The Role of Choline in Cognitive Performance and Cerebrovascular Health of Older Adults

Doctoral Thesis

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Abstract

Choline is an essential nutrient with multiple links to brain health. As a component of phospholipids, choline is involved in cell signaling, lipid transport, and cell membrane structure. In addition, choline is a precursor to the neurotransmitter acetylcholine and the methyl donor betaine. There is evidence that changes in DNA methylation play a role in early neuronal development as well as in the pathogenesis of neurodegenerative diseases by altering gene expression. In addition, dietary choline and betaine intakes have been differentially associated with risk of cardiovascular disease and may also be related to cognitive performance via cerebrovascular health.

Better understanding the role of choline in cognition and cerebrovascular health will be important factors in improving dietary intake recommendations for this nutrient in adults. To date, no human study has evaluated both dietary intake and plasma concentration of choline and choline metabolites in relation to cognitive function, cardiometabolic risk and cerebrovascular pathology. In this dissertation, the role of choline in cognitive performance, cerebrovascular health, and cardiometabolic risk in the older adult was explored through four separate studies.

In the first study, data and stored plasma samples from the Nutrition, Aging and Memory in Elders cohort were used to conduct a cross-sectional analysis evaluating relationships between dietary and plasma choline and related compounds, and cognitive performance, cerebrovascular pathology, and cardiometabolic risk. We found differences in associations between choline related compounds and outcome measures depending on whether dietary intake or plasma concentrations were used in the statistical model. There was no relationship between dietary intake and radiological evidence of cerebrovascular disease by magnetic resonance imaging (MRI) or self-reported cardiovascular disease; however, those individuals with lower plasma concentrations of choline had higher odds of small vessel infarct on brain MRI, and those with higher plasma concentrations had higher odds of large vessel infarcts and self-reported

cardiovascular disease, indicating that the relationship between choline and vascular disease is different when considering the location of blood vessels, cerebrovascular or cardiovascular, as well as the size of the blood vessel, small vessel versus large vessel disease. Dietary betaine intake was positively associated with executive function, while dietary choline intake was inversely related to memory; in contrast, plasma concentrations of choline were positively related to executive function and plasma betaine concentrations were positively associated with memory. These discrepancies may be due to variations in choline metabolism due to single nucleotide polymorphisms (SNPs), variations in intestinal microflora that utilize phosphatidylcholine, or other unknown factors that influence the use of dietary choline and the production of endogenous choline.

In the second study, we evaluated the relationship between dietary choline and cognitive outcomes, and whether SNPs in choline and folate metabolism modify this relationship, using data from the longitudinal Boston Puerto Rican Health study. Significant associations were found between 25 SNPs in choline and folate metabolism and cognitive outcomes, as well as interactions between dietary choline intake and 22 SNPs on cognitive outcomes. These relationships should be assessed in similar populations in order to draw significant conclusions regarding dietary choline recommendations based on genotype.

In the third study, stored brain tissue, serum, and associated cognitive data from the Georgian Centenarian study were analyzed to assess relationships between measures of methylation capacity from choline in serum and gene expression of genes related to memory in the frontal cortex, as well as global cognitive performance. We found that higher methylation capacity from choline, as assessed by the ratio of serum choline plus betaine to dimethylglycine, was associated with less cognitive impairment and better memory. The expression of *EGR-1*, a gene shown to be necessary for long-term memory in rodent models, was greater in those with methylation capacity in the upper 50% compared to those with methylation capacity in the lower

50%. This was the first study to look at gene expression related to methylation capacity in humans. While these relationships are correlational, they do provide some support of the hypothesis that choline is related to cognitive function via its role in methylation. Future studies should look at gene specific DNA methylation and determine whether differences in methylation are related to differences in gene expression.

The final study was a randomized controlled trial to assess the effects of a 6-month dietary intervention to increase dietary choline intake in adults on concentration of plasma choline-related compounds, cardiometabolic risk factors, and cognitive outcomes. Preliminary data have yielded significant associations between changes in concentration of plasma choline-related compounds and changes in scores on cognitive tests. In these preliminary results, we have not yet analyzed blood samples for high density lipoprotein cholesterol, low density lipoprotein cholesterol, or triglycerides in order to assess how the dietary intervention affects these cardiometabolic risk factors.

In summary, the role of choline in cognition and cerebrovascular health in the older adult was explored through four specific aims. From the first two aims, we were able to identify the best biomarker of dietary choline intake in blood and directly relate this parameter to measures of cognitive function and cerebrovascular pathology as well as identify single nucleotide polymorphisms that may modify these relationships. Aim 3 provided insight into the possible mechanism by which choline affects cognitive function by assessing methylation capacity and gene expression, and helped to generate hypotheses for further studies. From the fourth aim, we were able to test our hypothesis that increasing daily dietary choline intake will improve cognitive function. This intervention study is on-going and a progress report was presented in this dissertation.

Table of Contents

List of Tables and Figures

Chapter 1: Choline and choline-related compounds are associated with cardiometabolic risk factors, cognitive performance and cerebrovascular pathology in older adults

Chapter 2: The relationship between dietary choline and cognitive performance is modified by SNPs in genes related to choline and folate metabolism

Introduction

Advancing age has been reported as the strongest risk factor for cognitive decline.[1] As Americans are living longer,[2] the rising economic and societal costs associated with age-related cognitive decline create a need for the development of novel and cost-effective public health strategies that will delay, slow, or even prevent this decline. Observational studies have provided evidence that the risk of cognitive decline can be modified by dietary factors[3-5] and thus further investigation into the role of specific nutrients in cognition is critical.

Choline is an essential nutrient with multiple links to brain health. As a component of phospholipids, choline-containing compounds are involved in cell signaling, lipid transport, and cell membrane structure. In addition, choline is a precursor to the neurotransmitter acetylcholine and is oxidized to form the methyl donor betaine.[6] There is evidence that changes in DNA methylation play a role in early neuronal development[7] as well as in the pathogenesis of neurodegenerative diseases, such as Alzheimer's Disease and Parkinson's Disease, by altering gene expression.[8] In addition, dietary choline and betaine intakes have been differentially associated with risk of cardiovascular disease [9] and may also be related to cognitive performance via cerebrovascular health. Higher plasma choline concentration has been found to be positively associated with higher serum triglycerides, glucose, body mass index, percent body fat, and waist circumference, which would predict higher risk of cardiovascular disease. The opposite was found for plasma betaine concentrations. Whether this same relationship holds true for cerebrovascular disease risk has yet to be evaluated.

Significance

Choline is a nutrient with many links to brain function that relate to cognition as demonstrated by animal models [10-12] and observational studies.[13-15] No human study has evaluated both dietary intake and plasma concentration of choline and choline metabolites in relation to cognitive function and cerebrovascular pathology.

