HYPERHOMOCYSTEINEMIA AS AN EARLY MODIFIABLE BIOMARKER OF AGE-RELATED COGNITIVE DECLINE AND RISK OF ALZHEIMER DISEASE

By

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ABSTRACT OF THE THESIS

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Statement of Problem: Studies have demonstrated associations between elevated plasma homocysteine and cognitive impairment, Alzheimer's disease, and dementia. This study sought to explore the relationship between elevated plasma homocysteine, regional brain volumes, and cognitive function in older adults with no overt cognitive impairment and the potential of elevated homocysteine to serve as an early modifiable biomarker for age-related neurodegeneration and cognitive decline.

Objective: We evaluated the relationship between plasma homocysteine, regional brain volumes, and cognitive function in older adults with no cognitive impairment (age 69-90 y; N=243, 129 women, 114 men).

Methods: Blood samples were collected to determine concentration of plasma total homocysteine, RBC folate, serum vitamin B12 and serum creatinine. Cognitive function tests included assessment of global cognitive function, processing speed, verbal memory, and executive function. Regional brain volume scans were conducted using MRI imaging analyzed by Freesurfer. Associations between plasma homocysteine, cognitive function

and regional brain volumes were assessed using multiple regression analysis with control for age, sex, education, RBC folate, vitamin B12 and serum creatinine.

Results: Homocysteine was inversely correlated with 12 of 15 regional brain volumes ($P<0.05$) after adjusting for demographic and biochemical variables. Homocysteine was inversely correlated with 3 of the 12 cognitive function tests ($P<0.05$) after adjusting for demographic and biochemical variables. The three cognitive function tests that were significantly associated with homocysteine were tests of executive functions.

Conclusions: Elevated homocysteine can serve as an early modifiable risk factor for impaired cognitive function and neurodegeneration in healthy adults without overt clinical cognitive impairment. Further research is needed to confirm if lowering homocysteine with B vitamin supplements will slow the progression of neurodegeneration and cognitive decline in older adults.

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Last but not least, I would like to dedicate my thesis to my late grandmothers. To my grandmother Phyllis Macshane who passed away in June at a proud 95 years old and to Dolores Ferguson who had passed away from Alzheimer disease this past February.

Keelin Ferguson
Rutgers University
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CHAPTER ONE: INTRODUCTION

The prevention of dementia and Alzheimer Disease (AD) is a major public health challenge due to the prevalence, mortality rates, and financial impacts of these disorders. The number of people living with AD in the U.S. is estimated to become 13.8 million by mid-century.[1] This estimate is high because of the rapidly growing section of the population, those aged 65 years and older, who are at risk for developing dementia and AD. In 2013, AD became the fifth leading cause of death for those 65 years and older in the US.[1] With no drugs currently on the market that are able to stop or reverse dementia and AD progression, the financial and personal impacts on caregivers and society continues to increase. In 2016, the cost of healthcare, long-term care and hospice services for those 65 years and older with dementia was estimated to be $236 billion.[1] Finding a way to prevent dementia and AD or slow progression is currently the most effective strategy to combating this major public health challenge.

Researchers are seeking to identify modifiable risk factors to decrease the risk of dementia and AD.[2] A particular interest in this regard is diet and nutrition, including deficiencies of B vitamins such as folate, vitamin B6, and vitamin B12. Deficiencies of these B vitamins impair the metabolism of the sulfur amino acid, homocysteine, causing hyperhomocysteinemia, which is a confirmed risk factor for AD.[2]

Studies have demonstrated significant associations between elevated plasma homocysteine with dementia and AD. In a study by Smith et al,[3] elevated homocysteine was found to be associated with accelerated whole brain atrophy in older adults diagnosed with mild cognitive impairment. In addition, Smith et al demonstrated that lowering homocysteine with B vitamin supplements slowed whole brain atrophy.[3] This

research team then demonstrated that the attenuating effect of B vitamin supplementation on the rate of whole brain atrophy was strongest in the posterior brain regions, which are some of the most affected by AD.[4] They also found that brain atrophy rates were associated with cognitive decline, and that participants in the upper quartile and above the median of plasma homocysteine had significant attenuation of rate of cognitive loss with B vitamin supplementation.[5] However, hyperhomocysteinemia as an early biomarker of age-related decline has yet to be explored.

Thus, the aim of this study is to assess the relationship between elevated plasma homocysteine, regional brain volumes, and cognitive function in adults ages 65-90 years with neither dementia nor overt cognitive impairment. Our goal is to fill this research gap in existing literature by investigating the following research question:

Research Question 1: Can elevated plasma homocysteine be used as an early modifiable biomarker for dementia and AD?

Hypothesis 1: Elevated homocysteine is an early modifiable risk factor for neurodegeneration assessed by MRI as indicated by increased ventricular size and decreased regional brain volumes in healthy older adults without clinical cognitive impairment.

Hypothesis 2: Elevated homocysteine is an early modifiable risk factor for impaired cognitive function in healthy older adults without clinical cognitive impairment.

CHAPTER TWO: LITERATURE REVIEW

Alzheimer disease (AD), dementia, and age-associated cognitive impairment is highly prevalent in older adults. Globally, there is an estimated 55 million people living with dementia, with AD comprising 60-70% of those cases.[6] In 2014, there was an estimated 5 million U.S. adults with dementia, and this number is projected to rise to 14 million by 2060.[7] More recently in 2020, an estimated 5.8 million Americans 65 years or older had been diagnosed with AD.[8] The fastest growing section of the population is the elderly who are at most risk for Alzheimer disease and dementia. The global population of those 60 years or older is expected to double by 2050, estimated at 2.1 billion.[9] In the United States almost one in four people will be 65 years or older and the number of individuals 85 years or older will triple by 2060.[10] This population shift is important to acknowledge as the percentage of individuals 85 years or older diagnosed with AD is close to 50 percent.[11] This not only has an impact on those who are suffering from dementia, but also affects their caregivers, families, and institutional resources. Therefore, finding ways to combat this disease is a major health priority.

At this time there are no drugs that can alter AD and dementia progression. However, there are drugs like Tacrine, Donepezil and Memantine, that can treat certain symptoms such as memory, behavioral, and psychological problems that typically manifest from the disease.[12] Although some of these treatments may help temporarily preserve some cognitive function and quality of life, they currently offer little assistance in stopping or reversing Alzheimer disease-induced brain atrophy.

An alternative, and possibly more effective strategy, is to prevent AD and dementia or slow progression at the early stages. Thus, modifiable risk factors for AD and dementia need to be identified. Some known modifiable risk factors include physical inactivity, smoking, alcohol intake, and diet.[13] With respect to diet, healthy diets consisting of low amounts of saturated fat and added sugars and high in fruits and vegetables have been shown to reduce the risk of cardiovascular disease, diabetes, and obesity, all of which are associated with increased risk of AD and dementia.[13] Other important dietary factors include certain micronutrient deficiencies that are risk factors for age-associated cognitive decline, AD, and dementia including deficiencies of B vitamins such as folate, vitamin B12, and vitamin B6.[2] Deficiencies of these B vitamins impair the metabolism of homocysteine causing elevated levels of this sulfur amino acid in the blood known as hyperhomocysteinemia.[14] Hyperhomocysteinemia can lead to vascular endothelial cell damage, a risk factor for vascular disease.[15] Researchers have also repeatably confirmed hyperhomocysteinemia as a risk factor for AD.[2] Moreover, as is the focus of this thesis, hyperhomocysteinemia may be an early biomarker of age-related decline. If homocysteine can be used as an early biomarker, homocysteine-lowering treatments with B vitamins may be an effective intervention to decrease the risk or slow the progression of AD and dementia.

