred to as “nature’s most beautiful co-factor”[62, 80, 81]. This discovery also led to a Nobel Prize in Chemistry for Hodgkin “for her determinations by X-ray techniques of the structures of important biochemical substances”.

Vitamin B12 is unique in being synthesized only by bacteria and fungi, which have the necessary enzymes to produce all naturally-occurring B12. Plants are incapable of producing this vitamin, and herbivorous animals must obtain B12 from the bacteria in their rumens. Humans can obtain B12 from food of animal origin such as meat, liver, fish, poultry, eggs, and dairy products.

Vitamin B12 can be absorbed via passive diffusion which is an inefficient mechanism because only 1% of the oral dose is passively absorbed. The normal active physiological mechanism involves the ileum and is mediated by gastric intrinsic factor. The intrinsic factor is produced in the parietal cells of the stomach, the cells which also produce hydrochloric acid. In the ileum, the intrinsic factor-vitamin B12 complex binds to a specific cubilin receptor (cubam) and enters the ileal cells, where the intrinsic factor is destroyed[82, 83]. Vitamin B12 is then transported into the blood. About 20-30% of B12 in blood is bound to transcobalamin, forming holotranscobalamin (holoTC). HoloTC is able to enter cells and represents the active fraction of vitamin B12. The functions of the remaining haptocorrin-bound B12 are not clear[82, 84, 85].

Vitamin B12 is a co-factor for only two enzymes: methionine synthase (discussed above), and methyl-malonyl coenzyme A mutase. The latter enzyme is involved in the isomerization of methyl-malonyl conenzyme A to succinyl coenzyme A. This reaction is important for the catabolism of fatty acids with an odd number of carbon atoms, cholesterol, and several amino acids. Therefore, low B12 levels result in increased methyl-malonycoenzyme A concentrations, which are further converted to methyl-malonic acid (MMA)[86].

The most common causes of B12 deficiency include inadequate dietary intake, defective absorption, total or parietal gastrectomy, medications (i.e. metformin or drugs that block stomach acid), mutations in genes essential for transcobalamin, or auto-antibodies[66, 82].

Evaluating vitamin B12 levels in serum or plasma is often used to confirm the diagnosis of B12 deficiency. However, total B12 is predominantly a measure of haptocorrin-bound B12,

which is considered biologically inactive. Other B12 indicators such as Hcy, MMA, and holoTC could be considered as surrogate markers of B12 status. Although reasonably specific for B12 deficiency, MMA measurements are expensive, not widely available, and are affected by kidney function. Hcy is less specific because it may result from the deficiency of other vitamins such as folate, and is also affected by the kidney function. HoloTC appears to be promising as a more sensitive assay of B12 status, and decreased holoTC has been suggested as the first-line test for early diagnosis of B12 deficiency[82, 85, 87]. However, the availability of holoTC measurements is still somewhat restricted.

Vitamin B12 deficiency can result in reversible megaloblastic anemia, a condition which requires the interaction of both folate and vitamin B12. Other clinical and laboratory findings in vitamin B12 deficiency include altered mental status and cognitive deficits, myelopathy and spongy degeneration of the spinal cord, paresthesia, hyporeflexia, loss of proprioception, ataxic gate; developmental delay or hypotonia in infants and children, infertility, increased blood Hcy and methylmalonic acid levels, macrocytic red cells and macroovalocytes, leukopenia, thrombocystopenia or even pancytopenia[66, 82].

1.8 Hcy, vitamin B12 and folate in relation to AD and cognition in epidemiological studies

Epidemiological findings linking vitamin B12 and folate levels with late-life cognition began to be reported during the 1980s[88]. Associations between Hcy, B12 and neuropsychiatric symptoms were also described[89]. In the 1990s, a case-control study reported that total serum Hcy was higher in AD cases, and both Hcy and B12 were related to cognition[90]. Another case-control study indicated an association between Hcy, B12, folate, and pathologically confirmed AD[91]. Additional case-control or cross-sectional studies yielded mixed results[64].

An overview of the main available longitudinal studies (table 2) shows large variations in the duration of follow-up (from 2.3 to 30 years), as well as in the choice of cognition-related outcomes (from isolated cognitive tests to more complex neuropsychological batteries to the diagnosis of dementia/AD). It is thus not entirely surprising that findings on the relations between Hcy, B12, folate and late-life cognition are sometimes contradictory.

Table 2. Main longitudinal studies on Hcy, vitamin B12, folate, and cognitive performance or dementia/AD

Study (country)	Study population	Follow-up	Outcome	Results
Kalmijn et al, 1999[92] (Netherlands)	The Rotterdam study N = 702, mean age = 68y	2.7y	Decline in MMSE	No association with Hcy
McCaddon et al, 2001[93] (UK)	N = 32 community-dwelling elderly, median age = 74y	5y	Tests of verbal memory, orientation, praxis	All 3 tests showed inverse relations with Hcy
Wang et al, 2001[94] (Sweden)	The Kungsholmen project N = 370, age = 75+y	3y	Dementia/AD	Low folate alone, or low folate and low B12 were related to incident AD (only significant in subjects with baseline MMSE>26).
Seshadri et al, 2002[95] (USA)	The Framingham study N = 1092, mean age = 76y	8y	Dementia/AD	Hcy strongly associated with incident dementia and AD. No relation with B12 or folate
Dufouil et al, 2003[96] (France)	The Epidemiology of Vascular Aging (EVA) Study N = 1241, mean age = 67y	4y	MMSE, psychomotor speed, attention	Hcy related to worse cognitive performance. No association with folate or B12
Garcia et al, 2004[97] (Canada)	N = 180 community-dwelling elderly, mean age = 73y	2.3y	Multiple cognitive tests	Hcy inversely related to learning and executive functions. No association with B12 or RBC folate
Luchsinger et al, 2004[98] (USA)	The WHICAP study N = 679, mean age = 77.2y	4.7y	Multiple cognitive tests AD	No association with Hcy, B12, or folate
Mooijaart et al, 2005[99] (Netherlands)	The Leiden 85-Plus Study N = 341, age = 85+y	4y	Multiple cognitive tests	No relation with Hcy and B12; higher folate level related to faster rate of decline
Kado et al, 2005[100] (USA)	MacArthur Studies of Successful Aging N = 370, age = 74+y	7y	Multiple cognitive tests	Folate associated with cognitive decline, no associations with Hcy and B12
Tucker et al, 2005	The Veterans Affairs Normative	3y	Multiple cognitive	Hcy, B12, and folate associated with

(USA)	Study N = 321 men, age = 67y		tests	several cognitive tests. Only folate significant when all 3 vitamins and Hcy were considered together.
Ravaglia et al, 2005[101] (Italy)	The Conselice Study of Brain Aging N = 816, age = +74y	3.8y	Dementia/AD	Elevated Hcy and low folate were associated with incident dementia. No association with B12
Nurk et al, 2005[102] (Norway)	The Hordaland study N = 2189, baseline age = 65-67y	6y	Episodic memory	Higher Hcy and low folate had an inverse association with test scores. No relation with B12
Kang et al, 2006[103] (USA)	The Nurses' Health Study N = 391 women, mean age = 63y	4y	Multiple cognitive tests	No association
Clarke et al, 2007[104] (UK)	The Oxford Healthy Aging Project N = 691, mean age = 71.9y	10y	MMSE	HoloTC, Hcy, and MMA (but not folate) predicted decline. After adjusting for all vitamin markers simultaneously, only holoTC and MMA associated with decline.
Haan et al, 2007[105] (USA)	The Sacramento Area Latino Study on Aging N = 1779, mean age = 71y	4.5y	Dementia CIND	Hcy associated with both dementia and CIND. B12 modified the association between Hcy-dementia/CIND. No relation with RBC-folate.
Kim et al, 2008[106] (South Korea)	N = 518 community-dwelling subjects, mean age = 71.8y	2.4y	Dementia	Lower folate predicted dementia; no associations with B12 or Hcy. Dementia more common in subjects with decline in folate and B12 levels or a relative increase in Hcy
Feng et al, 2009[107] (Singapore)	The Singapore Longitudinal Aging Study N = 539, mean age = 64.9y	3.2y	Multiple cognitive tests	B12 related to cognition; stronger association in *APOE*ε4 carriers.
Kivipelto et al,	The Kungsholmen Project	6.7y	Dementia/AD	Elevated Hcy associated with

2009[108] (Sweden)	N = 228, mean age = 81y			increased dementia/AD risk; holoTC related to lower dementia/AD risk; no relation with folate
De Lau et al, 2009[109] (Netherlands)	The Rotterdam Scan Study N = 832, mean age = 72.2y	3-8y	Multiple cognitive tests	No association
Tangney et al, 2009[110] (USA)	The Chicago Health and Aging Project N = 516, mean age = 80y	6y	Multiple cognitive tests	Higher MMA and lower B12 associated with faster rate of cognitive decline; no relation with Hcy or cystathionine
Van den Kommer et al, 2010[111] (Netherlands)	The Longitudinal Aging Study Amsterdam N = 1076, age= 65+y	6y	Multiple cognitive tests	Hcy associated with faster rate of decline in several cognitive domains.
Zylberstein et al, 2011[112] (Sweden)	The Prospective Population Study of Women in Gothenburg N = 1368 women, mean age = 47y	30y	Dementia/AD	High midlife Hcy independent risk factor for late-life dementia/AD
Narayan et al, 2011 [113] (UK)	The Study on Cognition and Prognosis in the elderly N = 182 hypertensive subjects Mean age = 80y	3.7y	Multiple cognitive tests	Higher Hcy associated with cognitive decline
Ford et al, 2012[114] (Australia)	The Health in Men Study N = 4227 men, age = 70+y	5.8y	Dementia	Hcy associated with dementia
Whalley et al, 2013[115] (UK)	The Aberdeen 1921 Birth Cohort Study N = 201, age=77+y	5y	Dementia	Hcy increased dementia risk
Jochemsen et al, 2013[116] (Netherlands)	The Second Manifestations of ARTerial disease-Magnetic Resonance study N = 663, mean age = 57y	3.9y	Memory and executive functioning	Elevated Hcy increased the risk of decline in executive functioning

AD: Alzheimer's disease, *APOE*: Apolipoprotein E, CIND: Cognitive Impairment No Dementia, Hcy: homocysteine, holoTC: holotranscobalamin, MMA: Methyl Malonic Acid, MMSE: Mini-Mental State Examination.

1.9 Associations between Hcy, vitamin B12, folate, and structural brain changes on MRI or Alzheimer-related markers

Hcy and B-vitamins have been suggested to relate to cognitive impairment through brain atrophy[96]. However, few longitudinal studies have so far investigated associations with the rate of brain atrophy or white matter changes, with inconsistent results (table 3).

Experimental studies suggest that Hcy may increase the risk of dementia through several mechanisms, i.e. the impact on cerebrovascular pathology, potentiation of amyloid-β generation and its neurotoxicity, or effects on tau hyperphosphorylation[63, 64, 77, 117]. No longitudinal studies are currently available on the relation between Hcy, B-vitamins and AD-related neuropathological findings. The few cross-sectional studies focusing on plasma or CSF markers related to AD are summarized in table 4.

1.10 B-vitamins and cognition: randomized controlled trials (RCTs)

Few RCTs have so far investigated the usefulness of B-vitamin supplements in preventing cognitive decline, with mixed results (table 5). Limitations of statistical power, and variations in vitamin dosage or vitamin combinations, study durations, choice of target population, and cognitive assessments make such studies difficult to interpret.

A recent RCT including subjects with mild cognitive impairment (MCI) investigated the impact of B-vitamin supplementation on brain atrophy rate. High dose B-vitamin treatment was found to slow brain atrophy rate over 2 years as compared with placebo. This effect was even stronger in participants with elevated Hcy levels[118]. In addition, B-vitamin treatment reduced cerebral atrophy in regions particularly susceptible to AD neurodegenerative process, such as the medial temporal lobe. These beneficial effects of B-vitamins were confined to participants with Hcy above the median value of 11 μmol/L[119]. It is thus possible that people with moderately raised Hcy concentrations may benefit more from the effects of B-vitamin supplementation on cognition[120, 121] or brain atrophy[118, 119].

Table 3. Main studies on Hcy, vitamin B12, folate and structural brain changes on MRI

Study (country)	Study population	Follow-up	Outcome	Results
Clarke et al, 1998[91] (UK)	N = 43 subjects with dementia Mean age = 78.9y	3y	Rate of medial temporal lobe thinning	Elevated Hcy associated with progressive atrophy
Vogiatzoglou et al, 2008 (UK)[122]	N = 107 cognitively normal community-dwelling volunteers, age 61+y	5y	Rate of brain volume loss	Higher B12 and holoTC related to decreased atrophy rate. No association with Hcy, folate, or MMA
Firbank et al, 2011 (UK)	N = 80 hypertensive subjects, age 70+y	2y	Atrophy rate	Hcy related to white matter and hippocampal atrophy rate, but not global grey matter atrophy rate
Jochemsen et al, 2013[116] (Netherlands)	The Second Manifestations of ARTerial disease-Magnetic Resonance study N = 663, mean age = 57y	3.9y	Cortical, ventricular, and global brain volumes	Elevated Hcy related to progression of ventricular enlargement

Table 4. Main studies on Hcy, vitamin B12, folate and Alzheimer-related markers in plasma and CSF

Study (country)	Study population	Outcome	Results
Flicker et al, 2004[123] (Australia)	N = 299 men, mean age = 78.9y	Plasma Aβ40	Elevated Hcy was associated with increased plasma Aβ40
Irizarry et al, 2005[124] (USA)	N = 465 subjects evaluated in the Memory, Stroke, and Movement Disorders Units of Massachusetts General Hospital with neurological disorders, age 60+	Plasma Aβ40 and Aβ42	In the whole sample, Hcy was associated with increased plasma Aβ40 but not Aβ42
Obeid et al, 2007[125] (Germany)	N = 182 patients with different neurological disorders, age +41	CSF P-tau and Aβ42	CSF S-adenosyl homocysteine and CSF folate correlated with CSF P-tau; no associations with Aβ42
Luchsinger et al, 2007[126] (USA)	N = 327 community-dwelling elderly, mean age = 78y	Plasma Aβ40 and Aβ42	Plasma Hcy was related to Aβ40 but not Aβ42
Popp et al, 2009[127] (Germany)	N = 98 cognitively healthy subjects, mean age 50.1y N = 54 AD patients, mean age 73.0y	CSF Aβ42 and P-tau	CSF concentrations of SAH were positively and 5-MTHF were negatively associated with CSF P-tau levels; no relation with Aβ42
Alexopoulos et al, 2009[128] (Germany)	N = 88 patients from 3 clinics, mean age 68.9y	CSF Aβ40, Aβ42, T-tau and P-tau	No association
Smach et al, 2011[129] (Tunisia)	N =70 AD patients, 33 patients with other dementia types, 30 age-matched controls, age 58+y	CSF Aβ42 and T-tau	No association

Table 5. B-vitamins and cognition in older adults without cognitive impairment: main randomized controlled trials

Study (country)	Study population	Intervention	Follow-up	Results
Bryan et al, 2002[130] (Australia)	N = 221 healthy women randomly selected from the electoral roll (N = 75 elderly) Mean age = 74y	0.75mg folic acid	35 days	Positive effects on some measures of memory performance; no effects on mood
Durga et al[120], 2007 (Netherlands)	N = 818 healthy older men and women with elevated tHcy recruited via municipal and blood bank registries Mean age = 60y	0.8 mg folic acid	3 years	Significant improvement in cognitive domains that tend to decline with age
Eussen, 2006[131] (Netherlands)	N = 195 elderly with mild vitamin B12 deficiency recruited by post from community and residential care Mean age = 83y	0.4 mg folic acid 1 mg B12	24 weeks	No effects
Ford et al, 2010[132] (Australia)	N = 299 elderly hypertensive community-dwelling men Mean age = 79y	2 mg folic acid 0.5 mg B12 25 mg B6	2 years	No effects
Kang et al, 2008[133] (USA)	N = 2009 women with cardiovascular risk factors/conditions selected from a RCT of vitamin supplementation for secondary cardiovascular prevention Mean age = 71y	2.5 mg folic acid 1 mg B12 50 mg B6	5.4 years	No effects
Lewerin, 2005[134] (Sweden)	N = 195 community-dwelling participants Mean age = 76y	0.8 mg folic acid 0.5 mg B12 3 mg B6	4 months	No effects
McMahon et al, 2006[135] (New Zeeland)	N = 276 healthy volunteers with elevated tHcy, mean age = 74y	1 mg folic acid 0.5 mg B12 10 mg B6	2 years	No effects
Stott, 2005[136] (Scotland)	N = 185 hospital in-patients with ischemic vascular disease, MMSE≥19 Mean age = 73y	2.5 mg folic acid 0.4 mg B12 25 mg B6	12 weeks	No effects

2 AIMS

2.1 GENERAL AIMS

The general aim of this thesis was to investigate the relations of Hcy, vitamin B12, and folate with Alzheimer's disease, cognitive performance, and structural brain changes in older adults.