Our approach for exploring the role of choline in cognitive function in four different adult populations, looking at a range of biochemical, cognitive, anatomical, and genetic parameters, will help to fill this critical knowledge gap. Choline from animal products such as eggs, milk, and beef, is a major source of methyl groups in the diet,[11] however, analyses of several cohorts in the U.S. demonstrate choline intakes far below the Adequate Intake (AI).[16-18] Given that the AI for choline is currently based on one study evaluating the amount of choline necessary to prevent fatty liver damage,[6] it is unknown if this amount, or more or less, is needed for optimal cognitive function. Better understanding the role of choline in cognition and cerebrovascular disease will be an important factor in improving dietary intake recommendations for this nutrient and age group.

Specific Aims

The role of choline in cognition and cerebrovascular health in the older adult was explored through four specific aims. From the first two aims, a cross-sectional cohort and a longitudinal cohort, we were able to identify the best biomarker of dietary choline intake in blood and directly relate this parameter to measures of cognitive function and cerebrovascular pathology as well as identify single nucleotide polymorphisms that may modify these relationships. Aim 3 provided insight into the possible mechanism by which choline affects cognitive function by assessing methylation capacity and gene expression, and helped to generate hypotheses for further studies. From the fourth aim, we were able to test our hypothesis that increasing daily dietary choline intake will improve cognitive function. This intervention study is on-going and a progress report is presented in this dissertation.

Specific Aim 1: To describe the relationship between dietary and plasma choline and related compounds and cognition and cerebrovascular pathology. This aim involved secondary analysis of existing data and stored biological samples from the Nutrition, Aging, and Memory in Elders (NAME) study.[19]

Aim 1 Working hypotheses:

1.1: Total dietary choline (free choline (Cho), glycerophosphocholine (GPC), phosphocholine (PCho), phosphatidylcholine (PtdCho), sphingomyelin (SM)) and betaine will be positively related to cognitive performance and negatively related to brain pathology.

1.2: There will be a positive relationship between plasma choline-related compounds (Cho, Bet, PtdCho, SM) and total dietary choline and betaine.

1.3: Concentration of plasma choline-related compounds will be positively related to cognitive performance and negatively related to cerebrovascular pathology.

Specific Aim 2: To describe the relationship between dietary choline and cognitive outcomes and whether SNPs in choline and folate metabolism modify this relationship. This aim involved analysis of existing data from the Boston Puerto Rican Health Study (BPRHS).[20]

Aim 2 Working hypotheses:

2.1: Total dietary choline (free choline, glycerophosphocholine (GPC), phosphocholine (Pcho), phosphatidylcholine (PtdCho), sphingomyelin (SM)) will be positively related to cognitive performance

2.2: The relationship between dietary choline and cognitive performance will be modified by SNPs in genes related to choline and folate metabolism.

Specific Aim 3: To explore the relationship between methylation capacity from choline as measured by serum (free choline + betaine)/dimethylglycine ((Cho + Bet)/DMG) and global cognitive function and gene expression of genes related to memory. This aim involved secondary analysis of brain and serum samples as well as cross-sectional pre-mortem cognitive data from the Georgia Centenarian Study.[21]

Aim 3 Working hypotheses:

3.1: There will be a positive relationship between gene expression of genes related to long-term memory (*ARC, BDNF, REELIN, EGR-1, GADD45B*) in the frontal cortex and methylation capacity from choline as assessed by serum (Cho+Bet)/DMG

3.2: There will be a significant relationship between serum (Cho+Bet)/DMG and measures of global cognitive function

Specific Aim 4: To describe the effect of an intervention to increase dietary choline intake on concentration of plasma choline-related compounds and cognition.

Aim 4 Working hypotheses:

4.1: Consuming 2 whole eggs/day for 6 months will significantly increase the concentration of plasma choline-related compounds compared to consuming the equivalent amount of egg whites.

4.2: Consuming 2 whole eggs/day for 6 months will not significantly change the concentration of plasma trimethylamine-oxide (TMAO) compared to consuming the equivalent amount of egg whites.

4.3: There will be a positive relationship between change in plasma choline-related compounds and change in cognition over 6 months.

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Literature Review

Advancing age has been reported as the strongest risk factor for cognitive decline.[1] As Americans are living longer,[2] the rising economic and societal costs associated with age-related cognitive decline create a need for the development of novel and cost-effective public health strategies that will delay, slow, or even prevent this decline. Observational studies have provided evidence that the risk of cognitive decline can be modified by dietary factors[3-5] and thus further investigation into the role of specific nutrients in cognition is critical.

Choline is an essential nutrient with multiple links to brain health. As a component of phospholipids, choline is involved in cell signaling, lipid transport, and cell membrane structure. In addition, choline is a precursor to the neurotransmitter acetylcholine and the methyl donor betaine.[6] There is evidence that changes in DNA methylation play a role in early neuronal development[7] as well as in the pathogenesis of neurodegenerative diseases, such as Alzheimer's Disease, with global DNA hypomethylation in brain tissue of confirmed AD cases compared to neurologically normal brains.[8] In addition, dietary choline and betaine intakes have been differentially associated with risk of cardiovascular disease[9] and may also be related to cognitive performance via cerebrovascular health.

Dietary choline recommendations

The Dietary Reference Intake (DRI) recommendations for choline were set by the Institute of Medicine in 1998.[6] Due to insufficient data to set an Estimated Average Requirement (EAR), and thus calculate a Recommended Daily Allowance (RDA), an Adequate Intake (AI) was developed for choline based largely on the results of a single depletion/repletion study[10] using maintenance of serum alanine amino transferase levels as a marker of liver damage due to a choline deficient diet.[11]

Although humans have the ability to produce choline endogenously, involving the activity of phosphatidylethanolamine N-methyltransferase (PEMT) in the liver, this production is

not enough to meet daily needs and consumes the methyl donor S-adenosyl-methionine (SAM) in the process.[12] Single-nucleotide polymorphisms (SNPs) in PEMT may decrease its activity, making the AI values inadequate for a number of people.[12] One study predicted that more than 40% of women in the U.S. (of European origin) require additional dietary choline to prevent choline deficiency induced syndrome, due to a SNP in the PEMT gene that alters the estrogen responsiveness of the promoter.[13, 14] However, this was just one study and further evidence is necessary to determine if this should be a nutrient of concern for the U.S. population. Another SNP in PEMT is associated with susceptibility to choline deficiency as well as nonalcoholic fatty liver disease[14] and Alzheimer's disease in certain populations.[15] SNPs in other genes related to choline metabolism may also modify an individual's dietary choline needs.