One carbon metabolism is the biochemical process of chemical units consisting of a single carbon atom bound to hydrogen atoms, originating in the amino acid serine, being transferred between substrates to drive important metabolic reactions.[16] These metabolic reactions include the methionine and folate cycles.[16] The one-carbon units are

also used to make nucleotides used for DNA and RNA synthesis and the metabolic processing of homocysteine.[16]

Specific details of one-carbon metabolism are presented in **Figure 1** and include the following: In a vitamin B6 (pyridoxal-5'-phosphate or PLP)-dependent reaction, folate in the form of tetrahydrofolate receives a one-carbon unit from serine.[16] This reaction produces glycine and 5,10-methylenetetrahydrofolate.[16] The 5,10-methylenetetrahydrofolate can either donate the one-carbon unit to produce thymidylate and purines (required for DNA and RNA synthesis) or become reduced to 5-methyltetrahydrofolate with riboflavin (vitamin B2) serving as a cofactor in the form of flavin adenine dinucleotide (FAD).[17] The 5-methyltetrahydrofolate serves as a methyl donor for the conversion of homocysteine to form the amino acid, methionine, and tetrahydrofolate.[16] This reaction is catalyzed by the enzyme, methionine synthase, and vitamin B12 in the form of methylcobalamin serves as a cofactor for this reaction.[16]

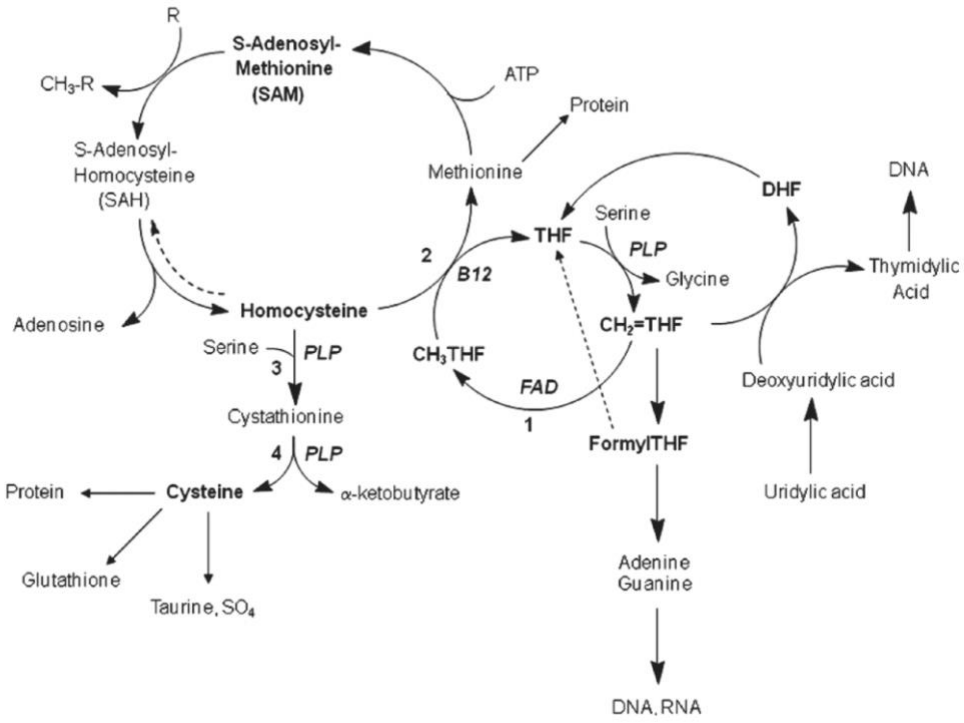

Figure 1: B vitamins, homocysteine, and one-carbon metabolism Key enzymes: 1, methylenetetrahydrofolate reductase; 2, methionine synthase, 3, cystathionine β-synthase; 4, cystathionine γ-lyase. Abbreviations: DHF, dihydrofolate; THF, tetrahydrofolate; CH_3 3, methyl; CH_2=, methylene; PLP, pyridoxal-5′-phosphate, FAD, flavin adenine dinucleotide.[16]

Methionine then has two important metabolic paths. The first path is the incorporation into protein. The second path is activation by the addition of an adenosyl group from ATP to make S-adenosylmethionine (SAM).[16] SAM, also known as the "universal methyl donor", participates in numerous methylation reactions.[16] Methyl acceptors in these reactions include important substrates such as DNA, RNA, histones, membrane phospholipids, neurotransmitters, and proteins among others.[16] In these methylation reactions, S-adenosylhomocysteine (SAH) is formed as a product which can then be metabolized back into homocysteine.[16] Homocysteine can also be catabolized

through cystathionine synthesis with PLP serving as a cofactor.[16] The cystathionine is then further metabolized to form the sulfur amino acid, cysteine, with PLP again serving as a cofactor.[16] Cysteine can be incorporated into proteins or metabolized further into the antioxidant, glutathione, and other metabolites.[16]

Given the role B vitamins play in one-carbon and homocysteine metabolism, deficiencies can have major effects on the intracellular levels of homocysteine, SAM and SAH. Deficiencies of B12 and folate can inhibit methionine synthesis leading to decreases in SAM, and deficiency of B6 can inhibit cystathionine synthesis.[16] Deficiencies of all three vitamins cause accumulation of SAH and homocysteine in cells and export of homocysteine into the blood and urine.[16] Hyperhomocysteinemia is an independent risk factor for vascular diseases leading to heart attacks and strokes, and is a risk factor for AD, dementia, and age-related cognitive dysfunction, as well as irritability, fatigue, and forgetfulness.[16] High homocysteine may affect brain function by damaging the cerebrovascular system. It also has been hypothesized that homocysteine can be metabolized to homocysteic acid in the brain leading to excitotoxicity and neuronal damage.[17] In addition, inhibition of one-carbon metabolism by B12 and folate deficiencies can impair one-carbon metabolism-dependent synthesis of DNA and RNA.[18] This can affect cell synthesis, in particular red and white blood cells, resulting in macrocytic or megaloblastic anemia.

An important aspect of one-carbon metabolism is that there are two main forms of dietary folate. The first source is reduced folates found naturally in foods, such as green leafy vegetables, citrus fruits, grains and meats. The second is folic acid, the synthetic oxidized form of the vitamin that is used in supplement pills and as fortification in foods.