2.2 SPECIFIC AIMS

The specific aims addressed in four different studies are summarized below:

1. To examine the relations between serum levels of Hcy, holoTC, folate, and the risk of incident AD in a longitudinal population-based study of Finnish elderly (CAIDE study) *(study I).*

2. To study the associations of serum Hcy, holoTC, and folate with cognitive functioning in a longitudinal population-based study of Finnish elderly (CAIDE study) *(study II)*

3. To investigate possible links between baseline Hcy, and post-mortem neuropathological and MRI findings up to 10 years later in a longitudinal population-based study of Finnish elderly aged $\geq$ 85 years (Vantaa 85+ study)*(study III)*

4. To examine the associations of plasma vitamin B12 and red blood cell folate with cerebral volumes in a longitudinal population-based study of Swedish older adults (SNAC-K study) *(study IV)*

3 METHODS

The data used in this thesis are derived from three projects: The Cardiovascular Risk Factors, Aging, and Dementia (CAIDE) Study, The Vantaa 85+ Study, and the Swedish National Study on Aging and Care in Kungsholmen (SNAC-K).

3.1 CAIDE Study (Studies I and II)

3.1.1 Study population

The Cardiovascular Risk Factors, Aging, and Dementia (CAIDE) study was carried out in Eastern Finland. CAIDE is a large population-based study focusing on the effects of cardiovascular and lifestyle-related factors on dementia and cognitive functioning. Participants in this study were derived from four independent, randomly selected, population-based samples originally studied within the framework of the North Karelia Project and the Finnish part of Monitoring Trend and Determinants in Cardiovascular Disease (FINMONICA) study in 1972, 1977, 1982 and 1987[137-139]. These surveys were carried out to assess the risk factors, morbidity, and mortality attributable to cardiovascular diseases in two eastern provinces of Finland: North Karelia and Kuopio. In 1972 and 1977, a random sample of 6.6 % of the population born in 1913-1947 and living in North Karelia and Kuopio provinces was drawn. In 1982 and 1987, the sample included the age group 25-64 years and was stratified so that in both areas at least 250 subjects were chosen of each sex and in each 10-year age group. This procedure was used to comply with the international WHO MONICA project protocol[140]. The participation rates in these baseline surveys were high, ranging from 77 % to 96 %[139].

A random sample of 2000 survivors was invited to participate in the first re-examination of the CAIDE study in 1998[141]. Altogether 1449 (72.5 %) individuals aged 65 to 79 years at the end of 1997, and living in two geographically defined areas in or near the towns of Kuopio and Joensuu agreed to participate. A total of 1409 participants completed the cognitive assessments. A second re-examination of the CAIDE study took place during 2005-2008. Of the original 2000 persons, 1426 were still alive and living in the geographical area, and were invited to participate. 909 (63.7 %) individuals agreed to participate, and 852 completed the cognitive assessments. During both re-examinations, survey methods followed those applied in the midlife surveys in all respects, and the cognitive status of the participants was additionally evaluated.

The 274 subjects included in Study I and Study II were selected based on the availability of serum samples from 1998 for tHcy, holoTC, and folate measurements. The mean (standard deviation) duration of follow-up of this sub-sample of CAIDE participants was 7.4 (0.3) years. There were no clinically significant differences between the 274 CAIDE participants and the entire dementia-free CAIDE cohort. None of the participants reported using B-vitamins or other vitamin supplements. No mandatory folic acid fortification is performed in Finland.

3.1.2 Data collection

Midlife examination. The survey methods used in the midlife visits were carefully standardized to comply with international recommendations. They followed the WHO MONICA protocols in 1982 and 1987[140], and the methods used in 1972 and 1977 were comparable[141]. Briefly, the baseline surveys included a self-administered questionnaire on health behavior, health status, and medical history of the participants. The questionnaire was sent to the participants prior to the examination, and a study nurse specifically trained for the survey checked the questionnaires during the visit to ensure that they were fully completed. Participants' systolic (SBP) and diastolic blood pressure (DBP) were measured from the right arm after a rest period of five minutes in a seated position. Their height, and weight were measured, and BMI was calculated as weight in kilograms divided by the square of height in meters.

First re-examination (1998). Similar to previous surveys, this survey comprised a self administered questionnaire on sociodemographic characteristics, health-related behaviors, medications, and medical history, including cerebrovascular and cardiovascular events, and history of any renal diseases. Height, weight, and blood pressure were measured, and BMI was calculated. The cognitive status of the participants was additionally assessed. The screening phase was conducted at the Department of Public Health and General Practice, University of Kuopio, and at the North Karelia Project Office in Joensuu. The clinical and differential diagnostic phases were conducted at the Memory Research Clinic of the Department of Neurology, University of Kuopio, and at the North Karelia Central Hospital in Joensuu.

Second re-examination (2005-2008). Survey methods were similar to those used in the 1998 re-examination. All three study phases in Kuopio were conducted at the Brain Research Unit of the Clinical Research Center, University of Kuopio. In Joensuu, the screening phase was carried out at the North Karelia Project Office, and the clinical and differential diagnostic phases at the North Karelia Central Hospital.

3.1.3 Diagnostic procedures

Cognitive status of the participants was determined at both re-examinations with a three-step protocol including a screening phase, a clinical phase, and a differential diagnostic phase:

1- During the screening phase, the subject and an informant were interviewed and a trained study nurse carried out a preliminary cognitive testing. These tests included:
   - Mini-Mental State Examination (MMSE)[142] in the first re-examination and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropsychological test battery[143] in the second reexamination
   - Immediate word recall tests [144, 145] (one word list regarded as a measure of episodic memory)
   - Category fluency test (measure of verbal expression)[146]
   - Bimanual Purdue Peg Board test and letter-digit substitution test[147, 148]; the mean of their normalized scores was used as a measure of psychomotor speed
   - Stroop test[149]; the time difference between the color word interference and naming tasks was used as a measure of executive functioning

2- If the subject scored 24 or less in the MMSE, a further assessment was made in the clinical phase. Additional screening criteria used in the second re-examination to improve sensitivity to detect very mild dementia were: 1) decline in MMSE of three or more points since the first re-examination, 2) delayed recall word list test < 70 % in the Finnish version of CERAD test battery, or 3) report of cognitive decline by the informant. In the clinical phase, subjects went through a detailed physical examination carried out by a physician as well as thorough neuropsychological testing conducted by a neuropsychologist. A review board consisting of the study physician and neuropsychologist, a senior neurologist and a senior neuropsychologist carefully evaluated all the available information and ascertained the preliminary diagnoses. Individuals who were judged to have possible dementia were invited to the differential diagnosis phase.

3- The differential diagnostic phase included brain imaging (CT/MRI), relevant blood tests, chest radiograph, electrocardiogram, and CSF analysis if needed. All accumulated data from the screening and clinical phases were carefully re-evaluated by the review board before establishing the final diagnosis.

3.1.4 Diagnosis of dementia and Alzheimer's disease

The diagnosis of dementia was based on the DSM-IV criteria[1] and diagnoses of probable and possible AD were based on the NINCDS-ARDRA criteria[23]. Individuals diagnosed with AD displayed generalized and/or medial temporal lobe atrophy, and none had any significant vascular pathology on MRI. Isolated, minor lacunae or moderate white matter changes were not considered as exclusion criteria for AD. People with AD scored four or less on the Hachinski Ischemia Scale[150]. Consensus criteria were used to diagnose other dementia types as follows: the NINDS-AIREN criteria for VaD[151], consensus diagnostic criteria for frontotemporal dementia[152], consortium criteria for dementia with Lewy bodies[153], and consensus criteria for alcohol related dementia[154].

3.1.5 Biochemical analyses

Venous blood samples were taken at the 1998 re-examination and serum specimens were stored at or below -20º C until analysis at the National Institute for Health and Welfare in Helsinki. Total serum Hcy was determined by Chemiluminescent Microparticle Immunoassay (CMIA) and serum folate was determined by Chemiluminescent Microparticle Folate Binding Protein assay by Architect *i* System (Abbott Laboratories, Abbott Park, IL, USA). The inter-assay coefficients of variation (CV) of homocysteine were 5.9% and 5.4% at the levels of 6.6 µM/L and 11µM/L and for folate 13% and 11% at the levels of 7.5 and 31 nM/L, respectively. Holotranscobalamin was measured by Microparticle Enzyme Immunoassay (MEIA) by AxSym System (Active-B12 (Holotranscobalamin), Axis-Shield, Dundee, UK, Abbott Laboratories). At the levels of 48 and 97 pM/L, the inter-assay CV were 7.1% and 8.0%.

Blood leukocyte samples were analyzed to determine *APOE* genotype in 1998. To extract DNA, a standard phenol-chloroform technique was used; *APOE* genotypes were analyzed by PCR and *Hha*I digestion, as described previously[155]. Participants were classified as positive for the *APOE*ε4 allele genotype if they had one or two ε4 alleles.

3.2 The Vantaa 85+ Study (Study III)

3.2.1 Study population

The Vantaa 85+ study included 553 participants who were clinically examined at baseline between April 1, 1991 and March 12, 1992. These 553 subjects represented 92% of the 601 individuals aged ≥85 years in 1991, and living in Vantaa, a city in southern Finland[156, 157].

Survivors were re-examined in 1994, 1996, 1999, and 2001. The entire study population is now deceased. 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.

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

3.2.2 Data collection

Evaluation included an interview by a trained nurse using a questionnaire concerning health, health-related behavior, 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.

3.2.3 Neuropathology

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

3.2.3.1 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 [158]. Paraffin sections were cut at a thickness of 8 µm for staining with methenamine silver for β-amyloid[159] and 10 µm for staining with modified Bielschowsky method[160] for neuritic plaques and NFTs. Methenamine silver staining has been shown to be as sensitive as β-amyloid immunostaining for detecting senile plaques, including diffuse plaques[159, 161]. To estimate the amount of β-amyloid 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[156]. Briefly, the contiguous cortical microscopic fields extending from the pial surface to the white matter were examined using a 10x objective under 10x 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 β-amyloid deposition in different brain regions. The final value (percentage) provided an estimate of the extent of β-amyloid deposition in the cerebral cortex.

After Bielschowsky silver staining, NFT in sections cut from the four specimens was counted[162]. In each specimen, 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 X 0.5 mm and a 20x objective under 10x ocular field. The average NFT number was determined by dividing the total NFT number in all four cortical sections by four. The CERAD scores and Braak stages were defined as originally described[13, 158].

3.2.3.2 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[163].

3.2.3.3 Cortical microinfarcts

Microinfarcts were analyzed 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[164]. Lesions located close to larger infarcts were considered to represent the border zone of the larger lesion, and were not counted as true microinfarcts.

3.2.3.4 Cerebral amyloid angiopathy (CAA)

CAA was analyzed in six brain regions (listed above)[165]. CAA diagnosis was based on Congo red staining and confirmed using immunohistochemistry against β-amyloid peptide (clone 4G8, detecting amino acids 17→24). The percentage of blood vessels with CAA was analyzed 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.

3.2.3.5 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[166]. 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 (DLB) consensus guidelines[153]. The type of α-synuclein pathology was determined for every subject[166]. 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 DLB do not recognize this entity.

3.2.4 Post-mortem MRI 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 pathologic 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 MR slices was defined parallel to the markers. Transverse images were obtained perpendicular to the coronal plane[163, 167]. Prior to scanning, specimens were rinsed in for at least 15 minutes and subsequently placed in 0.1 mM $MnCl_2$ solution and imaged with a 1.5 T Vision system (Siemens AG, Erlangen, Germany). The degree of medial temporal lobe atrophy (MTA) was determined using a five step (score 0-4) well-established

visual rating scale[168], by a highly trained observer blinded to clinical and neuropathological findings. The Scheltens scale[169] was used to visually rate the severity of white matter hyperintensities (WMH)[163].

3.2.5 Biochemical assessments

Non-fasting blood samples taken in 1991 were stored at -20ºC for 8 years, and Hcy levels were analyzed at the Department of Clinical Chemistry, Helsinki University Central Hospital by fluorescence polarization immunoassay using Abbott AxSYM analyser (Abbott Laboratories Inc, Abbott Park, IL). DNA mini-sequencing and DNA amplification with PCR followed by restriction enzyme digestion with *Hha*I, were independently used in two laboratories (Department of Medicine, University of Helsinki, and Mayo Clinic, Jackonsville, FL) with identical results[156, 157, 170].

3.3 The SNAC-K study (study IV)

The study population was derived from the Swedish National study on Aging and Care in Kungsholmen (SNAC-K), a population-based prospective study conducted in the Kungsholmen area of central Stockholm, Sweden[171, 172]. SNAC-K involves a random sample of persons aged ≥ 60 years who live either at home or in institutions. The sampling was stratified by age cohort[171]. In total, 11 age-specific cohorts were identified (60, 66, 72, 78 years, 81, 84, 87, 90, 93, 96 and 99+ years). Each age cohort was re-examined when it reached the age of the next cohort, resulting in a 6-year assessment interval for the younger cohorts and a 3-year assessment interval for the older cohorts. In 2001–2004, of the 4590 alive and eligible subjects randomly selected for SNAC-K, 3363 (73.3%) participated at the baseline examination.

3.3.1 Data collection

At baseline and each follow-up the SNAC-K participants underwent a thorough clinical examination, interview and assessments by a physician, a registered nurse and a psychologist. Data on socio-demographics characteristics, medical history, drug use and cognitive functions were collected according to a structured protocol (available at http://www.snac.org). Cognitive

functions were evaluated by an extensive cognitive test battery[172], and diagnosis of dementia was made according to the DSM–IV criteria[1] using a three-step procedure[172].