A SNP in methylene-tetrahydrofolate dehydrogenase (MTHFD1), which is involved in DNA synthesis from 5,10-methyene-tetrahydrofolate, is associated with increased susceptibility to organ dysfunction when people consume a choline deficient diet[14] and increased risk of having a child with a neural tube defect as well as being a weak risk factor for early onset Alzheimer's disease.[16] SNPs in choline dehydrogenase (CHDH), involved in the conversion of choline to betaine, have been shown to have variable associations (protective or increased susceptibility to choline deficiency) that may modify an individual's dietary choline needs.[17] Other SNPs are not related to susceptibility to choline deficiency, but due to relationships with vascular outcomes have the potential to interact with dietary choline intake to influence cognitive performance.

One study reported higher frequency of a SNP in betaine: homocysteine methyltransferase (BHMT), involved in the remethylation of homocysteine from betaine, in individuals ($\geq$60 yrs) with no or moderate coronary artery disease, suggesting a protective role.[18] More recently, BHMT was identified as part of a gene set involved in the pathways responsible for coronary heart disease.[19] Likelihood of stroke and hypertension was shown to be greater in people with certain SNPs in S-adenosylmethionine synthetase type 1 (MAT1A), which is

involved in the production of the universal methyl-donor s-adenosylmethionine.[20] In analysis of cohorts in the Cognitive Aging Genetics in England and Scotland consortium, a SNP in MAT1A was negatively associated with memory in elderly adults (~70 yr).[21]

Choline and vascular outcomes

Observational studies demonstrate a positive relationship between cardiovascular risk in mid-life and age-related cognitive decline.[22, 23] In addition, there is evidence from imaging studies, of a significant relationship between unrecognized myocardial infarction and increased risk of not only dementia, but also increased cerebral small vessel disease as indicated by volume of white matter lesions, in men.[24] Gender differences have also been noted in a longitudinal study describing the relationship between plasma lipid profile and incidence of cardiovascular and cerebrovascular events. In this study, Tohidi et al. (2013) reported that increased risk of stroke was associated with total cholesterol, low-density lipoprotein cholesterol, and non-high density lipoprotein cholesterol concentrations in women but not men, while all lipid components were associated with coronary heart disease in both men and women.[25]

Phosphatidylcholine biosynthesis in the liver is involved in the regulation of plasma lipoproteins, and when synthesis is decreased by impairment in either the de novo or endogenous pathways, plasma very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) are decreased while hepatic steatosis is increased.[26] In animal models of atherosclerosis, inhibition of the endogenous phosphatidylcholine pathway (catalyzed by PEMT) significantly decreases cardiovascular disease risk, but at the expense of fatty liver development.[26-28] In humans, *plasma* betaine and choline concentrations have been differentially associated with cardiovascular disease risk factors, with higher concentrations of betaine related to a lower cardiovascular risk factor profile and higher concentrations of choline related to a higher cardiovascular risk factor profile.[9] These conclusions were not similar to those of a longitudinal study that found a significant negative relationship between *dietary* choline intake and volume of cerebral white matter

hyperintensities, which is a marker of small vessel disease.[29] It may be important to note that these studies did not measure both dietary and plasma choline, thus failing to account for both endogenous and exogenous pathways. In addition, the plasma choline concentration was not fasting, thus perhaps reflecting recent dietary intake rather than habitual intake. In addition, they did not measure other potentially significant choline-related compounds, such as phosphatidylcholine and sphingomyelin. There is evidence that the enzyme sphingomyelinase is involved in the aggregation of low density lipoproteins (LDL) in atherosclerosis by hydrolyzing LDL-sphingomyelin and that the ratio of sphingomyelin to total phospholipids (sphingomyelin plus phosphatidylcholine) [SM:(SM+PC)] is an important factor in determining the susceptibility to sphingomyelinase.[30] Furthermore, SM:(SM+PC) has been found to be an independent predictor of coronary artery disease.[31] However, no study has looked at this ratio in relation to cerebrovascular disease.

When assessing relationships between dietary choline and vascular-related outcomes, it may also be important to take into account digestion by the gut microflora. Recent studies have highlighted possible relationships between dietary choline and vascular disease, not by a direct relationship, but rather by a relationship with trimethylamine (TMA), a metabolite produced by bacteria digestion of phosphatidylcholine in the colon. In 1989, Zeisel and colleagues[32] reported that regular dietary intakes from food sources were not likely to be large enough to overload choline absorption processes in the small intestine and significantly increase TMA production by colon micro flora. However, recent interest in the microbiome has prompted new research in this area. Wang et al. (2011) demonstrated a mechanistic link between the oxidation of TMA produced by microbial metabolism of dietary phosphatidylcholine and atherosclerosis in an animal model, as well as a significant relationship with increased risk of cardiovascular disease in humans.[33] Most recently, Tang et al. (2013) demonstrated a significant bacteria-dependent increase in plasma trimethylamine-N-oxide (TMAO) when humans are fed two-eggs and that

fasting TMAO levels predict risk of major cardiovascular disease events.[34] This work certainly points to a potential clinical significance of TMAO. However, it is important to note that the metabolite produced by the gut bacteria is TMA and it is the oxidized form of this metabolite that is associated with cardiovascular risk. Bennett et al. (2013) identified several factors that regulate the oxidation of TMA in the liver of both animals and humans, indicating that the relationship between dietary phosphatidylcholine and plasma TMAO is not a direct one.[35] At this point, the evidence supporting TMAO from dietary choline intake is still emerging and it is not clear whether TMAO is related to increased risk of cerebrovascular disease in addition to cardiovascular disease. However, TMAO may be an important covariate to consider when looking for relationships between dietary choline intake and cognitive function and cerebrovascular health.

Choline and B-vitamins

Another connection between cerebrovascular and cardiovascular disease and cognitive decline may be homocysteine. Hyperhomocysteinemia has been associated with increased risk of both coronary artery disease and stroke.[36] While supplementation with B-vitamins (folate, vitamin B-6, vitamin B-12 in combination or alone) has been shown to be effective in lowering homocysteine concentrations, this lowering has not been associated with reduced risk of cardiovascular events.[37] However, recent review of homocysteine lowering trials concludes that there may be an association between successful homocysteine lowering and a delay in cognitive decline.[38] Furthermore, these studies focused on supplementation with B-vitamins for lowering homocysteine. However, methyl groups from betaine can also remethylate homocysteine and may be important to take into account in addition to dietary intake of B-vitamins (folate, vitamin B-6, and vitamin B-12). Lever and colleagues conducted a series of studies to examine the relationship between plasma betaine and secondary risk factors in an acute coronary syndrome cohort.[39, 40] Both high and low plasma betaine were associated with increased risk of secondary

heart failure and acute myocardial infarction,[40] while lower plasma betaine was associated with higher triglycerides and non-high density lipoprotein cholesterol.[39]

The metabolic pathways of choline, folate, vitamin B-12, and vitamin B-6 intersect at the point of homocysteine in one-carbon metabolism. Both betaine, from the oxidation of choline, and 5-methyl-tetrahydrofolate can be used to remethylate homocysteine, while vitamin B-6 is involved in the transmethylation of homocysteine.[41] Thus, a fluctuation in concentration of any of these nutrients has the potential to alter utilization and concentration of the others.