If the form of folate is folic acid, it must first be metabolized to tetrahydrofolate via two rounds of reduction to become metabolically active.[18] This occurs by the action of the enzyme, dihydrofolate reductase, which first creates dihydrofolate and then tetrahydrofolate with vitamin B3 (niacin) in the form of NADPH serving as a cofactor.[18]

Alzheimer Disease is the most common cause of dementia, comprising 60-80% of all cases.[8] AD and dementia are the clinical consequences of neurodegeneration. Neurodegeneration or brain atrophy is a part of normal aging, but the rate and extent of degeneration is accelerated in those who are developing AD and dementia. The atrophy rate in those with mild cognitive impairment (an intermediate stage of cognitive decline between intact cognition and dementia) is at least twice as fast than it is with normal aging.[19] In the early stages, brain atrophy begins in the hippocampus interfering with specific cognitive functions, including memory, judgment, reasoning, language and other cognitive domains.[19]

Other types of dementia include vascular dementia, dementia with Lewy bodies and mixed dementia. Mixed dementia consists of a combination of different types of dementia such as AD and vascular dementia. Dementia is defined by symptoms of impaired cognition and memory loss. Memory loss is usually the first symptom of AD and dementia because the first neurons to become damaged in the brain are those responsible for memory.

In a healthy brain, neurons with branching extensions can signal other neurons via neurotransmitters released across synapses situated between neurons. These brain circuits underlie our thoughts, feelings, movement and memory. In AD, these neuronal circuits are interfered by two classical pathophysiological manifestations of the disease, beta-

amyloid and tau protein.[1] The protein beta-amyloid accumulates outside of neurons to form extracellular beta-amyloid plaques, which block synaptic signaling and lead to cell death.[1] The protein tau accumulates within neurons to form intracellular neurofibrillary tangles, which block nutrient transport leading to neuronal death. These plaques and tangles concentrate in the hippocampus and neocortex of the brain killing cholinergic neurons.[20] As neurons continue to die, the brain begins to shrink, and cognitive decline begins. However, these brain changes can begin for years before AD and dementia symptoms begin to show because the brain at first can compensate for these changes until a threshold of neurodegeneration is achieved and clinical impairment becomes apparent.

Vascular dementia occurs by blood vessel blockage or infarcts, and often causes initial symptoms of impaired judgment and decision making abilities.[1] Poor motor function may also be an initial symptom. About 50% of all AD cases have evidence of cerebrovascular disease, usually coinciding with AD pathology of beta-amyloid plaques and tangles.[21]

The first stages of AD are typically characterized by memory loss and confusion, but can later develop changes in behavior, decision making, and ability to recognize friends and family.[1] As the disease becomes more severe, activities of daily living such as eating, walking, and bathing may require assistance. The ability to eat and communicate may also become impaired.

Many studies[2,3,4,5,22,23] have demonstrated significant associations between elevated plasma homocysteine and both AD and dementia. However, it is unclear if hyperhomocysteinemia is a causative risk factor for AD and vascular dementia, or if it is simply a biomarker of these conditions. A seminal case-control study by Clarke et al in

1998[2] assessed blood levels of homocysteine, vitamin B12 and folate in 164 participants who were diagnosed with AD and 108 healthy control participants of similar age with no evidence of cognitive impairment. Patients diagnosed with dementia of the Alzheimer type (DAT) were found to have had faster disease progression amongst those with the highest plasma homocysteine concentrations at baseline after a 3-year follow up period. More importantly, this case-control study included postmortem histological analysis showing that 67 of the 103 patients who died had neuropathological evidence of AD. It also showed that B12 concentrations were lower in histologically confirmed AD cases. The odds ratio for confirmed AD was 4.5 (95% C.I.: 2.2, 9.2) when plasma homocysteine concentrations were in the top tertile ($\geq$14 umol/L) compared to the bottom tertile ($\leq$11 umol/L).[2] In addition, vitamin B12 and folate blood levels were significantly lower while homocysteine levels were significantly higher in those with AD than in those in the control group. The results of this study confirmed elevated plasma homocysteine is associated with increased risk of having AD in older adults. However, a limitation of this study is the cross-sectional design which cannot prove a cause-and-effect relationship.

Subsequent longitudinal studies aimed to distinguish whether high homocysteine levels in older adults can cause AD and dementia determined by a series of neuropsychological tests. In a study by Seshadri et al,[22] 1092 older adult participants without dementia were examined from baseline to an average 8-year follow-up to determine if elevated homocysteine was a risk factor for AD and dementia. The study showed that participants with plasma homocysteine levels in the highest quartile had the highest risk of AD and dementia compared to the three lower quartiles combined. This risk of AD was doubled when plasma homocysteine levels were greater than 14 umol/L

(fourth quartile). This observational study indicated that the elevation of plasma homocysteine has a direct association with risk of AD and dementia suggesting that high plasma homocysteine levels precede the onset of dementia.

After establishing the link between increased plasma homocysteine levels and risk of AD and dementia, the focus of research began to shift to how homocysteine can be influenced by folate and vitamin B12. A cohort study by Haan et al,[23] examined the incidence of cognitive impairment and dementia with homocysteine levels and B vitamins among participants in the Sacramento Area Latino Study on Aging (SALSA). Participants were recruited after the U.S. had initiated mandatory folic acid fortification in 1998 which had decreased the average plasma homocysteine concentrations in the general population due to folate's important role in homocysteine metabolism. The research concluded that homocysteine is an independent risk factor for cognitive impairment despite folic acid fortification.[23] However, plasma B12 levels in the lowest and highest tertiles had significantly higher and lower rates of homocysteine associated cognitive impairment and dementia.[23] Thus, researchers have focused on the importance of vitamin B12 in relation to homocysteine metabolism and how it can be modified.

Brain atrophy is found in older adults, with increased rates in those who have cognitive impairment, AD and dementia. One randomized double blind study by Smith et al[3] supplemented participants diagnosed with mild cognitive impairment (MCI) with folic acid (0.8 mg/d), vitamin B6 (20 mg/d) and vitamin B12 (0.5 mg/d) for over 2 years and assessed their rates of brain atrophy though MRI scans in comparison to a control group receiving placebo pills. This trial concluded that supplementation of homocysteine-lowering vitamins can slow the rate of brain atrophy in older adults with MCI.

Limitations to this study include that they were unable to pinpoint which of the B vitamins made the largest impact because the supplement contained folic acid, vitamin B6, and vitamin B12 combined. Another limitation to this study was the relationship between brain atrophy and cognitive decline; although significant associations were found, the study only focused on the rate of brain atrophy.

Continuing research on this same study sample of those 70 years and older with MCI was conducted to assess their cognition. In a study by de Jager et al,[5] B vitamin supplementation in those participants who were above the median homocysteine concentration at baseline exhibited slowing of cognitive and clinical decline. Clinical decline was assessed by the global dementia rating score (CDR) and the informant questionnaire of cognitive decline in the elderly (IQCODE) score. This clinical effect suggests that B vitamin treatment in those with MCI can result in maintenance of cognitive function or even reversal of cognitive impairment. An interpretation of the Smith et al and the de Jager et al study findings are that B vitamin supplements lower homocysteine, which slows brain atrophy and slows decline of cognitive function.