Diagnoses of chronic diseases were made by the examining physicians based on the clinical examination, medical history and laboratory data[173]. Presence of multimorbidity, defined as any co-occurrence of 2+ chronic diseases in the same individual, was evaluated according to a procedure fully described elsewhere[174]. Data on vitamin supplements use were collected from the subjects and verified by inspecting drug prescriptions and containers. Vitamin supplements were coded following the Anatomical Therapeutic and Chemical (ATC) classification system.

3.3.1.1 Brain imaging cohort

At baseline, 2204 persons randomly selected from the non-institutionalized, non-disabled, and non-demented participants in SNAC-K were invited to undertake a structural MRI scan. During September 2001-October 2003, 555 persons (25.2% of the total invited; 542 subjects with available plasma B12 and 543 with available RBC folate measurements) agreed to undergo the MRI scan. Compared to the whole SNAC-K cohort, the MRI sub-sample was on average younger (age, mean and standard deviation (SD): 71.3 (9.1) versus 74.6 (11.8) years, $p < 0.0001$), healthier (number of chronic diseases, mean (SD): 1.5 (1.4) versus 1.9 (1.5), $p < 0.0001$), had better MMSE total score (mean (SD): 29.0 (1.2) versus 27.1 (5.7), $p < 0.0001$) and more years of education (mean (SD): 12.6 (4.4) versus 11.7 (4.2) years, $p < 0.0001$).

Participants with poor MRI quality (n = 16), possible dementia (n = 3), Parkinson's disease (n = 4), bipolar disorder (n = 2) or major depressive disorder (n = 1), or MRI evidence of brain infarctions (n = 13) or arachnoid cysts (n = 3) or brain tumors (n = 1) were excluded from the present study, which thus included 501 subjects without dementia. Brain MRI scans were performed at baseline, and thereafter 3 years later for the older cohorts (i.e., those who were ≥78y at baseline, n = 93) and 6 years later for the whole cohort (n = 264). Therefore, 3 brain MRI scans were available for subjects ≥ 78 y at baseline and 2 brain MRIscans were performed for subjects aged < 78y at baseline.

3.3.2 Brain imaging methods

T1-weighted MRI images were acquired using the 3D FFE (fast field echo) sequence on a 1.5T (Philips Intera, Netherlands) for volumetric assessment. The acquisition parameters were: repetition time = 15 ms, echo time = 7 ms, Flip angle = 15°, Number of slices (axial) = 128,

Slice thickness = 1.5 mm, in-plane resolution = 0.9375 × 0.9375 mm, no gap, field of view = 240 × 240, Matrix = 196 × 256.

Preprocessing of the T1-weighted images was performed in SPM8 (Statistical Parametric Mapping, http://www.fil.ion.ucl.ac.uk/spm/, Wellcome Trust Centre for Neuroimaging, FIL, London, UK), implemented in Matlab 10 (The Mathworks Inc., MA, US). Segmentation of the T1 images into gray matter (GM), white matter (WM) and cerebrospinal fluid (CSF) was performed using the unified segmentation approach[175]. The "light cleanup" option was used to further remove odd voxels from the segments. Total brain tissue volume (TBT) was obtained by adding GM and WM volumes. Total intracranial volume (TIV) was finally calculated by adding the volumes of GM, WM and CSF. All segments were carefully checked. All volumetric measures from each subject were normalized by the subject's TIV.

3.3.3 Biochemical assessments

At baseline, venous blood samples were taken (fasting was not compulsory) and analyses were done within 2 hours at Sabbatsberg Hospital in Stockholm. Plasma levels of vitamin B12 were measured using a radioimmunoassay. RBC folate was assessed using chemiluminescence microparticle folate binding protein assay after mixing whole blood with ascorbic acid to hemolyze RBCs and stabilize released folate. Hemoglobin (Hb) and serum albumin were measured following standard procedures.

3.4 Statistical analyses

Data analyses were conducted with the Statistical Package for the Social Sciences, version 17.0 for Windows (SPSS Inc., Chicago, IL, USA) or the Stata version 9.0 or 12.0 for Windows (Stata-Corp. LP, TX, USA). The level of significance was < 0.05 in all analyses.

3.4.1. Specific analyses for each study

Study I: Differences between the AD and non-demented groups were assessed using binary logistic regression with diagnosis as the dependent variable. Results were presented as mean (standard deviation (SD)) for continuous variables or number (percentage) for categorical variables. Hcy and holoTC concentrations were further categorized using the corresponding median values: low tHcy was defined as serum Hcy concentrations $\leq$ 12.3 μmol/L, and low

holoTC was defined as serum holoTC concentrations ≤ 83.3 pmol/L and individuals were compared according to Hcy or holoTC categories defined by the median.

The associations between Hcy, holoTC, folate (as continuous variables) and the subsequent development of AD were examined using multiple logistic regression analyses. The results are presented as odds ratios (ORs) with 95% confidence intervals (CI). Analyses were adjusted for baseline age, sex, years of full-time education, and follow-up time (model 1), and then additionally for other potential confounding or mediating factors, including *APOE*ε4 status, BMI, SBP and DBP, MMSE score, history of stroke, and smoking (model 2). All variables were entered as continuous into the models except sex, *APOE*ε4, history of stroke, and smoking which were dichotomized. As creatinine values were not available, additional analyses were adjusted for presence of renal conditions (yes/no) during the study. None of the participants reported using B vitamin or other vitamin supplementations at baseline or follow-up.

Additional analyses were also conducted to investigate the effects of holoTC or folate on the relation between Hcy and AD, as well as the effects of Hcy or folate on the relation between holoTC and AD. Interaction terms were entered in the models in order to investigate possible interactions between Hcy, holoTC and folate in relation to AD risk, as well as Hcy-*APOE*, Hcy-sex, Hcy-age, holoTC-*APOE*, and holoTC-sex, holoTC-age interactions.

Study II: Because of skewness, cognitive test scores and all continuous variables were log transformed. Multiple linear regression analyses were done to investigate the associations of Hcy, holoTC, and folate at first re-examination with cognitive test scores at the second re-examination 7 years later. Each of the primary predictors was analyzed as continuous and quartile categories (with the lowest quartile as the reference category: ≤ 10.4 μmol/L for Hcy, ≤ 57.8 pmol/L for holoTC, and ≤ 4.9 nmol/L for folate). As in study I, models were adjusted for potential confounding or mediating factors known to influence cognition, including age, sex, education level, duration of follow-up, SBP, BMI, history of smoking, stroke, *APOE*ε4 allele, presence of renal conditions (as creatinine values were not available) (model 1), and cognitive measures in 1998 (model 2). Because folate and holoTC are intrinsically related to Hcy and are often correlated with each other, we examined their associations with the cognitive outcomes when adjusted for each other in a final set of models (model 3). All variables were entered as continuous into the models except for sex, history of stroke, smoking, *APOE*ε4 allele status, and presence of renal conditions which were dichotomized. Additional analyses were carried out to investigate the associations of holoTC and folate with cognitive test scores after excluding subjects with low levels of holoTC (< 35 pmol/L, n = 21) or folate (< 5.3 nmol/L, n = 97),

according to the laboratory cut-off values. All analyses were also repeated after excluding 20 subjects who developed dementia at follow-up. Results were presented as relative differences (RD) with 95% confidence intervals (CI) where numbers higher than 1.00 indicate better performance, and numbers lower than 1.00 indicate worse performance.

Study III: Individuals were compared according to Hcy levels below or above the proposed cut-off of 20 μmol/L in elderly > 65 years without folic acid supplementation[66]. 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. Spearman's rank-order correlations were assessed between dementia, and neuropathological and MRI variables.

Hcy and neuropathology. Hcy values were categorized into quartiles (calculated for the 265 subjects in the autopsy population) and the anticipated low-risk category (i.e. the lowest Hcy quartile (Q): ≤ 15.5 μmol/L) was set as reference. The tangle count, β-amyloid load and CAA were not normally distributed, and were categorized in three groups: 1) no NFT, β-amyloid load or CAA (reference group); 2) tangle count, β-amyloid load or CAA below median value (calculated for participants who had these pathologies); and 3) tangle count, β-amyloid load or CAA above the median. Because the tangle count, β-amyloid load, CAA, Braak and CERAD categories are ordered according to severity, ordinal logistic regressions were used to assess the associations of Hcy 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)[163]. The presence of α-synuclein pathology was treated as a dichotomous outcome (yes/no). Associations between Hcy, 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 plasma Hcy and

Alzheimer-related neuropathological outcomes. Interaction terms were entered in the models in order to investigate possible interactions between Hcy, age, gender, and *APOE* in relation to the outcomes.

Hcy, WMH and MTA on post-mortem MRI. Hcy 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 Hcy quartile: Q1 ≤ 16. 1 μmol/L) was set as reference. Because for some of the WMH or MTA scores the number of subjects was small, re-categorization was done to create categories of comparable size for each MRI outcome variable. Periventricular WMH (sum for caps and bands) were categorized as mild (score 0 - 3, *n* = 48 subjects), and more severe (score 4 - 6, *n* = 55). Deep WMH (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). MTA (highest of the left or right MTA 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 WMH, MTA and Hcy.

All analyses in the autopsy and post-mortem MRI populations were adjusted for age at death, gender, *APOE*ε4 status, and follow-up duration (model 1), and additionally for cardiovascular conditions, living in institutions, and use of B-vitamin supplements (model 2). Results were shown as odds ratios with 95% confidence intervals.

Study IV: Linear mixed models for repeated measures were used to estimate β and standard error (SE) for the association of plasma B12 and RBC folate levels with repeated measures of normalized brain volumes over 6 years. Models were adjusted for potential confounding or mediating factors known to influence cognition and B12 and folate levels, including age and education (both given continuous representation), sex, the use of vitamin supplements (yes/no) (model 1), and then additionally for hemoglobin and albumin concentrations (both given continuous representation) and multimorbidity (categorized as absence of chronic diseases, presence of < 2 or 2+ chronic diseases) (model 2). The interaction between time and each covariate was also added in all models. Because B12 and folate are intrinsically correlated with each other, we examined their associations with the outcomes while adjusting for each other in model 2.

In the linear mixed models, the β coefficient for B12 or RBC folate represents the cross-sectional association between the vitamin and baseline brain volume. The term for time

indicates the change per year on brain volume. The β coefficient for the vitamin B12 or RBC folate × time interaction represents the effect of B12 or folate on the rate of change in brain volume over time. A positive β coefficient indicates that an increase in B12 or RBC folate level was associated with a decreased rate of brain volume loss over time.

Additional analyses were carried out to investigate the association of plasma B12 and RBC folate levels with the rate of brain atrophy after excluding subjects with low levels of B12 (< 180 pmol/L, n = 8) and RBC folate (< 125 nmol/L, n = 17), according to the laboratory cut-off values. All analyses were also repeated after excluding 28 subjects who developed dementia during follow-up (diagnosed according to the DSM-IV criteria).

4 ETHICAL CONSIDERATIONS

4.1 The CAIDE Study

For the CAIDE Study, written informed consent was obtained from all participants. All phases of the CAIDE project received approval from the local ethics committees (University of Kuopio and Kuopio University Hospital).

- First re-examination: Dnr. 24/97
- Second re-examination: Dnr. 124/2004
- The Ethics Committee at the Karolinska Institute also approved the project (Dnr. 2004/2:3).

4.2 The Vantaa 85+ Study

The Vantaa 85+ study was approved by the Ethics Committee of the Health Centre of the city of Vantaa (Dnr 1/91, 29.05.1991). 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.

4.3 The SNAC-K Study

For the SNAC-K study, the Ethics Committee at Karolinska Institute and the Regional Ethical Review Board in Stockholm, Sweden, approved the protocols of each phase of the SNAC-K project, and informed consent was collected from all participants.

- Baseline: Dnr. KI 01-114
- First follow-up: Dnr. 04-929/3
- Second follow-up: Dnr. Ö26-2007

5 RESULTS

5.1 Characteristics of the CAIDE study population

The baseline sociodemographic and clinical characteristics of the study population are presented in table 6. As expected, Hcy was inversely correlated with holoTC (Pearson correlation coefficient = -0.46; $p < 0.001$) and with folate ($r = -0.42$; $p < 0.001$), indicating that elevated Hcy was a marker of low status of both vitamin B12 and folate. In addition, holoTC was positively correlated with folate ($r = 0.14$; $p = 0.02$).

Table 6. Baseline characteristics of the study population

Characteristic	All (n = 274)	No dementia (n = 254)	AD (n = 17)	P- value
Age, y	70.7 (3.6)	70.5 (3.5)	73.4 (4.3)	**0.003**
Sex, n (%) women	169 (61.7)	155 (61.0)	13 (76.5)	0.213
Education, y	9.2 (3.4)	9.1 (3.2)	9.4 (4.9)	0.73
MMSE	26.3 (2.0)	26.3 (2.0)	25.8 (2.1)	0.24
BMI, kg/m²	27.9 (4.0)	28.0 (4.0)	25.3 (3.4)	**0.005**
Systolic blood pressure, mmHg	152.6 (21.6)	153.3 (21.5)	142.2 (22.9)	**0.042**
Diastolic blood pressure, mmHg	82.3 (10.1)	82.8 (10.0)	77.2 (9.7)	**0.03**
***APOE* ε4 allele, n (%)**	92 (33.6)	82 (32.3)	10 (58.8)	**0.032**
History of stroke, n (%)	19 (6.9)	15 (5.9)	1 (5.9)	0.997
Ever smoked, n (%)	96 (35)	90 (35.4)	4 (23.5)	0.324
Hcy, µmol/L	12.7 (3.4)	12.6 (3.1)	14.9 (5.9)	**0.01**
HoloTC, ρmol/L	91.3 (50.7)	93.3 (51.6)	61.6 (27.4)	**0.01**
Folate, nmol/L	7.2 (3.8)	7.1 (3.9)	7.9 (3.4)	0.39
Follow-up, y	7.4 (0.3)	7.4 (0.3)	7.5 (0.4)	0.142

MMSE: Mini-Mental State Examination,BMI: Body Mass Index, APOE: Apolipoprotein E, Hcy: Homocysteine, holoTC: holotranscobalamin.

After 7.4 years of follow-up, 20 subjects (7.3 %) were diagnosed with dementia (17 AD, 2 VaD and 1 with AD/VaD) and 254 individuals represented the control group. The mean (SD) age of the whole sample was 70.7 (3.6) years and 61.7% were female.

As expected, people who developed AD were older at baseline and had lower BMI, lower BP, and higher frequency of *APOE*ε4 allele. They also had higher levels of Hcy and lower levels of holoTC compared to subjects without dementia.

Comparing participants based on the median Hcy levels (12.3 μmol/L) revealed that those with higher Hcy values were older (71.2 (3.9) vs 70.2 (3.4) years, p=0.027), less likely to be female (46.6% vs 73.5%, p <0.001), and had lower levels of holoTC (72.4 (36.6) vs 105.5 (55.8) pmol/L, p < 0.001) and folate (5.8 (2.9) vs 8.2 (4.2) nmol/L, p <0.001). No significant differences were observed for other clinical characteristics. However, when the cutoff was set at the 80th percentile of Hcy (15 μmol/L), more people with AD tended to belong to high Hcy group (12% vs 5%, p=0.064).