Changes in plasma concentrations of choline and related metabolites in response to dietary restriction or supplementation with B-vitamins have been demonstrated in both animal and human studies. Folate restriction in a group of pre-menopausal women resulted in decreased plasma phosphatidylcholine.[42] Rats fed a diet low in vitamins B-6, B-12, and folate then supplemented to repletion, had significantly higher concentrations of plasma free choline and lower homocysteine than rats maintained on the B-vitamin poor diet, despite adequate choline intake in both groups.[43] Similarly, plasma betaine concentration was increased and homocysteine decreased when elderly people consumed a vitamin B-12 and folate supplement.[44] In addition, those with the largest increases in plasma betaine concentration in this study also showed the greatest improvements in measures of memory function.[44] The relationship between choline-related compounds and memory is intriguing due to the role of choline in one-carbon metabolism and role of methylation in learning and memory.

Methylation in learning and memory

A recent review by Yu et al. (2011) summarizes the current state of knowledge regarding the role of changes in DNA methylation on learning and memory.[45] Briefly, DNA methylation in the brain may be transient or stable depending on the function of the gene as well as the location in the brain. For example, DNA in hippocampal cells, coding for plasticity genes has been shown to become demethylated following a learning task, allowing for the translation of new proteins

necessary for inter-neuronal connections. In addition, the methylation pattern of memory suppressor genes has been shown to increase following learning, thus decreasing their expression. The original methylation patterns, however, return after a short time. In order for memories to persist, there is evidence that stable changes in methylation patterns on DNA coding for other proteins occurs.[45]

Much of the work exploring the role of methylation in memory has focused on the hippocampus, which is necessary for memory formation. However, it is theorized that once formed, memories are only dependent on the hippocampus for a short time, and in order for memories to be stored long-term, persistent changes need to occur in the cortex.[46] Sui and colleagues (2012) demonstrated this phenomenon in an animal model, showing that methylation patterns of plasticity genes (brain derived neurotrophic factor and reelin) expressed in the medial-prefrontal cortex were necessary for long-term memory.[47] Further evidence supporting the role of methylation in cognitive function comes from studies in Bading's laboratory showing that expression of genes involved in methyl group transfer (*dnmt3a*) are decreased in mice with age-related cognitive impairment, and that over-expression of these genes resulted in changes in global methylation patterns as well as improved long-term memory.[48] Additional genes of interest that have shown to be necessary for formation of long-term memories in rodent models, but warrant further study in humans include activity-regulated cytoskeletal gene (*ARC*),[49] early growth response-1 (*EGR-1*),[50] and Growth arrest and DNA damage-inducible β (*GADD45β*).[51]

Choline and methylation

Choline from animal products such as eggs, milk, and beef, is a major source of methyl groups in the U.S. diet.[52] Animal models provide evidence that dietary choline deficiency during fetal development alters global and gene-specific DNA methylation in the hippocampus[53] and leads to compromised cognitive function and increased decline later in life.[54] Several animal studies have shown beneficial effects on memory of offspring when maternal diet is

supplemented with choline.[52] There is limited research, however, on how the consumption of choline or choline-rich foods affects cognition and cerebrovascular health in humans.

Dietary choline intake status

Analyses of several cohorts in the US (NHANES 2003-2004,[55] Framingham Offspring Study,[56] Atherosclerosis Risk in Communities Study[57]) demonstrate choline intakes far below the AI. Since the AI is based on the intake required to prevent liver damage,[6] it is not known if this is an adequate amount for optimal cognitive function. Adding just one egg per day would increase the percent of older Americans whose usual intake is greater than the AI from less than 10% to 20-35%.[55] Eggs are a major source of choline in the diet,[52] with just two large scrambled eggs providing half of the Adequate Intake (AI) recommended for men and over 60% of the AI for women.[6, 58]

Consumption of choline rich foods increases plasma choline concentrations.[59] Choline is transported bi-directionally across the blood brain barrier, entering the brain when plasma concentrations are high following a choline-rich meal, and leaving the brain to enter the plasma when plasma concentrations are low.[60] Proton magnetic resonance spectroscopy has shown that there is a significant increase in choline-containing compounds in human brain after ingestion of choline bitartrate,[61] demonstrating that dietary interventions can be effective and have the potential to influence brain function.

Choline and human cognition

There have only been a small number of studies that have sought to elucidate the role of choline in human cognition beyond development. In one of the first supplementation studies, Sitaram and colleagues[62] provided healthy volunteers with a high dose of choline chloride (10g) or placebo and looked at the effects on various learning and memory tasks. They found that participants did better on tasks of serial learning and recall of low imagery words but not high imagery words after receiving the choline supplement. Another study found a high dose of

choline in the form of phosphatidylcholine (25 g) was shown to enhance performance on a task of explicit memory in college-aged volunteers, while a lower dose (10 g phosphatidylcholine) did not enhance performance.[63] Similarly, when supplemental choline (2g choline chloride) was added to long-term (24+ weeks) total parenteral nutrition (TPN), patients scored better on visual memory tests compared to when a placebo was added to TPN, but there was no difference in intellectual functioning, verbal memory, visuospatial functioning and perceptual organization, verbal fluency, or manual dexterity and motor speed.[64] In another supplementation study, a drink containing choline citrate (50 mg/kg body weight choline) was given to volunteers before and after an exercise test.[65] This acute high dose of choline had no effect on physical tasks or cognitive performance (reaction time, logical reasoning, vigilance, spatial memory, or working memory) following the exercise test. In another study of college students, those who ingested a supplement containing choline (α-glycerophosphocholine (150 mg), choline bitartrate (125 mg)) and B-vitamins (niacin (vitamin B-3; 30 mg), pyridoxine HCl (vitamin B-6; 30 mg), methylcobalamin (vitamin B-12; 0.06 mg), folic acid (4 mg)), among other ingredients, were able to maintain reaction time, and had subjective feelings of focus and alertness to both visual and auditory stimuli.[66] However, since the supplement contained other ingredients, the cognitive measures cannot necessarily be attributed to choline and some habituation occurred after 4-weeks of supplementation. Others have shown positive results when supplementing with the choline containing compound citicoline as treatment to prevent or reverse vascular dementia in stroke patients, with citicoline supplementation benefiting several cognitive domains.[67, 68] In another study of elderly patients with memory complaints and vascular lesions, treatment with citicoline over 9 months either prevented further decline in general cognitive function or improved cognition, as measured by the Mini Mental State Exam.[69] While the aforementioned studies looked at supplementation with high doses of choline or choline-containing compounds, others

have found positive relationships between cognitive performance and choline intake coming from food alone.