Further analysis of this same study sample aimed to find the effect of B vitamin supplementation on the atrophy of specific regions of the brain was published by Douaud et al.[4] This experimental study aimed to find the effect of B vitamin treatment over a 2-year period on specific gray matter regions of the brain (as opposed to the whole brain as was studied in the Smith et al[3] publication). This study found that the group receiving the B vitamin supplements had a significant reduction of atrophy in the posterior brain regions which are usually affected by AD (i.e., bilateral hippocampus, parahippocampal gyrus, retrosplenial precuneus, lingual and fusiform gyrus and cerebellum). The study

also found average loss of gray matter in the treatment group was less (0.5%/year) compared to the placebo group (3.7%/year).

In summary, it is evident from prior work that B vitamin supplementation lowers elevated homocysteine levels resulting in a decrease in cognitive decline in older adults with MCI. In the Smith et al study,[3] high dose B vitamin supplementation was able to lower homocysteine and slow brain atrophy. In de Jager et al,[5] specifically those above the median and in the upper quartile of homocysteine, had significant attenuation of rate of cognitive loss with B vitamin supplementation. Finally, in the study by Douaud et al.[4] B vitamin supplementation reduced brain atrophy in brain regions usually affected by AD. The results of this randomized control trial demonstrate that the use of B vitamin supplementation is an effective treatment to lower homocysteine levels and in turn, decrease the rate of cognitive decline. Moreover, key findings from prior work[3-5] indicate that elevated homocysteine levels may be an early, modifiable biomarker of AD, dementia, and age-related cognitive decline.

The goal of the study/data analysis described below is to test this supposition in a group of healthy older adults with no clinical indication of neurodegenerative disease or cognitive impairment.

CHAPTER THREE: METHODS

Participants

A secondary data analysis was performed of this original cross-sectional study that was approved by the University of California San Francisco (UCSF) institutional review board where all participants provided written informed consent.[24,25] Participants were community dwelling older adults (age 69-90 y; N=243, 129 women, 114 men). Eligibility criteria were as follows: participants had to be over the age of 65 years with no dementia (Clinical Dementia Rating Score=0) and no overt cognitive impairment (Mini-Mental State Exam (MMSE) score >25 out of 30 points total). Eligibility screening also included an informant interview, a cognitive screen and a neurological exam. Exclusion criteria included current depression, medical illness, substance abuse, and any condition that impaired cognition such as dementia or a major psychiatric diagnosis. Within a 60-day period blood sampling, MRI brain imaging and cognitive assessment tests were conducted. The cognitive function tests were chosen to reflect the regions of the brain in and around the medial lobe that are typically affected in AD.

MRI Brain Scans

The brain scans were conducted using a 3.0 Tesla Siemans TIM Trio Scanner and T1 MPRAGE structural brain images were analyzed by Freesurfer. The TIM Trio Scanner is at the UCSF Neuroscience Imaging Center and has a 12-channel head coil. Volumetric magnetization prepared rapid gradient-echo sequencing was used to acquire whole brain images. Freesurfer is an open-source software suite for processing and

analyzing brain MRI images by segmenting T2-fluid attenuated inversion (FLAIR)[25] recovery images to show the quantitative measure of white matter hyperintensities (WMH). Each participant's reconstructed cortical surface models were inspected to ensure segmentation accuracy. The scans that had estimation errors were edited and rerun through the segmentation program. Before study recruitment, there was a final quality check for processing errors and image artifacts.

Blood Analytes

Blood samples were collected to determine concentrations of plasma total homocysteine, RBC folate, serum vitamin B12 and serum creatinine. All blood assays were carried out in the University of California Davis Medical Center clinical laboratory. Blood was collected into EDTA plasma tubes and serum separator tubes. After blood collection, the EDTA plasma tubes were placed on ice, while the serum separator tubes were left at room temperature for 30-60 minutes to clot. One EDTA plasma tube was saved for measurement of RBC folate. The rest of the tubes were centrifuged at room temperature at 2500 rpm for 15 minutes. Plasma and serum samples were stored at -80 ^{0}C until analysis. Plasma total homocysteine was measured by HPLC with post-column fluorescence detection.[26] RBC folate and serum vitamin B12 were measured by automated chemiluminescence using a Siemans Diagnostics ACS 180. Serum creatinine was measured by Jaffe Rate Reaction using a Beckman Coulter Synchron LX20 instrument. Abnormal values for each of the blood analytes were defined as: homocysteine >13 umol/L;[27] RBC folate <160 ng/ml (standard clinical cutoff); vitamin

B12 <148 pmol/L (standard clinical cutoff); and creatinine >1.3 mg/dL (standard clinical cutoff).

Cognitive Function Tests

Cognitive function tests included assessments of global cognitive function, processing speed, verbal memory, and executive functions using instruments commonly used in the 65 years and older population.

Global Cognitive Function: Global cognitive function was assessed using the MMSE which includes several different tests to screen for deficits in various domains of cognitive function.[28] The MMSE includes eleven questions and takes 5-10 minutes to complete. The maximum score is 30 indicating a perfect score and that no dementia or overt cognitive impairment is present. There are two sections, with the first consisting of vocal responses with a maximum score of 21. The second consists of reading and writing with a maximum score of 9. The first question of the MMSE is "What is the (year)(season)(date)(day)(month)?" One point is awarded for each part of the question answered correctly for a maximum of 5 points. The second question is "Where are we: (state)(county)(town)(hospital)(floor)?" (5 points maximum). These first two questions evaluate the individual's orientation. The next questions evaluate the individual's registration; they must name 3 objects with 1 second allowed to say each. Then the individual is asked to name all 3 after the examiner has said them. 1 point is given for each correct answer for a maximum of 3 points. The examiner then repeats the words until the individual learns all 3; the number of trials is counted and recorded. The next portion assesses attention and calculation. The individual is instructed to count down

from one hundred by sevens; this is called the Serial 7's test. The examiner stops after 5 answers, and the individual gets 1 point for each correct answer. An alternative to this test is to spell the word "world" backwards. The last portion of the first part of the MMSE is to assess recall. The examiner asks for the 3 objects that the individual repeated earlier. They get one point for each object recalled. The second part of the MMSE is worth 9 points and assesses language. The examiner will point to their wristwatch and ask the participant what it is and does the same for a pencil; the individual receives 1 point for each. Next, the examiner will ask the individual to repeat the same short sentence after the examiner says one (1 point). The examiner then gives the individual a piece of paper and gives them a 3-stage command such as, "Take a paper in your right hand, fold it in half, and put it on the floor". The individual receives 1 point for each part of the command done correctly. The next test the examiner writes a sentence on a piece of paper for the individual to read and obey such as "close your eyes". The individual receives 1 point if they close their eyes. The next test the individual is given a blank piece of paper and is instructed to write one sentence. If the sentence includes a subject, verb, and makes sense they receive 1 point. In the final test the examiner draws intersecting pentagons and has the participant copy the image. To receive a point all 10 angles must be present, and the two pentagons must intersect. Finally, the examiner rates the participant on a scale from alert to drowsy, stupor, or coma.