Individuals were further compared according to median holoTC (83.3 pmol/L) values; those with higher levels were younger (70.0 (3.3) vs 71.4 (3.9), p = 0.002) years) and had lower Hcy (11.6 (3.0) vs 13.8 (3.5) μmol/L, p < 0.001) and higher folate (7.7 (3.8) vs 6.7 (3.9) nmol/L, p = 0.047) values than those with lower holoTC levels. In addition, more AD people tended to belong to the low holoTC group (9% vs 4%, p = 0.077).

5.1.1 The associations of tHcy, holoTC, and folate with the risk of developing AD (study I)

The relationships between Hcy, holoTC, folate and AD risk are presented in table 6. The OR for AD for each increase of 1 μmol/L in serum Hcy values was 1.16 (95% CI 1.04-1.31). This association remained after adjusting for age, sex, education, and follow-up time (model 1). Furthermore, adjusting for *APOE*ε4, BMI, MMSE, SBP, DBP, history of stroke, smoking, and the presence of renal conditions did not influence the relationship. When analyses were stratified according to median holoTC, the association between Hcy and AD remained only in individuals who had holoTC below median values (OR = 1.19 (1.04-1.37), p = 0.014).

As presented in table 7, serum holoTC values were related to decreased risk of AD; OR was 0.980 (95% CI 0.965–0.995) for each increase of 1 pmol/L in baseline serum holoTC. This association remained after adjusting for all study covariates. No significant relation between folate and AD was detected.

Table 7. Serum Hcy, holoTC, folate, and OR (95% CI) for incident AD

Variable	Model 1	Model 2
Hcy	1.18 (1.02 – 1.36)	1.19 (1.01 – 1.39)
HoloTC	0.980 (0.963 – 0.997)	0.977 (0.957 – 0.997)
Folate	1.03 (0.91 - 1.16)	1.01 (0.87 – 1.18)

Model 1: adjusted for age, sex, education, and duration of follow-up.
Model 2: additionally adjusted for *APOE* ε4 allele, BMI, MMSE score, SBP, DBP, smoking, history of stroke, and presence of renal conditions.

Because folate and holoTC are intrinsically related to Hcy and are often correlated with each other, we ran additional analyses examining their associations with incident AD when adjusting for each other (table 8). The relation between Hcy and risk of AD was slightly attenuated by adjusting for holoTC: OR changed from 1.16 to 1.10 (0.95% CI 0.96 – 1.25). On the contrary, the association between holoTC and AD risk was less influenced by controlling for Hcy: OR for holoTC changed from 0.980 to 0.984 (0.95% CI 0.968 – 1.000). Adding folate to the models did not change the association between Hcy and AD or holoTC and AD.

Table 8. OR (95% CI) for the combined association of serum Hcy, holoTC, and folate with incident AD

	Crude model	Adjusted for			
		Hcy	**HoloTC**	**Folate**	**Both**
Hcy	1.16 (1.04-1.31)	-	1.10 (0.96-1.25)	1.17 (1.04-1.31)	1.10 (0.97–1.26)
HoloTC	0.98 (0.97-0.995)	0.98 (0.97-1.00)	-	0.98 (0.97-0.996)	0.985 (0.97-1.00)
Folate	1.05 (0.94 – 1.17)	1.07 (0.96-1.19)	1.06 (0.95-1.18)	-	1.06 (0.95-1.18)

No evidence of interaction was detected between Hcy, holoTC, or folate in relation to AD risk when interaction terms were entered into the models. In addition, no significant interactions were found between Hcy and *APOE*, Hcy and sex, Hcy and age, holoTC and *APOE*, and holoTC and sex in relation to the risk of AD. However, a significant interaction between holoTC and age was observed; the protective effect of holoTC became more pronounced with increasing age (adjusted OR for the interaction 0.994, 95% CI 0.989 – 0.998).

5.1.2 The associations of Hcy, holoTC, and folate with cognitive functioning (study II)

The relations between Hcy, holoTC, and folate (continuous or categorical variables) and cognitive performance 7 years later are presented in table 9. After adjusting for age, sex, education level, follow-up time, *APOE* ε4 status, BMI, SBP, history of stroke, smoking status and presence of renal conditions (model 1), higher concentrations of Hcy were significantly associated with lower performance in global cognition, episodic memory, executive functioning, verbal expression and psychomotor speed. Additional adjustment for baseline cognitive measures (model 2), attenuated the association between higher baseline Hcy and psychomotor speed. After controlling for holoTC and folate (model 3), the relationship between Hcy and MMSE was also no longer significant.

Higher baseline holoTC was significantly associated with better performance in global cognition, executive functioning and psychomotor speed, and approached significance in relation to verbal expression ($P < 0.1$) 7 years later, after taking into account baseline study covariates (table 8, model 2). In this model, the association between holoTC as a continuous variable and global cognition or verbal expression became stronger when excluding subjects with holoTC below the cut-off value of 35 pmol/L: RD changed from 1.07 to 1.11 (95% CI 1.01–1.21) for global cognition and from 1.05 to 1.08 (95% CI 1.00–1.18) for verbal expression. Including Hcy and folate into the models (model 3) resulted in associations that were no longer significant, although the size and direction of the associations did not change substantially. No significant relations between folate and any of the cognitive domains were detected, even after restricting the analyses to participants with serum folate levels higher than the cut-off value of 5.3 nmol/L.

Table 9. 7-year relative differences (RDs) and 95% confidence intervals (CIs) for the associations between Hcy, holoTC, folate and cognitive functions[1]

		Q1	Q2	Q3	Q4	Continuous
Global cognition (*n*=274)						
Hcy	Model 1	(Ref)	1.00 (0.91–1.09)	1.01 (0.91–1.11)	0.93 (0.84–1.02)	0.89 (0.80–1.00)*
	Model 2	(Ref)	1.00 (0.92–1.09)	1.00 (0.91–1.09)	0.92 (0.84–1.01)†	0.90 (0.81–0.99)*
	Model 3[2]	(Ref)	1.01 (0.93–1.10)	1.01 (0.92–1.11)	0.94 (0.85–1.05)	0.92 (0.81–1.03)
HoloTC	Model 1	(Ref)	1.03 (0.94–1.12)	1.06 (0.97–1.16)	1.10 (1.00–1.20)†	1.08 (1.00–1.17)†
	Model 2	(Ref)	1.03 (0.94–1.12)	1.06 (0.98–1.15)	1.09 (1.00–1.19)*	1.07 (0.99–1.15)†
	Model 3[3]	(Ref)	1.01 (0.93–1.10)	1.04 (0.96–1.14)	1.07 (0.97–1.17)	1.04 (0.96–1.13)
Folate	Model 1	(Ref)	1.09 (0.99–1.19)†	1.05 (0.96–1.15)	1.05 (0.95–1.15)	1.02 (0.96–1.08)
	Model 2	(Ref)	1.08 (0.99–1.17)†	1.08 (0.99–1.17)†	1.04 (0.96–1.14)	1.02 (0.97–1.09)
	Model 3[4]	(Ref)	1.07 (0.98–1.16)	1.07 (0.98–1.16)	1.00 (0.92–1.10)	1.00 (0.94–1.07)
Episodic memory (*n*=274)						
Hcy	Model 1	(Ref)	0.94 (0.84–1.05)	0.94 (0.84–1.06)	0.85 (0.75–0.95)*	0.82 (0.72– 0.93)*
	Model 2	(Ref)	0.95 (0.85–1.05)	0.93 (0.84–1.04)	0.89 (0.80–1.00)*	0.87 (0.77–0.99)*
	Model 3[2]	(Ref)	0.93 (0.83–1.03)	0.91 (0.81–1.02)	0.86 (0.76–0.98)*	0.83 (0.71–0.96)*
HoloTC	Model 1	(Ref)	1.09 (0.97–1.22)	1.02 (0.91–1.14)	1.10 (0.98–1.23)	1.04 (0.94–1.15)
	Model 2	(Ref)	1.11 (1.00–1.23)†	1.02 (0.92–1.13)	1.05 (0.94–1.17)	1.01 (0.92–1.11)
	Model 3[3]	(Ref)	1.08 (0.98–1.20)	0.99 (0.89–1.10)	1.01 (0.90–1.13)	0.96 (0.87–1.06)
Folate	Model 1	(Ref)	0.96 (0.85–1.07)	0.99 (0.89–1.11)	1.01 (0.90–1.13)	1.02 (0.94–1.10)
	Model 2	(Ref)	0.94 (0.84–1.04)	0.99 (0.89–1.09)	0.97 (0.87–1.08)	0.99 (0.92–1.07)
	Model 3[4]	(Ref)	0.92 (0.83–1.03)	0.96 (0.87–1.07)	0.92 (0.83–1.04)	0.96 (0.88–1.03)
Executive functions (*n*=260)						
Hcy	Model 1	(Ref)	0.90 (0.80–1.01)†	0.84 (0.74–0.95)*	0.86 (0.77–0.97)*	0.81 (0.70–0.93)*
	Model 2	(Ref)	0.87 (0.79–0.97)*	0.86 (0.77–0.96)*	0.89 (0.80–1.00)†	0.86 (0.75–0.98)*
	Model 3[2]	(Ref)	0.89 (0.79–0.99)*	0.87 (0.77–0.98)*	0.92 (0.81–1.05)	0.88 (0.75–1.03)
HoloTC	Model 1	(Ref)	1.08 (0.96–1.21)	1.09 (0.97–1.22)	1.10 (0.98–1.24)	1.13 (1.02–1.25)*
	Model 2	(Ref)	1.02 (0.92–1.14)	1.06 (0.95–1.18)	1.08 (0.96–1.20)	1.11 (1.01–1.21)*
	Model 3[3]	(Ref)	1.00 (0.90–1.12)	1.03 (0.92–1.15)	1.04 (0.92–1.16)	1.07 (0.96–1.18)
Folate	Model 1	(Ref)	1.02 (0.91–1.14)	1.10 (0.98–1.23)	1.06 (0.94–1.17)	1.02 (0.94–1.10)
	Model 2	(Ref)	1.00 (0.90–1.12)	1.04 (0.94–1.16)	1.06 (0.95–1.19)	1.01 (0.94–1.09)
	Model 3[4]	(Ref)	0.99 (0.89–1.11)	1.03 (0.92–1.14)	1.02 (0.91–1.14)	0.98 (0.91–1.06)
Verbal expression (*n*=273)						
Hcy	Model 1	(Ref)	0.94 (0.86–1.04)	0.99 (0.90–1.10)	0.89 (0.80–0.98)*	0.86 (0.78–0.97)*
	Model 2	(Ref)	0.92 (0.85–1.00)*	0.97 (0.89–1.06)	0.89 (0.82–0.97)*	0.89 (0.81–0.97)*
	Model 3[2]	(Ref)	0.93 (0.86–1.01)†	0.98 (0.90–1.07)	0.91 (0.83–1.00)*	0.90 (0.81–1.01)†

HoloTC	Model 1	(Ref)	1.04 (0.95–1.15)	1.08 (0.98–1.19)	1.10 (1.00–1.22)†	1.06 (0.98–1.16)
	Model 2	(Ref)	1.04 (0.96–1.12)	1.04 (0.96–1.13)	1.08 (0.99–1.17)†	1.05 (0.98–1.13)
	Model 3[3]	(Ref)	1.03 (0.95–1.11)	1.02 (0.94–1.11)	1.04 (0.96–1.14)	1.02 (0.94–1.10)
Folate	Model 1	(Ref)	1.09 (0.99–1.20)†	1.03 (0.94–1.14)	1.08 (0.98–1.20)	1.02 (0.95–1.09)
	Model 2	(Ref)	1.04 (0.97–1.13)	1.04 (0.96–1.12)	1.06 (0.98–1.15)	1.04 (0.98–1.09)
	Model 3[4]	(Ref)	1.04 (0.96–1.12)	1.02 (0.95–1.11)	1.03 (0.94–1.12)	1.01 (0.95–1.07)
Psychomotor speed (*n*=260)						
Hcy	Model 1	(Ref)	0.87 (0.78– 0.99)*	0.86 (0.75–0.99)*	0.80 (0.70–0.92)*	0.72 (0.62–0.84)*
	Model 2	(Ref)	0.94 (0.85–1.05)	0.94 (0.84–1.05)	0.95 (0.85–1.07)	0.91 (0.80–1.04)
	Model 3[2]	(Ref)	0.95 (0.85–1.06)	0.95 (0.84–1.07)	0.97 (0.85–1.10)	0.92 (0.78–1.08)
HoloTC	Model 1	(Ref)	1.21 (1.06–1.39)*	1.19 (1.04–1.36)*	1.28 (1.12–1.47)*	1.18 (1.05–1.03)*
	Model 2	(Ref)	1.11 (0.99–1.23)†	1.04 (0.93–1.15)	1.13 (1.01–1.26)*	1.08 (0.98–1.18)
	Model 3[3]	(Ref)	1.10 (0.99–1.23)†	1.03 (0.92–1.14)	1.11 (0.98–1.24)†	1.05 (0.95–1.17)
Folate	Model 1	(Ref)	1.07 (0.93–1.22)	1.03 (0.90–1.13)	1.06 (0.92–1.22)	1.03 (0.94–1.14)
	Model 2	(Ref)	1.04 (0.93–1.15)	1.02 (0.91–1.13)	0.97 (0.87–1.08)	0.99 (0.92–1.06)
	Model 3[4]	(Ref)	1.03 (0.92–1.14)	1.00 (0.90–1.12)	0.93 (0.83–1.05)	0.96 (0.89–1.04)

[1]Final cognitive scores regressed onto baseline serum measures.
Results are relative differences (Ref indicates reference groups, Q indicates quartiles). In cognitive tests, results greater than 1.0 indicate better performance and results less than 1.0 indicate worse performance.
Model 1: adjusted for age, sex, education, follow-up time, SBP, BMI, *APOE*-ε4, stroke, smoking and presence of renal conditions.
Model 2: additionally adjusted for baseline cognitive measures.
Model 3: additionally adjusted for holoTC and folate[2], Hcy and folate[3], or Hcy and holoTC[4]
Hcy concentration was ≤10.4 μmol/L for Q1, 10.5–12.3 μmol/L for Q2, 12.4–14.4 μmol/L for Q3 and ≥14.5 μmol/L for Q4.
HoloTC concentration was ≤57.8 pmol/L for Q1, 57.9–83.2 pmol/L for Q2, 83.3–114.7 pmol/L for Q3 and ≤114.8 pmol/L for Q4.
Folate concentration was ≤4.9 nmol/L for Q1, 5.0–6.3 nmol/L for Q2, 6.4–8.4 nmol/L for Q3 and ≥8.5 nmol/L for Q4.
*P<0.05; †P<0.1.