Usual choline intake over the previous year, as assessed by food frequency questionnaire, in a healthy cohort of older adults (Framingham Offspring Study) was positively associated with scores on tests of verbal memory and visual memory.[29] In a group of elderly persons in Madrid, those who made no errors on a test of general cognitive capacity consumed more eggs than those who made errors and after adjusting for energy intake and education level, there was a negative association between the number of errors and choline intake.[70] While the above studies assessed dietary intake, Nurk and colleagues[71] investigated the relationship between plasma choline and cognitive function of elderly subjects (Hordaland Health Study) and found that compared with low concentrations, high plasma choline (>8.4μmol/l) was associated with better scores of tests assessing perceptual speed, executive function, and global cognition.

Although these studies provide some evidence of the importance of choline for cognitive function later in life, they do not address the fact that choline can be produced endogenously as well as being obtained from the diet (i.e. they only assessed dietary intake or plasma concentrations, not both). Furthermore, these studies do not account for genetic polymorphisms that may influence how choline is metabolized as well as the endogenous production of choline. In addition, the significant relationship may not be with choline directly, but rather with one of its metabolites or choline containing compounds such as sphingomyelin, phosphatidylcholine, or betaine.

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Methods

Overall Study Design

The role of choline in cognitive performance and brain health in the older adult was explored through four separate studies: 1) A cross-sectional study evaluated relationships between dietary and plasma choline and related compounds, and cognitive performance and cerebrovascular pathology. 2) A longitudinal study evaluated the relationship between dietary choline and cognitive outcomes and whether SNPs in choline and folate metabolism modify this relationship. 3) An analytical study involved analysis of stored brain tissue (with associated pre-mortem cognitive measures) and serum for relationships between measures of methylation capacity from choline in serum and gene expression of genes related to memory in the frontal cortex, as well as global cognitive performance. 4) An intervention study, designed to increase dietary choline intake in the older adult, evaluated relationships between change in plasma choline and related compounds relative to change in cognitive outcomes. Information regarding methodology is included with each of the following chapters; details, such as sample size calculations and specific protocols are included in the appendix.

Chapter 1: Choline and choline-related compounds are associated with cardiometabolic risk factors, cognitive performance and cerebrovascular pathology in older adults

Introduction

Advancing age has been reported as the strongest risk factor for cognitive decline.[1] As Americans are living longer,[2] the rising economic and societal costs associated with age-related cognitive decline create a need for the development of novel and cost-effective public health strategies that will delay, slow, or even prevent this decline. Observational studies suggest that the risk of cognitive decline can be modified by dietary factors.[3-5] Further investigation into the role of specific nutrients in cognition is critical.

Choline is an essential nutrient with multiple links to brain health. As a component of phospholipids, choline-containing compounds are involved in cell signaling, lipid transport, and cell membrane structure. In addition, choline is a precursor to the neurotransmitter acetylcholine and the methyl donor betaine.[6] There is evidence that changes in DNA methylation play a role in early neuronal development[7] as well as in the pathogenesis of neurodegenerative diseases, such as Alzheimer's Disease and Parkinson's Disease, by altering gene expression.[8] In addition, plasma choline and betaine concentrations have been differentially associated with risk of cardiovascular disease and may also be related to cognitive performance via cerebrovascular health. Higher plasma choline concentration has been found to be positively associated with higher serum triglycerides, glucose, body mass index, percent body fat, and waist circumference, which would predict higher risk of cardiovascular disease. The opposite was found for plasma betaine concentrations.[9] Whether this same relationship holds true for cerebrovascular disease risk has yet to be evaluated.

The Dietary Reference Intake (DRI) recommendations for choline were set by the Institute of Medicine in 1998.[6] Due to insufficient data to set an Estimated Average

Requirement (EAR), and thus calculate a Recommended Daily Allowance (RDA), an Adequate Intake (AI) was developed for choline based largely on the results of a single depletion/repletion study[10] using maintenance of serum alanine amino transferase levels as a marker of liver damage due to a choline deficient diet.[11] Analyses of several cohorts in the US (NHANES 2003-2004,[12] Framingham Offspring Study,[13] Atherosclerosis Risk in Communities Study[14]) demonstrate choline intakes far below the Adequate Intake (AI). Since the AI is based on the intake estimated to prevent liver damage,[6] it is unknown if this is optimal for cognitive function.

Although humans have the ability to synthesize choline in the liver, through methylation of phosphatidylethanolamine to form phosphatidylcholine, catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT), this production is inadequate to meet daily requirements, thus making choline an essential nutrient.[15] Consumption of choline rich foods demonstrably increases plasma choline concentrations.[16] Choline is transported bidirectionally across the blood brain barrier, entering the brain when plasma concentrations are high following a choline-rich meal, and leaving the brain to enter the plasma when plasma concentrations are low.[17] Proton magnetic resonance spectroscopy has shown that there is a significant increase in choline-containing compounds in the human brain after ingestion of choline bitartrate,[18] demonstrating that dietary interventions can be effective in influencing choline availability in the brain and have the potential to influence brain function.

Previous studies provide some evidence of the importance of choline for cognitive function later in life. Intervention studies have shown choline-containing supplements enhanced learning and memory[19-21] and prevented or reversed cognitive decline in patients with cerebrovascular disease.[22-24] In addition, cross-sectional studies demonstrate associations between choline intake or foods high in dietary choline global cognition or memory.[25, 26] Only one study had the potential to capture both exogenous and endogenous sources by measuring plasma choline and found that higher concentrations of plasma choline were associated with

better scores on tests of perceptual speed, executive function, and global cognition.[27] However, if plasma betaine had also been assessed, the ratio of betaine to choline (Bet:Chol) could provide information on the activity of the endogenous pathway involving PEMT. An increase in PEMT activity would result in a lower betaine to choline ratio, as betaine would be used as a methyl donor in the production of phosphatidylcholine from phosphatidylethanolamine.[28] In addition, though previous studies show relationships with cognitive outcomes, only one also showed relationships between choline and cerebrovascular pathology.[25] However, this relationship was with dietary choline, and plasma choline or other choline containing compounds, such as sphingomyelin and phosphatidylcholine, were not assessed, which could be important when evaluating this relationship.