Processing Speed: Processing speed tests consisted of both verbal and spatial assessments. The participants' verbal processing was assessed by their ability to mentally arrange a random series of numbers and letters in numerical and alphabetical order.[29] Spatial processing was assessed by the participants' ability to process and match numbers

and symbols. The processing speed tests are scored on scales of 0-15 points, with higher scores indicating better processing speed.

Verbal Memory: Verbal memory tests consisted of word recognition and short and long delayed recall assessments. The word recognition test assessed the ability of the participants to immediately recall a list of words after a series of learning trials.[30] The examiner orally presents two word lists that each have 16 words; each is read 5 times before the individual attempts to recall the items.[31] Short delayed recall is assessed by having the individual recall a learned list of words after a brief period of distraction.[30] Long delayed recall is assessed by having the individual recall a learned list of words after an extended period of distraction.[30] After 20 minutes of nonverbal tasks being administered, the individual is asked to recall this list. These tests are summarized in a *T* score (M=50, SD=10).[31] The T score represents the number of standard deviations or units of measure above or below the average score. In this case the T score is based on norms established for different ages and for males and females.[31] Higher scores reflect better performance.

Executive Function: Executive functions are assessed using tests of timed word generation, cognitive switching, attention, working memory, and cognitive inhibition. The word generation I test assesses the individual's ability to name as many words as they can starting with a specific letter within one minute.[32] The word generation II test has the individual name as many animals as they can within one minute. Both word generation tests measure verbal fluency.[32] Cognitive switching is assessed using the trail making test. In this test the individual draws connections between numbers and letters in alternating numerical and alphabetical order.[32] Cognitive inhibition is tested by the

Stroop test, which assesses the ability of the individual to distinguish words for blue, green, and red and the color of the ink that the words are printed in.[33] Forward and backward digit span tests are used to assess attention and working memory, respectively.[32] The digit span tests consist of multiple random strings of numbers (starting with a string of 3 numbers) recited verbally over six trials with a number added on for each consecutive trial. The individual is instructed to repeat the strings of numbers in order (forward digit span) or reverse order (backward digit span). This continues until the participant fails on two consecutive trials of the same digit span length.[25]

Statistical Analyses

Descriptive statistics were performed for all participants using sex, age, education, homocysteine, RBC folate, vitamin B12 and creatinine as variables. Pearson correlations were performed to assess the associations of these variables with total plasma homocysteine. Multiple regression analysis was then used to assess the association between plasma total homocysteine (natural log transformed due to non-normal distribution with tailing toward higher values) as the primary independent variable and regional brain volumes as the dependent variables. The covariates for these analyses included age (years), sex, education (years), serum vitamin B12, RBC folate, serum creatinine and intracranial volume. Multiple regression analysis was also used to assess the association between total plasma homocysteine (natural log transformed) as the primary independent variable and cognitive function tests as the dependent variables. The covariates for these analyses included age, sex, education, RBC folate, serum B12 and serum creatinine. Covariates were not normally distributed so RBC folate, serum B12 and

serum creatinine were natural log-transformed before the analysis. All analyses were performed using STATVIEW for MACINTOSH (version 5.0.1; Abacus Concept, Berkeley, CA).[34]

CHAPTER FOUR: RESULTS

The main research question investigated is whether elevated plasma homocysteine is an early modifiable biomarker for risk of age-related cognitive decline, dementia and AD. The first hypothesis focuses on elevated homocysteine as a risk factor for neurodegeneration assessed by MRI as indicated by increased ventricular size and decreased regional brain volumes in healthy older adults without overt clinical cognitive impairment. The second hypothesis focuses on whether elevated homocysteine is a risk factor for impaired cognitive function in healthy older adults without overt clinical cognitive impairment.

Participant characteristics are presented in **Table 1**. The overall number of participants was N=243. The number of participants with complete data for the brain imaging analyses was N=190. The number of participants with complete data for the cognition analyses ranged from N=166 to N=235. The participants were 60-95 years of age (mean ± SD: 72.2 ± 6.6 years). Fifty-three percent of the participants were female. The participants were well educated with a mean number of years of education of 17.5 ± 2.2 years. The participants had no evidence of global cognitive impairment, as indicated by mean MMSE scores (29.3 ± 0.9 on a 30-point scale).

Primary predictors of total plasma homocysteine concentrations are presented in **Table 2**. Age (P=0.01) and serum creatinine (P<0.0001) were directly correlated with homocysteine, and education was inversely correlated (P=0.023). Sex was also associated with homocysteine, with women having lower homocysteine than men (P=0.001). These variables were included as covariates in the imaging and cognition multiple regression

models described below. RBC folate and serum vitamin B12 were not significantly correlated with homocysteine. RBC folate and serum vitamin B12 may be independently associated with brain structure and function, and therefore were included as covariates in the imaging and cognition multiple regression models *a priori*.

Table 1: Study Sample Characteristics

	N	Value	% Abnormal (cutoff value)
Sex (% Female)	243	53	---
Age (y)	243	72.2 ± 6.6	---
Education (y)	243	17.5 ± 2.2	---
Homocysteine (μmol/L)	241	8.60 ± 3.59	8.9% (>13)
RBC folate (ng/ml)	239	541 ± 148	0% (<160)
Vitamin B12 (pg/ml)	242	456 ± 244	1.1% (<200)
Creatinine (mg/dL)	242	0.97 ± 0.31	11.0% (>1.3)

Table 2: Predictors of Total Plasma Homocysteine

	Coefficient (95% C.I.)	*P*
RBC Folate (ng/ml)	0.003 (-0.124, 0.131)	0.963
Vitamin B12 (pg/ml)	-0.074 (-0.198, 0.054)	0.257
Creatinine (mg/dL)	0.360 (0.245, 0.466)	**<0.0001**
Age (y)	0.165 (0.039, 0.285)	**0.010**
Sex	-0.216 (-0.334, -0.092)	**0.001**
Education (y)	-0.147 (-0.268, -0.021)	**0.023**

Associations between homocysteine and each variable were assessed using Pearson correlations tests. Abbreviation: RBC, red blood cell

Imaging Results

A series of 3 regression models assessing the associations between homocysteine and regional brain volumes are presented in **Table 3.**

Before adjustment for confounding by demographic and biochemical variables (Model 1), but including control for intracranial volume, homocysteine was inversely

correlated with 11 out of 15 regional brain volumes ($P<0.05$), and borderline significant for the remaining 4 regional brain volumes ($P>0.05$ and <0.1). The R^2 values for these multiple regressions indicate that homocysteine, with controlling for intracranial volume, explained 12%-54% ($R^2 = 0.120$-0.540) of the variance in regional brain volumes among the study participants. Homocysteine remained inversely correlated with 9 of the 15 regional brain volumes ($P<0.05$) after the addition of the demographic variables age, sex, and education (Model 2). With the addition of these demographic variables the percentages of the variance in regional brain volumes explained by the model ranged from 23%-49% ($R^2 = 0.227$-0.490). After additional controlling for ln RBC folate, ln vitamin B12 and ln creatinine (Model 3), homocysteine was inversely correlated with 12 of the 15 regional brain volumes ($P<0.05$). After the addition of the blood biomarkers the percentages of the variance in regional brain volumes explained by the model ranged from 26%-50% ($R^2 = 0.263$-0.504) representing only modest improvement in the overall model compared with Model 2.