5.1.3 The associations of Hcy, holoTC, and folate with cognition in non-demented elderly

Analyses were repeated excluding 20 individuals with incident dementia at follow-up (table 10). These subjects had lower scores on episodic memory (4.9 (1.4) vs. 5.6 (1.5)), executive functioning (52.6 (18.4) vs. 37.6 (18.0)) and psychomotor speed (-0.46 (1.03) vs. 0.06 (0.80)) at baseline (move up to study I or say table 9). After controlling for all study covariates (model 2), increasing Hcy concentrations were significantly associated with worse performance in episodic memory, executive functioning and verbal expression, but not global cognition 7 years later: RDs for Hcy as a continuous variable were 0.87 (95% CI 0.76 – 0.99) for episodic memory, 0.88 (95% CI 0.78–0.99) for executive functioning and 0.91 (95% CI 0.83 – 1.00) for verbal expression. The relationship between Hcy and episodic memory remained unchanged even after including all three biomarkers into the model (model 3). In non-demented subjects, elevated holoTC concentrations were significantly related to better performance in executive functioning (holoTC as a continuous variable: 1.10 (1.00 – 1.21)) and psychomotor speed (highest holoTC quartile: 1.22 (1.08 – 1.39)), but not global cognition or verbal expression. These estimates changed slightly after controlling for other study covariates ($P < 0.10$). The associations of holoTC as a continuous variable with global cognition or executive functioning became slightly stronger after restricting the analyses to those with serum holoTC levels higher than the cut-off value of 35 pmol/L: RD changed from 1.05 to 1.08 (95% CI 0.99 – 1.17) for global cognition and from 1.09 to 1.11 (95% CI 1.01 – 1.24) for executive functioning. Additional adjustment for Hcy and folate attenuated the results, although the direction of associations remained unchanged.

Compared to the first quartile, the second and third quartiles of baseline folate were significantly associated with higher MMSE scores: RD was 1.12 (95% CI 1.03 – 1.21) for the second quartile and 1.09 (95% CI 1.01 – 1.18) for the third quartile; whilst the second and fourth quartiles were related to better performance in verbal expression: RD was 1.08 (95% CI 1.01 – 1.16) for the second quartile and 1.08 (95% CI 1.01 – 1.17) for the fourth quartile, after controlling for baseline study covariates. The association between the second folate quartile and MMSE remained significant even when Hcy and holoTC were incorporated into the models simultaneously: 1.11 (1.03 – 1.20). No relationship between folate and cognitive performance was found after excluding subjects with low folate levels.

Table 10. 7-year relative differences (RD) and 95% confidence interval (CI) for the associations between Hcy, holoTC, folate and cognitive functions in subjects without dementia[1]

		Q1	Q2	Q3	Q4	Continuous
Global cognition (N = 254)						
Hcy	Model 1	(Ref)	1.01 (0.93 - 1.10)	1.03 (0.94 - 1.13)	0.95 (0.87 - 1.04)	0.94 (0.84 - 1.04)
	Model 2	(Ref)	1.01 (0.93 - 1.09)	1.02 (0.94 - 1.11)	0.94 (0.87 - 1.02)	0.93 (0.85 - 1.03)
	Model 3[2]	(Ref)	1.02 (0.94 – 1.11)	1.04 (0.95 - 1.13)	0.97 (0.88 - 1.07)	0.97 (0.86 - 1.09)
HoloTC	Model 1	(Ref)	1.00 (0.92 - 1.09)	1.04 (0.96 - 1.14)	1.06 (0.97 - 1.15)	1.06 (0.98 - 1.14)
	Model 2	(Ref)	1.00 (0.92 - 1.09)	1.05 (0.97 - 1.14)	1.06 (0.98 - 1.15)	1.05 (0.98 - 1.13)
	Model 3[3]	(Ref)	1.00 (0.92 - 1.08)	1.05 (0.96 - 1.14)	1.04 (0.96 - 1.14)	1.04 (0.96 - 1.12)
Folate	Model 1	(Ref)	1.12 (1.03 - 1.22)*	1.06 (0.97 - 1.15)	1.07 (0.98 - 1.16)	1.03 (0.97 - 1.09)
	Model 2	(Ref)	1.12 (1.03 - 1.21)*	1.09 (1.01 - 1.18)*	1.06 (0.98 - 1.15)	1.03 (0.98 - 1.09)
	Model 3[4]	(Ref)	1.11 (1.03 - 1.20)*	1.08 (1.00 - 0.17)†	1.04 (0.95 - 1.13)	1.02 (0.96 - 1.08)
Episodic memory (N = 254)						
Hcy	Model 1	(Ref)	0.94 (0.84 - 1.05)	0.94 (0.84 - 1.06)	0.85 (0.75 - 0.96)*	0.82 (0.71 - 0.94)*
	Model 2	(Ref)	0.95 (0.86 - 1.06)	0.94 (0.84 - 1.05)	0.89 (0.80 - 1.00)*	0.87 (0.76 - 0.99)*
	Model 3[2]	(Ref)	0.93 (0.83 - 1.03)	0.91 (0.81 - 1.03)	0.85 (0.74 - 0.97)*	0.80 (0.69 - 0.94)*
HoloTC	Model 1	(Ref)	1.07 (0.95 - 1.20)	1.01 (0.90 - 1.13)	1.07 (0.95 - 1.20)	1.02 (0.92 - 1.13)
	Model 2	(Ref)	1.09 (0.98 - 1.21)	1.01 (0.90 - 1.12)	1.03 (0.92 - 1.15)	0.99 (0.90 - 1.08)
	Model 3[3]	(Ref)	1.07 (0.96 - 1.19)	0.98 (0.88 - 1.09)	0.99 (0.88 - 1.10)	0.94 (0.85 - 1.04)
Folate	Model 1	(Ref)	0.96 (0.86 - 1.08)	0.99 (0.88 - 1.11)	1.02 (0.91 - 1.15)	1.03 (0.95 - 1.11)
	Model 2	(Ref)	0.93 (0.84 - 1.03)	0.98 (0.88 - 1.09)	0.98 (0.88 - 1.09)	1.00 (0.92- 1.07)
	Model 3[4]	(Ref)	0.92 (0.83- 1.02)	0.96 (0.86 - 1.06)	0.93 (0.83- 1.04)	0.95 (0.88 - 1.03)
Executive function (N = 240)						
Hcy	Model 1	(Ref)	0.92 (0.83 - 1.02)	0.87 (0.78 - 0.97)*	0.89 (0.80 - 1.00)*	0.84 (0.74 - 0.96)*
	Model 2	(Ref)	0.89 (0.81 – 0.98)*	0.88 (0.80 - 0.98)*	0.91 (0.82 - 1.01)†	0.88 (0.78 - 0.99)*
	Model 3[2]	(Ref)	0.90 (0.82 - 1.00)†	0.90 (0.81 - 1.00)†	0.94 (0.83 - 1.07)	0.91 (0.78 - 1.05)
HoloTC	Model 1	(Ref)	1.03 (0.93 - 1.15)	1.05 (0.94 - 1.17)	1.10 (0.98 - 1.22)†	1.10 (1.00 - 1.21)*
	Model 2	(Ref)	0.97 (0.87 - 1.07)	1.02 (0.93 - 1.13)	1.07 (0.97 - 1.18)	1.09 (1.00 - 1.18)†
	Model 3[3]	(Ref)	0.96 (0.86 - 1.06)	1.00 (0.90 - 1.11)	1.04 (0.93 - 1.16)	1.06 (0.96 - 1.16)
Folate	Model 1	(Ref)	1.02 (0.92 - 1.13)	1.09 (0.98 - 1.22)	1.04 (0.94 - 1.16)	1.03 (0.96 - 1.11)
	Model 2	(Ref)	1.01 (0.91 - 1.11)	1.04 (0.95 - 1.15)	1.07 (0.97 - 1.18)	1.03 (0.96 - 1.10)
	Model 3[4]	(Ref)	1.00 (0.90 - 1.10)	1.03 (0.93 - 1.14)	1.03 (0.92 - 1.15)	1.00 (0.93 - 1.08)

Verbal expression (N = 253)

Hcy	Model 1	(Ref)	0.97 (0.88 - 1.06)	1.03 (0.93 - 1.13)	0.92 (0.83 - 1.01)†	0.91 (0.81 - 1.01)†
	Model 2	(Ref)	0.94 (0.88 - 1.01)†	1.00 (0.92 - 1.08)	0.92 (0.85 - 0.99)*	0.91 (0.83 - 1.00)*
	Model 3[2]	(Ref)	0.94 (0.88 - 1.02)	1.00 (0.92 - 1.09)	0.93 (0.85 - 1.02)	0.94 (0.84 - 1.04)
HoloTC	Model 1	(Ref)	1.04 (0.95 - 1.15)	1.09 (0.99 - 1.19)†	1.08 (0.98 -1.18)	1.05 (0.97 - 1.13)
	Model 2	(Ref)	1.03 (0.96 - 1.11)	1.05 (0.97 - 1.13)	1.06 (0.98 - 1.14)	1.03 (0.97 - 1.10)
	Model 3[3]	(Ref)	1.02 (0.95 - 1.10)	1.03 (0.96 - 1.12)	1.03 (0.95 - 1.12)	1.01 (0.94 - 1.08)
Folate	Model 1	(Ref)	1.12 (1.02 - 1.23)*	1.02 (0.94 - 1.12)	1.10 (1.00 - 1.21)*	1.03 (0.97 - 1.10)
	Model 2	(Ref)	1.08 (1.01 - 1.16)*	1.04 (0.96 - 1.12)	1.08 (1.01 - 1.17)*	1.04 (0.99 - 1.10)
	Model 3[4]	(Ref)	1.07 (1.00 - 1.16)†	1.03 (0.95 - 1.11)	1.06 (0.97 - 1.15)	1.03 (0.97 - 1.09)

Psychomotor Speed (N = 240)

Hcy	Model 1	(Ref)	0.89 (0.78 - 1.00)†	0.90 (0.79 - 1.03)	0.84 (0.74 – 0.96)*	0.78 (0.67 - 0.91)*
	Model 2	(Ref)	0.95 (0.86 - 1.04)	0.95 (0.86 - 1.06)	0.95 (0.86 - 1.05)	0.93 (0.83 - 1.05)
	Model 3[2]	(Ref)	0.95 (0.86 - 1.05)	0.95 (0.86 - 1.06)	0.95 (0.84 - 1.07)	0.92 (0.80 - 1.07)
HoloTC	Model 1	(Ref)	1.18 (1.04 - 1.34)*	1.17 (1.03 - 1.33)*	1.22 (1.08 - 1.39)*	1.14 (1.02 - 1.27)*
	Model 2	(Ref)	1.07 (0.97 - 1.19)	1.05 (0.95 - 1.15)	1.09 (0.99 - 1.20)†	1.05 (0.97 - 1.15)
	Model 3[3]	(Ref)	1.07 (0.97 - 1.18)	1.04 (0.94 - 1.15)	1.08 (0.97 - 1.20)	1.04 (0.95 - 1.14)
Folate	Model 1	(Ref)	1.12 (0.98 - 1.26)†	1.01 (0.90 - 1.14)	1.06 (0.93 - 1.21)	1.03 (0.94 - 1.12)
	Model 2	(Ref)	1.07 (0.97 - 1.18)	1.00 (0.91 - 1.10)	0.98 (0.89 - 1.08)	0.98 (0.92 - 1.05)
	Model 3[4]	(Ref)	1.06 (0.96 - 1.17)	0.99 (0.90 - 1.09)	0.95(0.85 - 1.05)	0.96 (0.89- 1.03)

[1]Final cognitive scores regressed onto baseline serum measures.
Results are relative differences (Ref indicates reference groups), Q indicates quartiles. In cognitive tests, results larger than 1.00 indicate better and results smaller than 1.00 indicate worse performance. Model 1: adjusted for age, sex, education, follow-up time, SBP, BMI, APOE-ε4, stroke, smoking, and presence of renal conditions.
Model 2: additionally adjusted for baseline related cognitive measure.
Model 3: additionally adjusted for holoTC and folate[2], Hcy and folate[3], or Hcy and holoTC[4]
Hcy concentration was ≤ 10.4 µmol/L for Q1, 10.5-12.3 µmol/L for Q2, 12.4-14.4 µmol/L for Q3, and ≥ 14.5 µmol/L for Q4.
HoloTC concentration was ≤ 57.8 pmol/L for Q1, 57.9-83.2 pmol/L for Q2, 83.3-114.7 pmol/L for Q3, and ≤ 114.8 pmol/L for Q4.
Folate concentration was ≤ 4.9 nmol/L for Q1, 5.0-6.3 nmol/L for Q2, 6.4-8.4 nmol/L for Q3, and ≥ 8.5 nmol/L for Q4.
*$p < 0.05$; †$p < 0.1$

5.2 Characteristics of the Vantaa 85+ study population (study III)

Socio-demographic and clinical characteristics according to Hcy levels are shown in Table 11 for the autopsy population (265 subjects) and Table 12 for the post-mortem MRI population (103 of the 265 subjects). The follow-up duration was somewhat shorter in subjects with Hcy > 20 μmol/L, but there were no other differences according to Hcy levels. Because brain MRI scans were done in the first participants who died and were autopsied, the post-mortem MRI population was younger at time of death (mean (standard deviation) age 90.8 (3.4) versus 93.8 (3.2), $p < 0.001$), and had slightly higher Hcy 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 NFT count (Spearman-rho 0.316; $p < 0.001$), Braak stage (0.271; $p < 0.001$), β-amyloid load (0.348; $p < 0.001$), CERAD score (0.272; $p < 0.001$), cerebral macroinfarcts (0.180; $p = 0.004$), CAA (0.234; $p < 0.001$), α-synuclein pathology (0.177; $p = 0.004$), MTA score (0.317; $p = 0.001$), but not with periventricular (-0.084; $p = 0.400$) or deep (0.099; $p= 0.322$) WMH. Relations between clinical dementia syndromes, neuropathologic and MRI findings in the Vantaa 85+ population have been previously described in detail[157, 163, 164, 166, 167].

Table 11. Characteristics of the autopsy population according to Hcy values

Characteristics	Hcy ≤ 20 µmol/L *n* = 154	Hcy > 20 µmol/L *n* = 111	*P* - value	No.
Age at baseline, y	88.4 (2.8)	88.9 (3.2)	0.15	265
Age at death, y	92.6 (3.3)	92.6 (3.9)	0.93	265
Follow-up, y*	3.7 (2.3 – 6.0)	3.3 (1.6 – 5.5)	*0.06*	265
Women (*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 institutions (*n*, %)	53 (34.6%)	48 (43.2%)	0.16	264
Mini Mental State Examination (MMSE) 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*, %) • None • <Median of those with amyloid β • >Median of those with amyloid β	 24 (15.6%) 66 (42.9%) 64 (41.6%)	 20 (18.0%) 45 (40.5%) 46 (41.4%)	0.86	265
CERAD score (*n*, %) • None or sparse • Moderate • Frequent	 50 (32.5%) 85 (55.2%) 19 (12.3%)	 41 (36.9%) 59 (53.2%) 11 (9.9%)	0.68	265
CAA (*n*, %) • None • <Median of those with CAA • >Median of those with CAA	 47 (30.9%) 49 (32.2%) 56 (36.8%)	 33 (30.3%) 42 (38.5%) 34 (31.2%)	0.52	261
Tangle count (*n*, %) • None • <Median of those with tangles • >Median of those with tangles	 57 (37.0%) 50 (32.5%) 47 (30.5%)	 34 (30.6%) 39 (35.1%) 38 (34.2%)	0.56	265
Braak stage (*n*, %) • 0-2 • 3-4 • 5-6	 46 (29.9%) 69 (44.8%) 39 (25.3%)	 27 (24.3%) 60 (54.1%) 24 (21.6%)	0.33	265
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 Hcy was chosen according to proposed upper reference limit in elderly > 65 years without folate supplementation [66].