Observational studies demonstrate a positive relationship between cardiovascular risk in mid-life and age-related cognitive decline.[29, 30] In addition to being an endogenous source of choline, phosphatidylcholine biosynthesis in the liver is critical for the synthesis and secretion of lipoproteins. Some evidence suggests that sphingomyelinase is involved in the aggregation of low density lipoproteins (LDL) in atherosclerosis by hydrolyzing LDL-sphingomyelin and that the ratio of plasma sphingomyelin to total choline-containing phospholipids (sphingomyelin plus phosphatidylcholine) [SM:(SM+PC)] is an important factor in determining the susceptibility to sphingomyelinase.[31] Furthermore, there is some evidence that plasma SM:(SM+PC) has been found to be an independent predictor of coronary artery disease.[32] However, this ratio in relation to cerebrovascular disease or cognitive function has not previously been assessed.

Another connection between cerebrovascular and cardiovascular disease and cognitive decline may be homocysteine. The metabolic pathways of choline, folate, vitamin B-12, and vitamin B-6 intersect at the point of homocysteine in one-carbon metabolism. Both betaine, from the oxidation of choline, and 5-methyl-tetrahydrofolate can be used to remethylate homocysteine, while vitamin B-6 is involved in the transmethylation of homocysteine.[33] Thus, a fluctuation in

concentration of any of these nutrients has the potential to alter utilization and concentration of the others and all should be taken into account when assessing relationships between choline and cerebrovascular health or cognitive function.

Hyperhomocysteinemia has been associated with increased risk of both heart disease and stroke.[34] While supplementation with B-vitamins has been shown to be effective in lowering homocysteine concentrations, this lowering has not been consistently associated with reduced risk of cardiovascular events.[35] However, recent review of homocysteine lowering trials concludes that there may be an association between successful homocysteine lowering and a delay in cognitive decline.[36] Furthermore, these studies focused on supplementation with B-vitamins, involved in one-carbon transfer (folate and vitamin B-12), for lowering homocysteine. However, methyl groups from betaine can also remethylate homocysteine and may be important to take into account in addition to dietary intake of B-vitamins. Another possible link between choline and cardiometabolic risk may be in relation to insulin resistance. A recent review by Zeisel (2014) has also proposed signaling pathways for crosstalk between choline metabolism and insulin sensitivity; both increased and decreased plasma choline and betaine concentrations have been associated with increased insulin sensitivity.[37] Furthermore, executive function and visuospatial impairment has been reported in a cohort of older people (77 + 8.5 yr) with type 2 diabetes mellitus.[38]

Therefore, the aim of this study was to contribute to this growing body of evidence by evaluating the associations of dietary and plasma choline and choline-related compounds with cardiometabolic risk factors, cognition, cerebrovascular pathology and regional brain volume measurements in an elderly cohort and include cardiovascular risk factors and B-vitamin intake as covariates in the analysis. Our hypothesis is that there is a positive association between dietary intake and the concentration of plasma choline or Bet:Chol and cognitive performance and an

inverse association with cerebrovascular pathology, while SM:(SM+PC) is positively related to cerebrovascular pathology and inversely related to cognition.

Methods

Subjects and Study Design

This study involved secondary analysis of a subset of the Nutrition, Aging, and Memory in Elders (NAME) cohort[39] who had undergone magnetic resonance imaging, cognitive testing, and had archived plasma. The NAME study was a cross-sectional study designed to identify relationships between micronutrient status and cognitive capacity and cerebrovascular pathology in older adults. The study cohort consisted of community-based elders living in the greater Boston, Massachusetts area age 60 years and older. Data that had been collected previously and were utilized in the present study include: demographics, comorbidities (presence of hypertension, presence of diabetes, and body mass index (BMI)), and cognitive measures of memory, attention, and executive function analyzed by principle components analysis (PCA) and volumetric magnetic resonance imaging (MRI) measures of white matter hyperintensities, brain atrophy, and brain infarcts. In addition, previously assessed plasma concentrations of high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), triglycerides, glucose, vitamin B-12, pyridoxal-5'-phosphate (PLP), folate, and total homocysteine (Hcy) were included as covariates in analysis.

Dietary Intake

The previously recorded Willett Food Frequency Questionnaire (FFQ) was assessed for choline and betaine intake using the USDA database for the choline content of common foods, release 2 (https://catalog.data.gov/dataset/usda-database-for-the-choline-content-of-common-foods).

Plasma Biomarkers

Plasma samples collected between 2003 and 2007 were stored in a -80°F freezer until assayed for concentrations of free choline, betaine, phosphatidylcholine, and sphingomyelin using liquid chromatography-stable isotope dilution-multiple reaction monitoring-mass spectrometry (LC-SID-MRM-MS)[40] at the University of North Carolina Chapel Hill Nutrition Research Institute. See Appendix A for further details.

Statistical Methods

Multiple linear regression models and logistic regression models were used to examine relationships between a) dietary intake and plasma concentrations of choline and choline-related compounds, b) dietary intake and cognitive performance, c) dietary intake and cardiometabolic risk factors, d) dietary intake and cerebrovascular pathology and regional brain volume measurements, e) plasma compounds and cognitive performance, f) plasma compounds and cardiometabolic risk factors, g) plasma compounds and cerebrovascular pathology and regional brain volume measurements. Independent variables were examined as continuous variables and as categorical data (tertiles) when the outcome variable was dichotomous. When continuous data were not normally distributed, the values were log transformed prior to analysis (plasma choline, plasma betaine, volume of white matter hyperintensities, ventricular volume).

Covariates considered for inclusion into the model include a) age, sex, education, b) body mass index, presence of hypertension, presence of diabetes, plasma HDL-c, LDL-c, triglycerides, blood glucose and c) other nutrients or biomarkers (dietary vitamin B-12, vitamin B-6, folate, calories, cholesterol, saturated fat; plasma homocysteine, vitamin B-12, PLP, folate, HDL- and LDL- cholesterol). Results were considered significant at $p \leq 0.05$.

Results

Population Characteristics

Demographics and other cohort characteristics of the participants included in our analyses are presented in Table 1. The mean age was 75 (± 8.6) years and participants were predominantly female (75%). The mean BMI was in the obese range (31.5 ± 8.6 kg/m^2) with a high prevalence of hypertension (85%). Attained level of education was moderate, with 33% high school graduates and 32% having attended at least some college.

Dietary Intakes and Plasma Concentrations

Dietary intake and plasma concentrations of choline and choline-related compounds are found in Table 2. Dietary choline and betaine intakes as well as plasma choline and betaine concentrations were greater in men than women, while plasma phosphatidylcholine and sphingomyelin were greater in women than men. The average dietary choline intake was below the AI for men (550 mg/day) and women (425 mg/day). Out of the 1084 participants, only 100 met or exceeded the AI for dietary choline intake.

Dietary choline intake was positively associated with plasma betaine concentration and negatively associated with plasma phosphatidylcholine concentration. There was no significant relationship found between dietary choline intake and plasma choline concentration or plasma sphingomyelin concentration. However, dietary choline intake was positively associated with plasma ratios of SM:(SM+PC) and betaine:choline (see Table 3). There were no significant relationships found between dietary betaine intake and any of the plasma measurements (data not shown).