Cognitive Function Results

A series of 3 regression models describing the associations between homocysteine and cognitive function tests are presented in **Table 4.**

Before adjustment for confounding by demographic and biochemical variables (Model 1), homocysteine was inversely correlated with 5 out of 12 cognitive function tests ($P<0.05$). The R^2 values for this simple regression indicate that homocysteine explained 0.1%-14% ($R^2 = 0.001$-0.143) of the variance in cognitive function scores within the sample. Homocysteine remained inversely correlated with 3 of the 12

cognitive function tests after the addition of the demographic variables, age, sex, and education (Model 2). With the addition of these demographic variables the percentages of the variance in cognitive function scores explained by the model increased to 4.7%-18% (R^2= 0.047-0.176). After additional controlling for ln RBC folate, ln vitamin B12 and ln creatinine, homocysteine remained inversely correlated with 3 of the 12 cognitive function scores ($P<0.05$) (Model 3). After the addition of the blood biomarkers the percentage of the variance in cognitive function scores explained by the model increased modestly to 5.8%-19% (R^2= 0.058-0.189). In the final model (Model 3), the three cognitive function tests that remained significantly associated with homocysteine were Word Generation II, Cognitive Switching, and Working Memory ($P\leq0.025$). These three represent 3 of the 6 tests of executive functions conducted on the study sample. In contrast, no significant associations of homocysteine with global cognitive function (MMSE), processing speed (Verbal and Spatial), and verbal memory (Word Recognition, Short and Long Delayed Recall) were observed.

Table 3: Multiple Linear Regression Models for Homocysteine (Independent) vs. Regional Brain Volumes (Dependent)

	Model 1			Model 2			Model 3		
Brain Regions	R^2	Coefficient ± SE	P	R^2	Coefficient ± SE	P	R^2	Coefficient ± SE	P
Left Lateral Ventricle	0.314	5290 ± 1447	**<0.001**	0.491	3688 ± 1351	**0.007**	0.494	3410 ± 1419	**0.017**
Right Lateral Ventricle	0.310	4037 ± 1266	**0.002**	0.477	3077 ± 1194	**0.011**	0.485	3500 ± 1248	**0.006**
Left Thalamus Proper	0.319	-299 ± 113	**0.009**	0.412	-222 ± 113	0.051	0.454	-248 ± 115	**0.033**
Right Thalamus Proper	0.347	-291 ± 112	**0.010**	0.478	-220 ± 109	**0.045**	0.504	-260 ± 112	**0.021**
Left Caudate	0.303	-173 ± 73	**0.018**	0.303	-176 ± 79	**0.026**	0.309	-193 ± 82	**0.021**
Right Caudate	0.292	-137 ± 74	0.064	0.293	-126 ± 80	0.115	0.297	-128 ± 84	0.127
Left Putamen	0.165	-198 ± 107	0.065	0.227	-173 ± 111	0.122	0.265	-247 ± 114	**0.032**
Right Putamen	0.169	-184 ± 94	0.052	0.228	-148 ± 98	0.135	0.263	-201 ± 101	**0.049**
Left Pallidum	0.237	-71 ± 37	0.052	0.377	-74 ± 36	**0.041**	0.405	-99 ± 37	**0.008**
Right Pallidum	0.307	-66 ± 29	**0.023**	0.408	-79 ± 29	**0.006**	0.430	-98 ± 30	**0.001**
Left Hippocampus	0.120	-227 ± 77	**0.004**	0.431	-117 ± 67	0.083	0.452	-132 ± 70	0.059
Right Hippocampus	0.128	-213 ± 79	**0.008**	0.389	-119 ± 72	0.099	0.409	-133 ± 74	0.075
Left Amygdala	0.180	-90 ± 39	**0.022**	0.349	-88 ± 38	**0.020**	0.392	-111 ± 38	**0.004**
Right Amygdala	0.160	-69 ± 32	**0.033**	0.307	-65 ± 32	**0.040**	0.335	-65 ± 33	**0.048**
Brain Stem	0.305	-845 ± 418	**0.045**	0.440	-945 ± 407	**0.021**	0.454	-1019 ± 423	**0.017**

Multiple linear regression models are as follows: Model 1 = ln (homocysteine); Model 2 = model 1 + age + sex + education; Model 3 = Model 1 + Model 2 + ln (B12) + ln (RBC folate) + ln (creatinine). R^2 values are those for the entire model including ln (homocysteine) and the covariates. Coefficients and *P* values are for ln (homocysteine) within each model. Intracranial volume is controlled for in each model. Number of subjects with complete data in the models: N=190.

Table 4: Multiple Linear Regression Models for Homocysteine (Independent) vs. Cognitive Function Tests (Dependent)

	Model 1			Model 2			Model 3		
Cognitive Test	R^2	Coefficient ± SE	P	R^2	Coefficient ± SE	P	R^2	Coefficient ± SE	P
Global									
MMSE[1]	0.020	-0.37 ± 0.18	**0.042**	0.125	-0.16 ± 0.18	0.393	0.122	-0.18 ± 0.20	0.368
Processing Speed									
Verbal[2]	0.013	0.38 ± 0.22	0.078	0.087	0.18 ± 0.22	0.431	0.088	0.18 ± 0.24	0.456
Spatial[3]	0.019	0.50 ± 0.23	**0.033**	0.176	0.29 ± 0.23	0.200	0.179	0.22 ± 0.24	0.355
Verbal Memory									
Word Recognition[4]	0.018	-0.26 ± 0.14	0.054	0.141	-0.03 ± 0.14	0.834	0.144	-0.04 ± 0.15	0.782
Short Delayed Recall[5]	0.016	-1.14 ± 0.62	0.070	0.176	-0.04 ± 0.61	0.954	0.189	-0.08 ± 0.65	0.900
Long Delayed Recall[6]	0.018	-1.17 ± 0.60	0.053	0.122	-0.22 ± 0.61	0.719	0.137	-0.45 ± 0.65	0.496
Executive Function									
Word Generation I[7]	0.001	0.44 ± 0.99	0.659	0.038	0.82 ± 1.04	0.432	0.058	0.30 ± 1.11	0.788
Word Generation II[8]	0.074	-4.04 ± 1.00	**<0.0001**	0.145	-3.09 ± 1.03	**0.003**	0.154	-2.97 ± 1.10	**0.008**
Cognitive Switching[9]	0.043	-7.07 ± 2.36	**0.003**	0.145	-4.71 ± 2.39	**0.050**	0.155	-6.37 ± 2.53	**0.013**
Attention[10]	2.7E-6	-0.01 ± 0.23	0.982	0.047	-0.04 ± 0.24	0.868	0.058	-0.16 ± 0.26	0.540
Working Memory[11]	0.024	-0.54 ± 0.24	**0.029**	0.068	-0.52 ± 0.26	**0.042**	0.089	-0.62 ± 0.27	**0.025**
Cognitive Inhibition[12]	0.010	-3.28 ± 2.32	0.160	0.127	-1.06 ± 2.34	0.650	0.125	-1.70 ± 2.51	0.500