Table 12. Characteristics of the post-mortem MRI population according to Hcy values

Characteristics	Hcy ≤ 20 µmol/L *n* = 55	Hcy > 20 µmol/L *n* = 48	*P*-value	No.
Age at baseline, y	89.1 (2.9)	88.9 (3.7)	0.76	103
Age at death, y	91.1 (3.0)	90.5 (3.7)	0.40	103
Follow-up, y*	2.0 (1.2 – 2.9)	1.3 (0.7 – 2.8)	0.03	103
Women (*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 (MMSE) 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[66].

5.2.1 Hcy and neuropathology

Associations between baseline Hcy and neuropathological measurements are shown in table 13. For individuals in the highest Hcy quartile, OR (95% CI) was 2.60 (1.28 – 5.28) for having a higher NFT count, even after all adjustments (Model 2). After adjusting for age at death, gender, follow-up duration and *APOE*ε4 status (Model 1), OR (95% CI) for a more severe Braak stage was 1.96 (1.05 – 3.68) for the highest Hcy 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 13, Model 2). The pattern of association for the highest Hcy quartile remained even after excluding subjects with dementia at baseline: OR (95% CI) was 3.44 (1.22 - 9.68) for higher NFT count, and 2.32 (0.91 – 5.91) for more severe Braak stage.

Table 13. OR (95%CI) for the associations of Hcy (quartiles) with neuropathology and post-mortem MRI measurements

	Q1	Q2	Q3	Q4
Tangle count[1]				
• 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[1]				
• 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[1]				
• 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[1]				
• 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[1]				
• 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[1]				
• 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[1]				
• 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[1]				
• 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[1]				
• 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[2]				
• 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[2]				
• 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[2]				
• 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 marked in bold, trends ($P < 0.10$) are marked in italic.
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.
[1]Hcy 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
[2]Hcy 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

In stratified analyses investigating the impact of dementia status at death, cerebral infarcts and follow-up time (table 14), the highest Hcy quartile was related to higher NFT 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 Hcy measurement and death: 3.25 (1.19 - 8.90).

Table 14. OR (95%CI) for the associations of Hcy (quartiles) with NFT count according to dementia status, presence of infarcts, and time between baseline assessment and death

	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 marked in bold, trends ($P < 0.10$) are marked in italic. 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. Hcy 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

Higher Hcy was not significantly related to increased β-amyloid burden or CERAD score (Table 13), and this was not influenced by dementia status at death or cerebral infarcts (Table 15). However, participants with longer time between the baseline Hcy measurement and death tended to have a higher β-amyloid burden (p = 0.085): for the highest Hcy quartile, OR (95% CI) was 2.52 (0.88 - 7.19) (Table 15). No significant relations between Hcy and CAA, cerebral infarcts, or α-synuclein pathology were found (Table 13).

Table 15. OR (95%CI) for the associations of Hcy (quartiles) with β-amyloid load according to dementia status, presence of infarcts and time between baseline assessment and death

	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 ≤ 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 marked in bold, trends ($P < 0.10$) are marked in italic. 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. Hcy 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

5.2.2 Hcy, WMH and MTA on post-mortem MRI

After adjusting for age at death, gender, follow-up time, *APOE*ε4 status, history of cardiovascular conditions, use of vitamins, and living in institutions, higher Hcy levels were associated with higher MTA and periventricular WMH scores (Table 13). For the highest Hcy quartile, OR (95% CI) were 3.78 (1.12 - 12.79) for more severe MTA, and 4.69 (1.14 - 19.33) for more severe periventricular WMH. No significant association was found between homocysteine and deep WMH scores.

5.3 Characteristics of the SNAC-K MRI study population (study III)

Characteristics of the 501 study participants are presented in table 16. The mean (SD) age of the subjects was 70.9 (9.1) years and 59.5 % were female. The mean plasma levels of vitamin B12, RBC folate, hemoglobin, and albumin were all within normal range. Vitamin B12 and folic acid supplements were used by 97 (19.4) and 75 (14.9%) of the participants, respectively.

Table 16. Characteristics of the SNAC-K study population at baseline (n = 501)[a]

Characteristic	Value
Age, y	70.9 (9.1)
Sex, women (N, %)	298 (59.5)
Education, y	12.6 (4.5)
MMSE	29.2 (2.4)
Hemoglobin, g/L	139.1 (11.5)
Albumin, g/L	43.0 (3.4)
Vitamin B12 supplements (N, %)	97 (19.4)
Folic acid supplements (N, %)	75 (14.9)
Vitamin B12, pmol/L	402.9 (202.3)
RBC Folate, nmol/L	265.8 (124.1)

[a]If not otherwise specified, data are presented as mean (SD). MMSE = Mini-Mental State Examination; RBC = Red Blood Cell.

5.3.1 Vitamin B12, RBC folate, and the rate of brain volume loss

Linear mixed models were used to examine the cross-sectional and longitudinal associations of vitamin B12 and RBC folate concentrations with GM, WM, and TBT volumes. After adjusting for age, sex, education level, and use of vitamins (model 1), RBC folate had a borderline significant cross-sectional relation with GM volume (β (SE): 0.0159 (0.008); p = 0.055) (table 17, model 1). Additional adjustment for hemoglobin, albumin, chronic diseases, and vitamin B12 did not influence the results: β (SE) became 0.0160 (0.008); p = 0.056 (table 17, model 2). No significant cross-sectional associations were detected between B12 and brain volumes.

In the prospective analysis over 6 years, higher baseline concentrations of plasma vitamin B12 was related to a decreased rate of TBT volume loss (β (SE): 0.0020 (0.001); p = 0.002) after adjusting for all study covariates. This association was stronger for GM (β (SE): 0.0013 (<0.001); p = 0.016) than WM (β (SE): 0.0007 (0.001); p = 0.251). The associations between B12 and TBT and GM remained unchanged after excluding subjects with plasma vitamin B12 below the cut-off value of 180 pmol/L (β (SE): 0.002 (0.001); p = 0.005 for TBT and 0.0012 (0.001); p = 0.026 for GM). No significant associations were detected between RBC folate and brain volumes over 6 years, even after restricting the analyses to participants with RBC folate levels higher than the cut-off value of 125 nmol/L.

Table 17. B12 and RBC folate in relation to changes in grey matter, white matter, and total brain volumes during 6 years (n = 501)[a]

		Cross-sectional β (SE); p-value	**Time** β (SE); p-value	**Vitamin × time** β (SE); p-value
Vitamin B12	**Total Brain Volume**			
	Model 1	-0.0010 (0.007); 0.891	-5.589 (0.641); <0.001	0.0021 (<0.001); 0.001
	Model 2[b]	-0.0024 (0.007); 0.735	-6.924 (2.423); 0.004	0.0020 (0.001); 0.002
	Grey Matter Volume			
	Model 1	0.0046 (0.005); 0.359	-1.556 (0.516); 0.003	0.0011 (<0.001); 0.022
	Model 2[b]	0.0042 (0.005); 0.406	-1.900 (1.947); 0.329	0.0013 (<0.001); 0.016
	White Matter Volume			
	Model 1	-0.0063 (0.007); 0.366	-4.167 (0.569); <0.001	0.0009 (0.001); 0.094
	Model 2[b]	-0.0075 (0.007); 0.296	-5.410 (2.161); <0.012	0.0007 (0.001); 0.251
RBC Folate	**Total Brain Volume**			
	Model 1	0.0074 (0.012); 0.523	-4.980 (0.693); <0.001	0.0002 (0.001); 0.868
	Model 2[c]	0.0089 (0.012); 0.448	-6.726 (2.431); 0.006	-0.0002 (0.001); 0.841
	Grey Matter Volume			
	Model 1	0.0159 (0.008); 0.055	-1.189 (0.553); 0.032	0.0001 (<0.001); 0.893
	Model 2[c]	0.0160 (0.008); 0.056	-1.785 (1.953); 0.361	-0.0002 (0.001); 0.850
	White Matter Volume			
	Model 1	-0.0077 (0.012); 0.508	-3.650 (0.609); <0.001	-0.0006 (0.001); 0.547
	Model 2[c]	-0.0058 (0.012); 0.619	-5.330 (2.167); <0.014	-0.0005 (0.001); 0.597

β represents the coefficient for vitamin B12 and folate and SE represents the standard error.

[a]Associations were examined by linear mixed models. The term *cross-sectional* represents the cross-sectional association between B12 or folate and brain volumes at baseline. The term *time* indicates the decline in brain volumes during follow-up. The term *Vitamin × time* represents the effect of B12 or folate on the rate of change in brain volumes over time. A positive coefficient for Vitamin × time indicates that an increase in the vitamin value was associated with the decreased rate of brain atrophy over time.

Model 1: adjusted for age, sex, education, and the use of vitamins supplements and their interactions with time.

Model 2: additionally adjusted for hemoglobin, albumin, chronic diseases, [b]folate and [c]vitamin B12 and their interactions with time.

5.3.2 Vitamin B12, RBC folate, and rate of brain volume loss in individuals without dementia

Analyses were repeated after excluding 28 subjects with incident dementia at follow-up (table 18). These subjects were older (mean age (SD): 79.7 (6.8) versus 70.3 (9.0) years), less educated (mean (SD): 9.4 (2.8) versus 12.8 (4.5) years), and had a lower baseline MMSE total score (mean (SD): 28.1 (1.6) versus 29.2 (2.4)) compared with non-demented participants. After controlling for all study covariates, increasing B12 concentrations were significantly related to longitudinal TBT and GM volume changes (β (SE): 0.0019 (<0.001); p = 0.003 for TBT and 0.0014 (<0.001); p = 0.010 for GM). These associations did not change after restricting the analyses to participants with plasma B12 higher than the cut-off value of 180 pmol/L (β (SE): 0.0017 (0.001); p = 0.008 for TBT and 0.0013 (0.001); p = 0.017 for GM). No significant longitudinal relationship was observed between RBC folate and brain volumes over 6 years, even after excluding subjects with low RBC folate values.

Table 18. Vitamin B12 and RBC folate in relation to changes in grey matter, white matter, and total brain volumes during 6 years in subjects without dementia (n = 473)[a]

		Cross-sectional β (SE); p-value	**Time** β (SE); p-value	**Vitamin × time** β (SE); p-value
Vitamin B12	**Total Brain Volume**			
	Model 1	0.0076 (0.007); 0.312	-5.576 (0.628); <0.001	0.0021 (0.001); 0.001
	Model 2[b]	0.00642 (0.008); 0.413	-8.011 (2.387); 0.001	0.0019 (<0.001); 0.003
	Grey Matter Volume			
	Model 1	0.0064 (0.005); 0.239	-1.569 (0.520); 0.003	0.0013 (<0.001); 0.014
	Model 2[b]	0.0057 (0.006); 0.310	-2.022 (1.983); 0.308	0.0014 (<0.001); 0.010
	White Matter Volume			
	Model 1	0.0015 (0.007); 0.840	-4.156 (0560); <0.001	0.0009 (0.001); 0.102
	Model 2[b]	0.0010 (0.008); 0.894	-5,753 (2.138); 0.007	0.0006 (0.001); 0.290
RBC Folate	**Total Brain Volume**			
	Model 1	0.0076 (0.012); 0.527	-4.937 (0.677); <0.001	-0.00003 (0.001); 0.980
	Model 2[c]	0.0065 (0.012); 0.591	-7.823 (2.394); 0.001	-0.0004 (0.001); 0.740
	Grey Matter Volume			
	Model 1	0.0181 (0.009); 0.036	-1.144 (0.558); 0.040	-0.00001 (0.001); 0.995
	Model 2[c]	0.0171 (0.009); 0.052	-1.902 (1.988); 0.339	-0.0003 (0.001); 0.742
	White Matter Volume			
	Model 1	-0.0101 (0.012); 0.395	-3.679 (0.599); <0.001	-0.0005 (0.001); 0.602
	Model 2[c]	-0.0100 (0.012); 0.404	-5.661 (2.142); 0.008	-0.0004 (0.001); 0.671

β represents the coefficient for vitamin B12 and folate and SE represents the standard error.
[a]Associations were examined by linear mixed models. The term *cross-sectional* represents the cross-sectional association between B12 or folate and brain volumes at baseline. The term *time* indicates the decline in brain volumes during follow-up. The term *Vitamin* × *time* represents the effect of B12 or folate on the rate of change in brain volumes over time. A positive coefficient for Vitamin × time indicates that an increase in the vitamin value was associated with the decreased rate of brain atrophy over time.
Model 1: adjusted for age, sex, education, and the use of vitamins supplements and their interactions with time.
Model 2: additionally adjusted for hemoglobin, albumin, chronic diseases, [b]folate and [c]vitamin B12 and their interactions with time

6 DISCUSSION

The results of this research project indicate that Hcy, vitamin B12, and folate are related to cognitive performance, risk of AD, and structural brain changes in older adults.

6.1 tHcy, holoTC, folate and AD and cognition

Study I showed that elderly with elevated serum Hcy concentrations had an increased risk of developing AD 7 years later. This association was independent of several potential confounders, including common vascular risk factors. In addition, higher holoTC values were independently related to a reduced risk of AD. The protective effect of holoTC was more pronounced with increasing age.

These results are in line with previous longitudinal studies on late-life Hcy and risk of AD[95, 101, 108]. High midlife Hcy increased the risk of late-life AD 35 years later in the Prospective Population Study of Women in Gothenburg[112]. In addition, the Sacramento Area Latino Study on Aging reported that elevated Hcy associated with low B12 status had the strongest association with combined incidences of dementia and cognitive impairment without dementia[105]. In contrast, in the Washington Heights-Inwood Columbia Aging Project no significant association between Hcy and AD was detected after adjustments[98]. Possible explanations for these differences could be the relatively short follow-up time (4.7 years), and the rather homogenously high Hcy concentrations in this sample, which did not allow enough variability to detect an association[98, 101].

The association between holoTC and AD has been previously less investigated. One case-control study reported lower holoTC values in AD patients[176]. Another case-control study which additionally examined TC776C>G polymorphism (a genetic determinant of holoTC that may influence AD risk[177]) found no difference in holoTC values between patients with AD and controls, although genotype influenced the age at disease onset[178]. Furthermore, results from the Kungsholmen Project showed that moderate (third quartile) but not high (fourth quartile) holoTC levels were associated with reduced AD risk at follow-up[108], probably reflecting concomitant systemic disease in some individuals in the highest level of holoTC[62].

Little is currently known about the interactions between Hcy and holoTC in relation to AD risk. In study I, interaction terms were not significant. However, the Hcy-AD association was

attenuated by adjusting for holoTC. This could be due to the small sample size, but it may also point to an important effect of holoTC on Hcy, suggesting that holoTC itself may explain the Hcy-AD relationship. Interestingly, the holoTC-AD link was less influenced by adjusting for Hcy. This supports the hypothesis that factors other than Hcy may also explain the association between holoTC and AD.

No association between folate and AD was found in study I, and there were no significant interactions between folate, Hcy, and holoTC in relation to AD. Low folate levels have been indicated as a risk factor for AD in some (but not all) studies[64]. Differences in results could be due to the population differences. However, similar to our results, no association between folate and AD risk was found in the Kungsholmen Project, suggesting that Hcy and holoTC may be a better and earlier marker for AD[108].