Diet and Cardiometabolic Risk Factors

Relationships between dietary choline or betaine intake and blood measures of cardiometabolic risk factors are shown in Table 4. There was a positive relationship between HDL-c and both dietary choline and betaine, while homocysteine was positively associated with dietary choline intake and inversely associated with dietary betaine intake. There was also an inverse relationship between dietary choline intake and blood triglyceride concentration.

Plasma and Cardiometabolic Risk Factors

Results of multiple linear regressions looking at the relationships between cardiometaboic risk factors and plasma concentrations of choline and choline-related compounds are found in Table 5. LDL-c was inversely associated with betaine concentration, and positively associated with concentration of plasma phosphatidlycholine, sphingomyelin, and SM:(SM+PC). HDL-c was inversely associated with plasma concentration of choline and positively associated with phosphatidylcholine, sphingomyelin, and Bet:Chol. Concentration of trigylcerides was inversely associated with plasma betaine concentration and the ratio of SM:(SM+PC) and Bet:Chol and positively associated to phosphatidylcholine concentration. Homocysteine concentration was positively related to plasma choline concentration and the ratio of SM:(SM+PC) and inversely associated with the ratio of Bet:Chol. There were no significant relationships between blood glucose concentration and plasma concentrations of choline and choline-related metabolites. BMI was positively associated with plasma choline concentration and SM:(SM+PC) and inversely associated with Bet:Chol.

Diet and Cardiovascular Disease

The odds of self-reported cardiovascular disease related to dietary intake are presented in Table 6. The odds of reporting cardiovascular disease were 28% lower in those with dietary betaine intakes in the third tertile (328.8 – 329.2 mg/day) compared to the first tertile (45.3 – 247.7 mg/day). This did not remain significant when all measured cardiometabolic risk factor covariates were added to the model.

Plasma and Cardiovascular Disease

The odds of self-reported cardiovascular disease related to plasma choline-related compounds are presented in Table 7. The odds of reporting cardiovascular disease were greater in those with plasma choline concentrations in the second (247.7 – 328.8 mg/day) or third tertile versus the first tertile. These relationships remained significant when adjusting for all measured

cardiometabolic risk factor covariates, with the odds of cardiovascular disease in those with plasma choline concentrations in the second tertile being 2.3 times and those in the third tertile 3.2 times the odds of those in the first tertile.

Diet and Cognitive Outcomes

Dietary betaine intake was positively associated with executive function score, while dietary choline intake was inversely related to memory scores (see Table 8). There were no other statistically significant relationships between dietary intake and cognitive outcomes.

Plasma and Cognitive Constructs

Relationships between plasma choline and choline metabolite concentrations and cognitive constructs are presented in Table 9. Executive function scores were positively associated with plasma concentrations of choline and negatively associated with plasma Bet:Chol. After controlling for age, sex, and education, the concentration of plasma phosphatidylcholine was negatively associated with memory scores and betaine was positively associated with memory scores. The relationship with phosphatidylcholine remained significant when all other covariates were added to the model, while the relationship with betaine was no longer significant when additional covariates were added. Attention scores were not significantly related to the concentration of any of the plasma measurements assessed.

Diet and Cerebrovascular Pathology and Regional Brain Volume

Dietary betaine was positively related to hippocampal volume and inversely associated with ventricular volume. There were no significant relationships found between dietary choline or betaine and amygdala volume, volume of white matter hyperintensities, or risk of a small or large vessel infarct (see Table 10).

Plasma Concentrations and Cerebrovascular Pathology and Regional Brain Volume

Relationships between plasma measurements and MRI volumetric measurements are presented in Table 11. Plasma sphingomyelin concentration was positively related to

hippocampal volume when controlling for age sex, and education. However, this relationship was attenuated when all covariates were added to the model. There was a significant positive relationship between plasma choline concentration and amygdala volume which was maintained when all covariates were added to the model. After controlling for age, sex, and education, ventricular volume was inversely associated with both plasma betaine concentration and SM:(SM+PC). The relationship with betaine remained significant when all covariates were added to the model, but the relationship with SM:(SM+PC) was no longer significant. There were no significant relationships between hippocampal volume and plasma choline or any of the choline-related compounds measured.

The volume of white matter hyperintensities was negatively related to SM:(SM+PC). There was no significant relationship between volume of white matter hyperintensities and concentration of plasma sphingomyelin or phosphatidylcholine alone, or choline or betaine. The odds of a small vessel infarct were lower in those with plasma choline concentrations in the second (8.1-10.4 μM) or third tertile (10.4-26.9 μM) versus the first tertile (3.6-8.1 μM). This relationship remained significant when adjusting for all covariates with the odds of a small vessel infarct in those with plasma choline concentrations in the second tertile being 57% lower and those in the third tertile 64% lower than those with concentrations in the first tertile. In addition, as shown in Figure 1, after adjusting for all covariates, the odds of a small vessel infarct in those with plasma betaine concentrations in the third tertile (43.7-239.4 μM) were 59% less than those in the first tertile (13.7-33.3 μM) .

Conversely, the odds of a large vessel infarct were greater in those with plasma choline concentrations in the second or third tertile versus the first tertile. As shown in Figure 2, these relationships remained significant when adjusting for all covariates, with the odds of a large vessel infarct in those with plasma choline concentrations in the second tertile being 10.5 times and those in the third tertile 12.4 times the odds of those in the first tertile.

Discussion

Dietary Intake and Plasma Choline and Choline-Related Compounds

The demographics of the NAME study participants are representative of the population of interest. Dietary choline intakes in the NAME population were similar to other cohorts (NHANES 2003-2004 (less than 10% met or exceeded the AI),[12] Framingham Offspring Study (313 $\pm$ 61 mg/day),[13] Atherosclerosis Risk in Communities Study (332 $\pm$ 125 mg/day for men, 294 $\pm$ 112 mg/day for women)[14]). However, dietary betaine intakes in the NAME population were in between intakes measured in other similar populations (Framingham Offspring Study (208 $\pm$ 90 mg/day),[13] Atherosclerosis Risk in Communities Study (118 $\pm$ 55 for men, 102 $\pm$ 47 for women)[14]). Plasma choline and betaine concentrations were comparable to what others have found in similar populations,[9, 27, 28, 41, 42] while the concentration of SM:(SM+PC) was lower that what has been previously reported.[32]