Multiple linear regression models are as follows: Model 1 = ln (homocysteine); Model 2 = model 1 + age + sex + education; Model 3 = Model 1 + Model 2 + ln (B12) + ln (RBC folate) + ln (creatinine). R^2 values are those for the entire model including ln (homocysteine) and the covariates. Coefficients and *P* values are for ln (homocysteine) within each model.
Number of subjects with complete data in the models: [1]N=199, [2]N=235, [3]N=231, [4]N=201, [5]N=201, [6]N=200, [7]N=166, [8]N=203, [9]N=197, [10]N=196, [11]N=196, [12]N=191. Abbreviation: MMSE, mini-mental state examination

CHAPTER FIVE DISCUSSION

The goal of this investigation was to assess homocysteine as an early indicator of neurodegeneration and cognitive decline. We assessed this in an elderly cohort with no overt cognitive impairments and few health issues. In this cohort, homocysteine was inversely correlated with 12 of 15 regional brain volumes and 3 of 12 cognitive function tests after controlling for sex, age, education, ln RBC folate, ln vitamin B12, and ln creatinine. This suggests that measuring homocysteine in older adults can be an early screen for neurodegenerative disease and cognitive decline.

These findings are generally consistent with previous studies that assessed the relationship between homocysteine and cognitive function in those with mild cognitive impairment, dementia or AD. One of the early seminal studies that assessed this association was by Clarke et al in 1998.[2] This study compared those with AD with controls of similar age with no cognitive impairment. This study focused on the later stages of AD when there is significant brain injury and serious loss of cognitive function. The study found that participants with elevated homocysteine had a higher odds ratio for having AD. The diagnosis of AD was pathologically confirmed through autopsies. This was one of the first studies that demonstrated elevated plasma homocysteine is associated with increased risk of having AD in older adults. However, this was a case-control, cross-sectional study that could not establish a cause-and-effect relationship between homocysteine and AD.

Subsequent studies by Seshadri et al[22] and Haan et al[23] looked at the longitudinal association between elevated homocysteine and development of cognitive impairment and dementia. The Seshadri et al[22] study was an 8-year observational study that assessed the association between homocysteine and incidence of clinical dementia diagnosis in the Framingham Study cohort. Plasma homocysteine in the highest quartile, compared with the three lower quartiles, was associated with increased risk of dementia. Haan et al[23] made a similar observation in the Sacramento Area Latino Study on Aging cohort: homocysteine was directly correlated with increased risk of incident cognitive impairment (defined as dementia or cognitively-impaired but not dementia) over a 4.5-year follow-up. In addition, Haan et al[23] found that plasma B12 levels in the lowest and highest tertiles had respectively higher and lower rates of homocysteine associated cognitive impairment and dementia compared with those in the middle tertile of plasma B12, suggesting that elevated homocysteine due to low B12 status explained at least part of the association between homocysteine and incident cognitive impairment/dementia. In contrast to Clarke et al[2], the Seshadri et al[22] and Haan et al[23] studies focused on participants who were at earlier stages of cognitive impairment before development of dementia and AD, and established that elevated homocysteine preceded the onset of clinical dementia. However, they were still unable to conclusively address the issue of cause-and-effect because neurodegenerative processes underlying the ultimate development of dementia may occur over many years or decades.

Evidence that lowering homocysteine with B vitamin supplements can slow the progression of neurodegeneration and age-related cognitive decline comes from the work of Smith and colleagues.[3-5] This study looked at older adults with mild cognitive

impairment who were given folic acid, vitamin B6 and vitamin B12 supplements and assessed over 2 years. The Smith et al[3] study assessed their global brain atrophy using MRI scans and cognitive testing. This trial concluded that supplementation of homocysteine lowering vitamins can slow the rate of brain atrophy. The de Jager et al[5] study assessed cognition in the same cohort of older adults with mild cognitive impairment using the global dementia rating score (CDR) and the informant questionnaire of cognitive decline in the elderly (IQCODE) score. This study found that B vitamin supplementation can result in maintenance of cognitive function or even reversal of cognitive impairment in those with mild cognitive impairment. The Douaud et al[4] study aimed to find the effect of B vitamin supplementation on atrophy of specific gray matter regions of the brain. This study found significant reduction of atrophy in posterior brain regions which are usually affected by AD. The results of these three studies indicate that B vitamin supplementation that lowered homocysteine levels can slow global and regional brain atrophy and prevent loss of cognitive function as indicated by MRI scans, CDR and IQCODE. These study results suggest that elevated homocysteine levels or low B vitamin status may be a causative or contributing agent of neurodegeneration.

The studies cited above clearly established a significant association between elevated homocysteine, neurodegeneration, and age-associated cognitive impairment. However, these studies focused on cohorts that were either at the middle or later stages of decline. In contrast, the present study suggests that homocysteine is predictive of subtle cognitive impairment and changes in regional brain volumes even before overt clinical impairment becomes apparent. Data from this study coupled with the findings of those

studies cited above highly suggest that homocysteine could be used as a modifiable blood biomarker screen for the early stages of neurodegeneration and age-related cognitive decline. Consistent with this is the finding in the present study that the cognitive functions that were associated with homocysteine were executive functions. Word generation II, cognitive switching, and working memory, each a measure of different aspects of executive function, were inversely correlated with homocysteine, while other cognitive domains were not correlated with homocysteine, including processing speed and verbal memory. Executive functions are arguably the more complex cognitive functions, which may be the most sensitive as indicators of cognitive decline. It can be surmised that at the early stages of decline in cognitive function in adults it will be the executive functions that will start to show the first effects of neurodegeneration. These may also be the functions that are not as overtly apparent in older adults.

A limitation of this study is that the possibility of reverse causality cannot be dismissed due to its cross-sectional design. Elevated homocysteine may be a consequence of neurodegenerative disease. People with impaired cognitive function may have altered diets which may lead to deficiencies that can cause elevated homocysteine. Alternatively, it is possible that the neurodegenerative process may cause elevated homocysteine. Neuroinflammatory oxidative stress may affect homocysteine metabolism and cause hyperhomocysteinemia.[35] We also cannot rule out the possibility of other confounding factors that were not controlled for in our analyses, such as ApoE4 genotype which is a major risk factor for Alzheimer Disease and dementia, but was not assessed in this study sample. Another limitation of this study is that we did not account for multiple comparisons in our statistical analyses of the associations between homocysteine and the

regional brain volumes and cognitive function tests. However, because the homocysteine was associated with multiple brain regions and cognitive function tests, this suggests that the associations were not due to chance.