In the CAIDE study, higher Hcy concentrations were related to worse cognitive performance 7 years later, irrespective of several potential confounders. In addition, higher holoTC had a protective role for several cognitive domains. The protective effect of holoTC was observed over the whole normal range of holoTC (Study II). These findings are consistent with previous longitudinal studies reporting an association between Hcy and cognitive decline[93, 96, 102, 104, 111, 113, 116, 179-181]. In contrast, no association between Hcy and cognition were found in the Rotterdam study (follow-up 2.7 years)[92], the Leiden 85-Plus Study (follow-up 5 years)[99], the Mac Arthur Study of Successful Aging (follow-up 7 years)[100] or the Chicago Health and Aging Project (follow-up 6 years)[110]. Differences in follow-up periods, cognitive measurement methods, other characteristics of the study population, and implementing the study after mandatory folic acid fortification[110] can explain some of the discrepancies across studies.

Compared with Hcy, the association between holoTC and cognitive decline has been previously less studied. Results from study II are in line with findings from the Oxford Healthy Aging Project, where lower levels of holoTC were associated with greater cognitive decline over 10 years[104]. However, no relations between holoTC and cognition were detected in the Rotterdam scan study[109].

In study II, the associations between Hcy or holoTC and MMSE scores were somewhat weaker in study participants without dementia. It may be possible that Hcy and holoTC need longer time to affect cognition, and their effects become manifest closer to dementia onset. Interestingly, in CAIDE participants without dementia higher folate values were significantly related to measures of global cognition and verbal expression. A possible explanation could be

that the protective effects of folate are no longer manifest when dementia-related disease processes become too advanced. These findings are in agreement with an earlier prospective study reporting an association of low folate or B12 levels with increased AD risk particularly in subjects with baseline MMSE > 26[94]. However, studies on associations between folate and cognition are notoriously inconsistent[96, 99, 100, 102-104, 179-181], and such findings need to be further investigated.

6.2 Hcy, Alzheimer and cerebrovascular pathology

In elderly aged over 85 years (Study III), elevated Hcy was associated with increased neurofibrillary tangle burden at the time of death up to 10 years later. Having Hcy levels in the highest quartile was related to about 2.5-fold increased OR for higher NFT count. The association was observed particularly in subjects with dementia, in the presence of cerebral infarcts, and with longer time between the baseline Hcy assessment and death. There was no significant association between Hcy and amyloid-β accumulation, although elevated Hcy tended to relate to amyloid-β accumulation in people with longer follow-up time. All observed associations were independent of several potential confounders, including common vascular risk factors.

The impact of Hcy on AD-type pathology has previously been less investigated. Very few studies have reported associations of Hcy with plasma/CSF β-amyloid[123, 124, 126] or CSF tau [125, 127]. Two cross-sectional studies including community-dwelling elderly and one clinical study including patients with AD, mild cognitive impairment, Parkinson's disease, cerebral amyloid angiopathy, and healthy controls found a positive association between Hcy and plasma Aβ levels[123, 124, 126]. In addition, two recent studies focusing on participants with different neurological diseases (including AD) reported significant relations between metabolites of Hcy cycle and CSF P-tau, although no association with CSF Aβ was detected[125, 127]. In contrast, two other clinical studies of participants with different neurological diseases did not find any significant correlations between Hcy and CSF β-amyloid or tau level[128, 129]. The cross-sectional nature of these studies, differences in Hcy status, other population characteristics, and approach (i.e. focus on plasma rather than CSF Aβ) could explain some of the discrepancies between results. In the Vantaa 85+ study, the association between elevated baseline Hcy, NFT burden and even amyloid-β load became stronger in participants with

longer follow-up, suggesting that the impact of Hcy on NFT formation and possibly also Aβ accumulation is a long-term process.

The association of Hcy with NFT count in the Vantaa 85+ population was stronger compared to Braak staging. Such a discrepancy between individual NFT counts and the Braak stages is not entirely surprising, because occasional neocortical tangle counts can be seen in any Braak stages less than V[163].

Although a relation between Hcy and vascular disease has long been recognized, no associations between Hcy and cerebral macro- or microinfarcts were found in our study. As elevated Hcy is associated with increased mortality, the lack of significant associations may be related to selective survival; individuals with higher Hcy or more severe cerebrovascular lesions may have died before the beginning of the Vantaa 85+ study. However, Hcy was related to NFT burden particularly in individuals with cerebral infarcts, suggesting that blood perfusion of the brain may modulate the impact of Hcy on tau pathology.

6.3 Hcy, B12, folate and structural brain changes on MRI

Higher Hcy levels were associated with more severe medial temporal lobe atrophy and periventricular white matter hyperintensities on post mortem MRI in elderly aged over 85 years (Study III). In addition, in the longitudinal population-based study of Swedish older adults (Study IV), higher plasma concentrations of vitamin B12 were independently related to decreased rate of brain volume loss over 6 years. The protective effect of vitamin B12 was present over the whole normal range of plasma B12 levels.

Associations between Hcy and medial temporal atrophy or hippocampal atrophy have been reported in some[91, 182, 183] but not all[184] previous studies using in vivo MRI. Medial temporal atrophy is indicative of primary neurodegenerative pathology in the medial temporal lobe[167], and is pathologically characterized by a high NFT burden according to a previous study in the Vantaa 85+ population[163]. Hcy may thus be directly involved in the pathology of brain areas affected by AD.

Few longitudinal studies have investigated the associations between vitamin B12 or folate and the rate of brain volume loss. Similar to study IV findings, lower levels of vitamin B12 but not folate were associated with an increased rate of brain volume loss over 5 years in the Oxford Project to Investigate Memory and Aging (OPTIMA), which included 107 elderly subjects aged

61-87 years without dementia at baseline[122]. In contrast, data from the Nun Study based on 30 subjects who were 78-101 years old at the time of death indicated a significant relationship between low serum folate and atrophy of cerebral cortex at autopsy[185]. Differences in follow-up time, vitamin status, atrophy assessment methods, and other characteristics of the study populations can explain the differences in results.

In the Vantaa 85+ population, Hcy was related to periventricular but not deep white matter hyperintensities on post-mortem MRI. A stronger relation between Hcy and periventricular rather than deep white matter hyperintensities on *in vivo* MRI has been previously reported[186], although other in vivo MRI studies did not support this pattern[187, 188]. Although deep white matter hyperintensities are considered markers for cerebral small vessel disease and related problems in white matter perfusion and ischemia, different pathogenetic mechanisms (i.e. blood-brain barrier dysfunction, disturbances in CSF production) may lead to periventricular white matter hyperintensities[189]. It has been suggested that periventricular white matter hyperintensities may be an epiphenomenon of brain atrophy and may not be independently related to AD[190]. However, based on the rather small number of Vantaa 85+ participants with Hcy measurements and post-mortem MRI scans, an association of Hcy with deep white matter hyperintensities cannot be completely ruled out.

Consistent with the proposed etiological differences between deep and periventricular WMH, the presence of multiple cerebral infarcts was associated with deep WMH, but not with periventricular WMH[163] in the Vantaa 85+ study population. In contrast, periventricular WMH were associated with NFT burden[163], which is in agreement with our results relating Hcy with both periventricular WMH and NFT.

Based on results from study III, it seems less likely that the association between Hcy and neurofibrillary pathology might come directly via Hcy-associated cerebrovascular disease, because no significant association between Hcy and cerebral infarcts or deep WMH was found. These findings support a direct association between Hcy and neurofibrillary formation, and the association between Hcy and periventricular WMH 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 fiber tracts, which run near the lateral ventricles.

6.4 Biological plausibility

Vitamin B12 and folate are essential factors for the remethylation of Hcy to methionine and the subsequent formation of S-adenosylmethionine (SAM), the primary methyl donor for many biochemical reactions involved in normal brain functions (i.e. the production of cell membrane phospholipids, myelin, catecholamine neurotransmitters, proteins and nucleic acid). Subsequently, S-adenosylmethionine is converted into S-adenosyl homocysteine (SAH), a potent competitive inhibitor of several methyl transferases, after donating its methyl group to other cellular components. SAH is further hydrolyzed into homocysteine in a reversible reaction that favors SAH formation when homocysteine concentrations increase[66, 125] (figure 1).

The effects of B12/holoTC or folate on AD risk may be partly mediated by Hcy, since Hcy concentrations are dependent on vitamin B12 and folate status. High Hcy levels have been related to endothelial dysfunction, impaired nitric oxide activity, atherosclerosis[191, 192], and subsequent increase in the risk of various cardiovascular and cerebrovascular events which may increase the risk of dementia and AD[193-196].

Experimental studies have shown that elevated Hcy may cause DNA damage[197] or impair DNA repair in neurons[198]. Alternatively, the effects of B12/holoTC or folate may be mediated through SAM. Deficiency of SAM has been linked to white matter damage and brain atrophy[122], factors association with cognitive decline and dementia[199]. In addition, low concentrations of SAM or low SAM:SAH ratio have been associated with insufficient methylation and decreased activity of protein phosphatase 2A (PP2A), an enzyme involved in the dephosphorylation of phospho-tau. The insufficient phosphorylation 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[63, 64, 77, 117, 125, 127, 200-202], which in turn is associated with increased rate of grey matter atrophy[203].

Furthermore, elevated Hcy may also influence amyloid-β generation and its neurotoxicity through several mechanisms. High Hcy may stimulate the endoplasmic reticulum stress response in endothelial cells, neurons and glia, activating presenilin-induced amyloid-β generation[204]; deficient methylation upregulates presenilin gene function and amyloid-β generation[205]; Hcy 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[63, 206].

6.5 Clinical implications

The results of this project indicate that vitamin B12, folate, and Hcy are related to cognitive performance and structural brain changes in older adults. The observed associations were present even in non-demented elderly and over the whole normal range of holoTC/vitamin B12. The association of Hcy with AD pathology (particularly NFT burden), was observed especially when study participants were followed-up for a longer time; it also seems to be more pronounced in the presence of cerebrovascular pathology, despite the lack of direct association between cerebrovascular pathology and Hcy.

This project emphasizes the need for further studies on the role of sensitive markers of B12 and folate status in identifying individuals who are at increased risk of dementia. Few randomized controlled trials have so far investigated the usefulness of B-vitamin supplements in preventing cognitive decline, with mixed results (table 5)[118, 119, 207, 208]. Limitations of statistical power, vitamin dosage, use of vitamin combinations, very different study durations, choice of target population, and failure to include measures for monitoring in-vivo biological response makes such studies difficult to interpret. NFT and amyloid-β accumulation in AD are a long-term process, starting earlier in life when individuals are still cognitively normal[14]. Supplementation may be most effective for prevention during a critical time window, and adequately timed and powered randomized controlled trials are necessary to formulate efficient guidelines (dose, treatment start and duration, target population) for better cognitive performance later in life.

The need to more closely monitor B-vitamin status at older ages has been recognized for some time. High Hcy, and low B12 and folate levels are surprisingly common conditions in the elderly, both in developed and developing countries[64, 209, 210]. It has been estimated that vitamin B12 deficiency affects about 10-15% of people > 60 years. Elderly frequently lack the classical sign and symptoms of such a deficiency, and neurological signs of deficiency may occur in patients who do not show the classical anemia signs such as increased mean corpuscular volume[89, 210, 211]. A deficiency of B12 or folate may have several causes, such as inadequate dietary intake, defective absorption, total or partial gastrectomy, medications (i.e. anti-folates, metformin, or drugs that block stomach acid), mutations in genes essential for folate or transcobalamin, or auto-antibodies[82, 212, 213]. It is important to regularly assess vitamin B12 and folate status in elderly, and Hcy, holoTC or MMA should be included as additional laboratory markers[82, 214]. Of course, the kidney function should be taken into account when interpreting the tHcy and MMA values in the elderly[82].

Our findings also highlight the need for reviewing the blood values chosen as cutoffs for B12, as associations with cognition and related brain changes were present over the whole normal range of holoTC/B12. Based on current evidence, the widely used cutoff value of 148 pmol/L (200pg/mL) is too low[211] as it misses patients with clinical deficiency[215]. It has been shown that Hcy and MMA levels start to increase at B12 levels considerably above the typical cutoff value of 148 pmol/L. A range of values above the conventional cutoffs should be considered when deciding to monitor or treat patients. Determining 'the best cutoff' is still a matter of discussion. Patients with symptoms of B12 deficiency and B12 levels above 148 pmol/L (particularly in the low-normal range up to 300 pmol/L) may need further assessments of B12 status[211].

Since 1998, USA, Canada, and several other countries have started fortifying flour and pasta products with folic acid in order to reduce the number of infants with neural tube defects. It has been estimated that several hundred thousand people would be exposed to an increased folic acid intake for each infant saved[216]. Several concerns have been raised during the last 15 years, for example that the masking of hematologic signs of overt B12 deficiency may result in missing the diagnosis. Also, many drugs used in the treatment of cancer, bacterial infections, rheumatoid arthritis, malaria, and psoriasis are anti-folates, and the status of these conditions may be affected by folate fortification [216-218]. Furthermore, a combination of high folate level and low B12 status may be associated with an increased risk of cognitive impairment in the elderly[219, 220]. This is due to the inhibitory effects of high folic acid concentrations on 5-Methyl-THF formation, which leads to reduced methionine synthesis. Methionine synthesis is already impaired because of low B12 status, and this may be worsened by high folic acid concentrations, leading to a negative impact on cognition[216].

6.6 Methodological issues

The main strengths of studies included in this project are the population-based design and long duration of follow-up ranging from 6 to 10 years. Also, a large number of potential confounders were taken into account in each study.

In all studies, Hcy, vitamin B12/holoTC, and folate were measured in plasma or serum. Unlike dietary intake assessment, micronutrient plasma/serum measurement is an objective measure and is independent of the capacity to estimate/remember intake over a period of time. In

addition, plasma level assessments consider individual variations in metabolism, giving a reliable evaluation of micronutrient bioavailability.

The Hcy, B12/holoTC and folate assessments were available at only one time point. Due to possible intra-individual variations, this may underestimate the associations with cognition-related outcomes due to regression dilution[221]. Selective survival may also have contributed to an underestimation of the associations, because elevated Hcy or low B12/folate status are related to increased mortality[66].

The follow-up intervals were long, but due to the long preclinical phase of AD it cannot be completely excluded that Hcy, B12 and folate status may have been affected by potential changes in dietary habits caused by early-stage cognitive impairment. However, all studies included extensive diagnostic protocols for detecting even milder forms of cognitive impairment. Also, findings remained significant even in additional analyses excluding participants who developed dementia during follow-up. Finally, although adjustment for several relevant covariates did not alter the results, the possibility of residual confounding cannot be fully excluded.

6.6.1 CAIDE study

CAIDE is a well-characterized longitudinal study specifically designed to investigate risk factors for dementia and AD, and includes population-based random samples of individuals investigated on three different occasions. Participation rates were high, ranging from 77% to 96% at baseline, 72% at first re-examination, and 63% at the second reexamination[222]. Blood samples from the first re-examination (1998) were used for Hcy, holoTC, and folate assessment in 274 subjects, and the follow-up period was at least 7 years. Compared to vitamin B12, holoTC is a more sensitive assay of B12 status, comparable and possibly superior to Hcy and MMA[87, 176]. The long follow-up period, comprehensive evaluation and diagnostic protocol at each examination, recruitment of subjects without dementia in 1998, and adjustments for cognitive status in 1998 make the findings from studies I and II less prone to the influence of reverse causality (i.e., effects of preclinical AD on Hcy and holoTC).

In the 2005-2008 re-examination, the sensitivity for detecting very mild cognitive impairment was improved by including in the clinical phase subjects with MMSE $\leq$ 24, decline on MMSE of 3 or more points since 1998, delayed recall word list of < 70% in the CERAD neuropsychological test battery, or reported cognitive decline by the informant. Individuals

diagnosed with dementia in the clinical phase underwent brain imaging in the differential diagnosis phase. Autopsy data were not available to confirm the clinical diagnoses, but a previous neuropathological study in the clinic in Kuopio has shown that the accuracy of clinical AD diagnosis is good (96% for probable AD and 86% for possible AD)[223].

The main limitation of studies I and II is the relatively small sample size, which may have affected statistical power. Hcy was measured in serum. Even if serum is optimally prepared, it yields slightly higher Hcy values compared to plasma. This is because serum is left to sit at room temperature for 30-60 minutes to allow clotting before centrifugation and separation of the serum from the blood cells. During this time, cells release Hcy into the serum, causing an artificial elevation of Hcy level[62, 66]. As a result, the strength of the association between homocysteine and the outcome may be attenuated. Although the stability of Hcy in longtime-stored samples has been reported previously[66, 112], folate levels decline during storage[224]. This may have underestimated the folate-cognition associations compared to studies with folate measured in fresh samples. Since creatinin values were not available, self-reported history of renal conditions was considered in the analyses. Although holoTC may be an earlier and more sensitive marker of B12 deficiency, the best indicator or combination of indicators of B12 status (i.e., B12, MMA) in relation to AD or cognition remains to be determined.

6.6.2 Vantaa 85+ study

The entire Vantaa 85+ study population comprised 92% of the people aged 85 years or above living in a geographically well-defined area, and the consented post-mortem examination was conducted in 291 subjects who died during the 10-year follow-up until the 1st of April 2001. This cohort has high generalizability for the oldest-old[225]. The Vantaa 85+ autopsy rate is the second highest among all population-based autopsy studies on dementia worldwide, after the Japanese Hisayama study[226]. The characteristics of the brain donors show no evidence of systematic bias[227]. While it has been shown that neuropathological assessments can be reliably made by a single rater[228], it is an advantage that all scorings were interpreted by the same neuropathologist.

The high age of the study cohort can be considered as strength as well as shortcoming. Given the increased mortality in subjects with vascular risk factors or conditions (including Hcy), many such individuals were probably lost before beginning of the study[66]. Another limitation was the lack of data on creatinine values. Furthermore, the role of vitamin B12 and folate in

relation to neuropathology or MRI measurements could not be investigated. Quantitative, systematic methods were used 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[18].

6.6.3 The SNAC-K study

The SNAC-K study is a prospective survey of a community-based cohort of elderly aged 60+, including individuals from the Kungsholmen area of central Stockholm, Sweden. The response rate in SNAC-K at baseline was 73%. MRI data were available for a sub-sample of 555 subjects. The MRI scans were available on at least 2 time points over 6 years and it was possible to examine the simultaneous relation of B12 and folate with the outcome. The long follow-up duration, the comprehensive evaluation and diagnostic protocol, and the recruitment of dementia-free subjects at baseline make findings of study IV less prone to the influence of reverse causality (i.e. effects of preclinical cognitive impairment on B12 and folate levels). In addition, results remained unchanged after excluding subjects with incident dementia. However, we could not assess the role of functional indicators of folate and vitamin B12 status, such as Hcy and holoTC. The MRI sub-sample was younger, healthier, and had a better cognitive status compared to the rest of study population. This might have led to an underestimation of the observed associations.

7 CONCLUSIONS

The aim of this thesis was to investigate the impact of Hcy, vitamin B12, and folate on Alzheimer's disease, cognitive performance, and structural brain changes in older adults using data from well-designed population-based studies with a follow-up duration ranging from 6 to 10 years. Based on the findings, the following conclusions can be drawn:

I- Both Hcy and holoTC are involved in the development of AD. The association between Hcy and AD may be partly explained by holoTC. The protective effects of holoTC seem to be more pronounced with increasing age.

II- Hcy, holoTC and folate are associated with cognitive performance even in non-demented elderly, irrespective of several potential confounders. The protective role of holoTC was present over the whole normal range of holoTC levels.

III- Elevated plasma Hcy is related to an increased NFT burden. This association appears to be more pronounced in people with dementia, in the presence of cerebral infarcts, and with longer time between the Hcy assessment and death. Hcy also tended to relate to amyloid -β accumulation in people with longer follow-up time. The impact of Hcy on NFT formation and possibly Aβ accumulation is thus a long-term process.

Higher Hcy levels were also associated with more severe medial temporal lobe atrophy and periventricular white matter hyperintensities.

IV- Higher plasma vitamin B12 concentrations are independently associated with decreased rate of brain volume loss over 6 years, even in elderly without dementia. The protective effects of vitamin B12 are present over the whole distribution of vitamin B12 concentrations.

8 FUTURE DIRECTIONS

Vitamin B12, folate, and Hcy were discovered more than 80 years ago, and some of their roles in various pathological processes are already well characterized. Still, low B12 or folate and high Hcy levels are common conditions both in developed and developing countries, especially in more vulnerable sections of the population such as the elderly.

Results of the current project highlight the importance of these factors in the development of dementia/AD and related structural brain changes. They emphasize the need for further long-term population-based studies to clarify the role of functional indicators of vitamin B12 and folate status such as Hcy, holoTC, and methyl malonic acid in individuals at increased risk of dementing disorders dementia risk. A balanced B12 and folate status can be important for neuroprotection. Hence, a key point is the evaluation of all functional indicators together, taking into account their potential interactions and effects on cognitive impairment and related brain changes.

Interestingly, recent reports indicate that dementia incidence may be declining[229-232], although this needs to be confirmed in more studies. New epidemiological studies will be necessary to investigate risk factors for dementia/AD in new generations of older people. Conditions in the population can change considerably during the course of long-term observational studies. During the 20th century, significant changes occurred in many areas, including perinatal care, education, work and retirement conditions, housing and hygiene, dietary habits, health care, and survival. Factors related to dementia/AD may thus differ between birth cohorts. More information is needed on possible changes in B12 and folate status across birth cohorts, and their impact on the risk of late-life cognitive impairment.

Future studies will also need to focus more on the effects of B12 and folate status already at midlife, given that AD-related pathological processes can start up to decades before dementia onset. A life-course approach is essential in epidemiologic studies of chronic disorders with a long preclinical phase, in order to identify optimal windows for prevention. In addition, repeated measurements in the same individual over time are required for detecting patterns of change that may be more relevant than single measurements.

The present project additionally highlights the need for re-evaluating current laboratory cut-off values for defining vitamin B12/holotranscobalamin deficiency at older ages. Elderly who show

symptoms of B12 deficiency in a low-normal range of B12 values may particularly benefit from adequate treatment and monitoring. Regular assessments of B12 and folate status (including functional indicators such as Hcy, holoTC, and methyl malonic acid) are recommendable in elderly at risk of dementia. Elderly frequently lack the classical sign and symptoms of B12 and folate deficiencies, and insidious neurological signs of B12 deficiency may occur in patients who do not show the classical anemia signs such as increased mean corpuscular volume.

It is important to keep in mind that dietary intake involves a complex combination of nutrients. Evaluation of patterns instead of single nutrients can provide additional information on the relations of micro- and macro-nutrients with late-life cognition. Integrating the assessment of dietary intake, blood values of vitamin B12 and folate, and data on other micro- and macro-nutrients intake/levels could help unravel the synergistic and antagonistic effects of different nutrients on cognition.

Because dementia/AD is a multifactorial condition, and vitamin B12, folate, and Hcy are only pieces of a more complex puzzle, prevention trials targeting several risk factors simultaneously may be more likely to succeed. Such multi-domain trials are already ongoing (i.e. the FINGER study[51], www.edpi.org), and their results can provide necessary information for formulating effective dementia prevention strategies.

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11. APPENDIX

List of dissertations from the Aging Research Center and the Stockholm Gerontology Research Center, 1991-2013:

1991

Herlitz Agneta: Remembering in Alzheimer's disease. Utilization of cognitive support. (Umeå University)

1992

Borell Lena: The activity life of persons with a dementia disease.

1993

Fratiglioni Laura: Epidemiology of Alzheimer´s disease. Issues of etiology and validity.

Almkvist Ove: Alzheimer´s disease and related dementia disorders: Neuropsychological identification, differentiation, and progression.

Basun Hans: Biological markers in Alzheimer´s disease. Diagnostic implications.

1994

Grafström Margareta: The experience of burden in care of elderly persons with dementia. (Karolinska Institutet and Umeå University)

Holmén Karin: Loneliness among elderly - Implications for those with cognitive impairment.

Josephsson Staffan: Everyday activities as meeting-places in dementia.

Stigsdotter-Neely Anna: Memory training in late adulthood: Issues of maintenance, transfer and individual differences.

Forsell Yvonne: Depression and dementia in the elderly.

1995

Mattiasson Anne-Cathrine: Autonomy in nursing home settings.

Grut Michaela: Clinical aspects of cognitive functioning in aging and dementia: Data from a population-based study of very old adults.

1996

Wahlin Åke: Episodic memory functioning in very old age: Individual differences and utilization of cognitive support.

Wills Philippa: Drug use in the elderly: Who? What? & Why? (Licentiate thesis)

Lipinska Terzis Beata: Memory and knowledge in mild Alzheimer's disease.

1997

Larsson Maria: Odor and source remembering in adulthood and aging: Influences of semantic activation and item richness.

Almberg Britt: Family caregivers experiences of strain in caring for a demented elderly person. (Licentiate thesis)

1998

Agüero-Eklund Hedda: Natural history of Alzheimer's disease and other dementias. Findings from a population survey.

Guo Zhenchao: Blood pressure and dementia in the very old. An epidemiologic study.

Björk Hassing Linda: Episodic memory functioning in nonagenarians. Effects of demographic factors, vitamin status, depression and dementia. (In collaboration with the Department of Psychology, University of Gothenburg, Sweden)

Hillerås Pernilla: Well-being among the very old. A survey on a sample aged 90 years and above. (Licentiate thesis)

1999

Almberg Britt: Family caregivers caring for relatives with dementia – Pre- and post-death experiences.

Robins Wahlin Tarja-Brita: Cognitive functioning in late senescence. Influences of age and health.

Zhu Li: Cerebrovascular disease and dementia. A population-based study.

2000

Hillerås Pernilla: Well-being among the very old. A survey on a sample aged 90 years and above. (In collaboration with H. M. Queen Sophia University College of Nursing, Stockholm, Sweden)

von Strauss Eva: Being old in our society: Health, functional status, and effects of research.

2001

Jansson Wallis: Family-based dementia care. Experiences from the perspective of spouses and adult children.

Kabir Nahar Zarina: The emerging elderly population in Bangladesh: Aspects of their health and social situation.

Wang Hui-Xin: The impact of lifestyles on the occurrence of dementia.

2002

Fahlander Kjell: Cognitive functioning in aging and dementia: The role of psychiatric and somatic factors.

Giron Maria Stella: The rational use of drugs in a population of very old persons.

2003

Jönsson Linus: Economic evaluation of treatments for Alzheimer's disease.

2004

Berger Anna-Karin: Old age depression: Occurrence and influence on cognitive functioning in aging and Alzheimer´s disease

Cornelius Christel: Drug use in the elderly - Risk or protection? Findings from the Kungsholmen project

Qiu Chengxuan: The relation of blood pressure to dementia in the elderly: A community-based longitudinal study

Palmer Katie: Early detection of Alzheimer's disease and dementia in the general population. Results from the Kungsholmen Project.

Larsson Kristina: According to need? Predicting use of formal and informal care in a Swedish urban elderly population. (Stockholm University)

2005

Derwinger Anna: Develop your memory strategies! Self-generated versus mnemonic strategy training in old age: Maintenance, forgetting, transfer, and age differences.

De Ronchi Diana: Education and dementing disorders. The role of schooling in dementia and cognitive impairment.

Passare Galina: Drug use and side effects in the elderly. Findings from the Kungsholmen Project.

Jones Sari: Cognitive functioning in the preclinical stages of Alzheimer's disease and vascular dementia.

Karp Anita: Psychosocial factors in relation to development of dementia in late-life: a life course approach within the Kungsholmen Project.

Nilsson Jan: Understanding health-related quality of life in old age. A cross-sectional study of elderly people in rural Bangladesh.

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Klarin Inga: Drug use in the elderly – are quantity and quality compatible.

Nilsson Erik: Diabetes and cognitive functioning: The role of age and comorbidity.

Ngandu Tiia: Lifestyle-related risk factors in dementia and mild cognitive impairment: A population-based study.

Erika Jonsson Laukka: Cognitive functioning during the transition from normal aging to dementia.

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Ferdous Tamanna: Prevalence of malnutrition and determinants of nutritional status among elderly people. A population-based study of rural Bangladesh. (Licentiate thesis)

Westerbotn Margareta: Drug use among the very old living in ordinary households-Aspects on well-being, cognitive and functional ability.

Rehnman Jenny: The role of gender in face recognition. (Stockholm University)

Nordberg Gunilla: Formal and informal care in an urban and a rural population. Who? When? What?

Beckman Gyllenstrand Anna: Medication management and patient compliance in old age.

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Gavazzeni Joachim: Age differences in arousal, perception of affective pictures, and emotional memory enhancement. (Stockholm University)

Marengoni Alessandra: Prevalence and impact of chronic diseases and multimorbidity in the aging population: A clinical and epidemiological approach.

Rovio Suvi: The effect of physical activity and other lifestyle factors on dementia, Alzheimer's disease and structural brain changes.

Weili Xu: Diabetes mellitus and the risk of dementia. A population-based study.

Meinow Bettina: Capturing health in the elderly population – complex health problems, mortality, and the allocation of home help services. (Stockholm University)

Agahi Neda: Leisure in late life. Patterns of participation and relationship with health.

Haider Syed Imran: Socioeconomic differences in drug use among older people. Trends, polypharmacy, quality and new drugs.

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Thilers Petra: The association between steroid hormones and cognitive performance in adulthood.

Rana AKM Massud: The impact of health promotion on health in old age: results from community-based studies in rural Bangladesh

Paillard-BorgStéphanie: Leisure activities at old age and their influence on dementia development.

Livner Åsa: Prospective and retrospective memory in normal and pathological aging.

Atti Anna-Rita: The effect of somatic disorders on brain aging and dementia: Findings from population-based studies.

2010

Fors Stephan: Blood on the tracks. Life-course perspectives on health inequalities in later life.

Keller Lina: Genetics in dementia. Impact in sequence variations for families and populations.

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Schör Per: Gender matter. Differences and changes in disability and health among our oldest women and men.

Caracciolo Barbara: Cognitive impairment in the nondemented elderly: Occurrence, risk factors, progression.

Rieckmann Anna: Human aging, dopamine, and cognition. Molecular and functional imaging of executive functions and implicit learning.

2012

Haasum Ylva: Drug use in institutionalized and home-dwelling elderly persons.

Mangialasche Francesca: Exploring the role of vitamin E in Alzheimer's disease.An epidemiological and clinical perspective.

Lovén Johanna: Mechanism of women's own-gender bias and sex differences in memory for faces.

2013

Debora Rizzuto: Living longer than expected: protective and risk factors related to human longevity.