A strength of this study is that it includes both imaging and cognitive function data. It is uncommon that published studies include both types of data and have sufficient power to see associations between a primary independent variable (in this case homocysteine) and both brain structures and cognitive function test scores. This study was also able to control for the primary determinants of homocysteine, including folate, vitamin B12, and renal function (as indicated by serum creatinine) and still have sufficient power to see significant associations. Another strength is that the study participants were those who were in generally good health at the time of their recruitment, which allowed for the assessment of associations before clinical cognitive impairment was manifested.

In summary, the findings from this study suggest elevated homocysteine can be used as an early modifiable risk factor for impaired cognitive function and neurodegeneration in healthy older adults without overt clinical cognitive impairment. Further research is needed to confirm if lowering homocysteine with B vitamin supplements will slow the progression of neurodegeneration and cognitive decline in older adults. Nonetheless, the studies by Smith and colleagues[3-5] suggest screening for high levels of homocysteine in older adults is warranted, and if elevated, supplementation with B vitamins may be beneficial.

REFERENCES

1. 2016 Alzheimer's disease facts and figures. *Alzheimers Dement.* 2016;12(4):459-509.
2. Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease. *Arch Neurol.* 1998;55(11):1449-1455.
3. Smith AD, Smith SM, de Jager CA, et al. Homocysteine-Lowering by B Vitamins Slows the Rate of Accelerated Brain Atrophy in Mild Cognitive Impairment: A Randomized Controlled Trial. *PLoS ONE.* 2010;5(9):1-10.
4. Douaud G, Refsum H, de Jager CA, et al. Preventing Alzheimer's disease-related gray matter atrophy by B-vitamin treatment. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(23):9523-9528.
5. de Jager CA, Oulhaj A, Jacoby R, Refsum H, Smith AD. Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial. *International Journal of Geriatric Psychiatry.* 2012;27(6):592-600.
6. Dementia. Global action plan on the public health response to dementia 2017-2025 Web site. https://www.who.int/news-room/fact-sheets/detail/dementia. Updated September 2021. Accessed2021.
7. US burden of Alzheimer's disease, related dementias to double by 2060. https://www.cdc.gov/media/releases/2018/p0920-alzheimers-burden-double-2060.html. Published 2018. Updated September 20,2019. Accessed2021.
8. Alzheimer's Disease and Related Dementias. Division of Population Health. https://doi.org/10.1016/j.jalz.2018.06.3063. Updated October 26, 2020. Accessed2-21.
9. Ageing and Health. https://www.who.int/news-room/fact-sheets/detail/ageing-and-health. Published 2021. Updated October 4, 2021. Accessed.
10. Vespa J. The U.S. Joins Other Countries With Large Aging Populations. https://www.census.gov/library/stories/2018/03/graying-america.html Published 2018. Updated October 9, 2021. Accessed October 25, 2021.
11. Evans DA, Funkenstein HH, Albert MS, et al. Prevalence of Alzheimer's disease in a community population of older persons. Higher than previously reported. *Jama.* 1989;262(18):2551-2556.
12. Briggs R, Kennelly SP, O'Neill D. Drug treatments in Alzheimer's disease. *Clin Med (Lond).* 2016;16(3):247-253.
13. Edwards Iii GA, Gamez N, Escobedo G, Jr., Calderon O, Moreno-Gonzalez I. Modifiable Risk Factors for Alzheimer's Disease. *Front Aging Neurosci.* 2019;11:146.
14. Green R, Miller JW. Vitamin B12 deficiency is the dominant nutritional cause of hyperhomocysteinemia in a folic acid-fortified population. *Clin Chem Lab Med.* 2005;43(10):1048-1051.
15. Ganguly P, Alam SF. Role of homocysteine in the development of cardiovascular disease. *Nutrition Journal.* 2015;14(1):6.
16. Miller J. Homocysteine, B vitamins, and cognitive function. In: Kanarek R LH, ed. *Diet, Brain, and Behavior.* Boca Raton, FL: CRC Press; 2012.

17. Miller J. Homocysteine. In: B. C, ed. *Encyclopedia of Human Nutrition.* Vol 2. Waltham MA: Academic Press.
18. Miller JW. <2011 FolicAcidFortification.pdf>. *Vitamins in the Prevention of Human Diseases.* 2011:273-293.
19. Tabatabaei-Jafari H, Shaw ME, Walsh E, Cherbuin N. Regional brain atrophy predicts time to conversion to Alzheimer's disease, dependent on baseline volume. *Neurobiol Aging.* 2019;83:86-94.
20. Nelms M, Sucher KP. Nutrition Therapy and Pathophysiology Book Only. 2019.
21. Iadecola C, Gottesman RF. Cerebrovascular Alterations in Alzheimer Disease. *Circ Res.* 2018;123(4):406-408.
22. Seshadri S, Beiser A, Selhub J, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *The New England journal of medicine.* 2002;346(7):476-483.
23. Haan MN, Miller JW, Aiello AE, et al. Homocysteine, B vitamins, and the incidence of dementia and cognitive impairment: results from the Sacramento Area Latino Study on Aging. *The American journal of clinical nutrition.* 2007;85(2):511-517.
24. Bettcher BM, Watson CL, Walsh CM, et al. Interleukin-6, age, and corpus callosum integrity. *PLoS One.* 2014;9(9):e106521.
25. Frazier DT, Bettcher BM, Dutt S, et al. Relationship between Insulin-Resistance Processing Speed and Specific Executive Function Profiles in Neurologically Intact Older Adults. *J Int Neuropsychol Soc.* 2015;21(8):622-628.
26. Gilfix BM, Blank DW, Rosenblatt DS. Novel reductant for determination of total plasma homocysteine. *Clin Chem.* 1997;43(4):687-688.
27. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *N Engl J Med.* 1999;340(19):1449-1454.
28. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res.* 1975;12(3):189-198.
29. Wechsler D. *Wechsler Adult Intelligence Scale.* 4th ed ed. San Antonio: Pearson; 2008.
30. Delis DCK, J.H. Kaplan, E. & Ober,B.A. Manual for the California Verbal Learning Test (CVLT-II). *The Psychological Corporation.* 2000;San Antonio, TX.
31. Donders J. A Confirmatory Factor Analysis of the California Verbal Learning Test—Second Edition (CVLT-II) in the Standardization Sample. *Assessment.* 2008;15(2):123-131.
32. Stebbins GT. *Textbook of Clinical Neurology.* 3rd ed. Philadelphia: Saunders; 2007.
33. Stroop JR. Studies of interference in serial verbal reactions. *J Exp Psychol.* 1992;121 (1):15-23.
34. Ramos MI, Allen LH, Mungas DM, et al. Low folate status is associated with impaired cognitive function and dementia in the Sacramento Area Latino Study on Aging. *Am J Clin Nutr.* 2005;82(6):1346-1352.

35. Ho PI, Collins SC, Dhitavat S, et al. Homocysteine potentiates beta-amyloid neurotoxicity: role of oxidative stress. *J Neurochem.* 2001;78(2):249-253.