Cognitive Constructs

There have only been a small number of studies that have sought to elucidate the role of choline in human cognition beyond development. In one of the first supplementation studies, Sitaram and colleagues[20] provided healthy adult volunteers with a high dose of choline chloride or placebo and looked at the effects on various learning and memory tasks. They found that participants did better on tasks of serial learning and recall of low imagery words after receiving the choline supplement. Another study found a high dose of choline in the form of phosphatidylcholine was shown to enhance performance on a task of explicit memory in college-aged volunteers.[19] Similarly, when supplemental choline was added to long-term total parenteral nutrition (TPN), adult patients scored better on visual memory tests compared to when a placebo was added to TPN.[21] In another supplementation study, a drink containing choline citrate was given to volunteers before and after an exercise test.[43] This acute high dose of choline had no

effect on cognitive performance following the exercise test. In another study of college students, those who ingested a supplement containing choline and B-vitamins, among other ingredients, were able to maintain reaction time, and had subjective feelings of focus and alertness to both visual and auditory stimuli.[44] However, since the supplement contained other ingredients, the cognitive measures cannot be attributed to choline alone. Others have found promising results when supplementing with the choline containing compound citicoline as treatment to prevent or reverse vascular dementia in stroke patients.[22, 23] In another study of elderly patients with memory complaints and vascular lesions, treatment with citicoline over 9 months either prevented further decline in general cognitive function or improved cognition, as measured by the Mini Mental State Exam.[24] While the aforementioned studies looked at supplementation with high doses of choline or choline-containing compounds, others have found positive relationships between cognitive performance and choline intake coming from food alone.

Choline intake, assessed by food frequency questionnaire at the same time as cognitive tests were administered, in a healthy cohort of older adults (Framingham Offspring Study) was positively associated with scores on tests of verbal memory and visual memory.[25] In a group of elderly persons in Madrid, those who made no errors on a test of general cognitive capacity consumed more eggs than those who made errors and after adjusting for energy intake and education level, there was a negative association between the number of errors and choline intake.[26] While the above studies assessed dietary intake, Nurk and colleagues[27] investigated the relationship between plasma choline and cognitive function of elderly subjects (Hordaland Health Study) and found that compared with low concentrations, high plasma choline (>8.4μmol/l) was associated with better scores of tests assessing perceptual speed, executive function, and global cognition. By assessing plasma choline rather than dietary intake, this study had the potential to capture both endogenous and exogenous choline. However, if they had also measured plasma betaine, the ratio of betaine to choline could provide information on the activity of the

endogenous pathway involving PEMT. An increase in PEMT activity would result in a lower betaine to choline ratio, as betaine would be used as a methyl donor in the production of phosphatidylcholine from phosphatidylethanolamine.[28]

Although these studies provide some evidence of the importance of choline for cognitive function later in life, they do not address the fact that choline can be produced endogenously as well as being obtained from the diet (i.e. they only assessed dietary intake or plasma concentrations, not both). In addition, the significant relationship may not be with choline directly, but rather with one of its metabolites or choline containing compounds such as sphingomyelin, phosphatidylcholine, or betaine.

In the NAME population, executive function was positively related to plasma choline concentration and dietary betaine concentration and inversely related to plasma Bet:Chol. Executive function is a cognitive construct that can be described as mental flexibility, the ability to multi-task, plan and organize. It is a higher order cognitive process, dependent on connections between subcortical structures and the prefrontal cortex. Any disruption in this pathway, by for example small vessel infarcts, or disruption in myelin integrity, would have the potential to alter executive function. Based on previous research demonstrating a lower betaine to choline ratio indicates an increase in PEMT activity,[28] we might infer that the inverse relationship between executive function and plasma Bet:Chol may indicate that a greater reliance on the endogenous choline pathway is associated with better executive function. In addition, we found that people with a lower Bet:Chol also have lower dietary choline intake, further supporting the idea that they would need to rely more on the endogenous pathway. However, we also found that lower Bet:Chol was associated with higher plasma homocysteine concentration, which one would not expect if betaine was used as a methyl donor to remethylate homocysteine and form SAM to be used in the endogenous pathway.

Memory was inversely related to dietary choline and plasma phosphatidylcholine concentrations. Dietary choline intake was also inversely related to plasma phosphatidylcholine concentrations. Phosphatidylcholine is a major component of plasma lipoproteins, especially VLDL. It may be that high plasma PC is a reflection of increased cardiovascular disease markers. In the NAME population, plasma phosphatidylcholine was positively associated with both LDL-c and HDL-c, as well as triglyceride concentrations. However, we included plasma concentrations of LDL-c and HDL-c as covariates in the analyses with cognitive constructs. Another possibility is the specific fatty acid chains contained within PC are what is actually related to memory rather than the entire PC molecule. daCosta et al. (2011) found that the ratio of plasma phosphatidylcholine containing docoshexaenoic acid (DHA) to total PC may be a good surrogate marker of PEMT activity.[45] Observational evidence demonstrates mainly inverse relationships between serum concentrations of DHA and cognitive impairment, while supplementation studies have mixed results.[46] Future studies in the NAME population may want to examine the side chains of PC in order to see if there is a similar relationship.

Cardiometabolic Risk, Cardiovascular and Cerebrovascular Pathology

Observational studies demonstrate a positive relationship between cardiovascular risk in mid-life and age-related cognitive decline.[29, 30] In addition, there is evidence from imaging studies, of a significant relationship between unrecognized myocardial infarction and increased risk of not only dementia, but also increased cerebral small vessel disease as indicated by volume of white matter lesions, in men.[47] Gender differences have also been noted in a longitudinal study describing the relationship between plasma lipid profile and incidence of cardiovascular and cerebrovascular events. In this study, Tohidi et al. (2013) reported that increased risk of stroke was associated with total cholesterol, low-density lipoprotein cholesterol, and non-high density lipoprotein cholesterol concentrations in women but not men, while all lipid components were associated with coronary heart disease in both men and women.[48]

Phosphatidylcholine biosynthesis in the liver is involved in the regulation of plasma lipoproteins, and when synthesis is decreased by impairment in either the exogenous or endogenous pathways, hepatic secretion of very low-density lipoprotein (VLDL) and HDL-c into plasma is decreased while hepatic steatosis is increased.[49] In animal models of atherosclerosis, inhibition of the endogenous phosphatidylcholine pathway by PEMT significantly decreases cardiovascular disease risk, but increases fat accumulation in the liver.[49-51] In humans, *plasma* betaine and choline concentrations have been differentially associated with cardiovascular disease risk factors, with higher concentrations of betaine predicting lower risk and higher concentrations of choline predicting higher risk.[9] In addition, an inverse relationship has been shown between plasma betaine concentration and BMI, [9, 52] while a positive association between plasma choline and BMI was found. [9] In the NAME population, we saw a similar relationship with plasma choline concentrations; those with choline concentrations in the upper two tertiles had significantly greater odds of self-reported cardiovascular disease compared to those with plasma choline concentrations in the lower tertile, even after adjusting for age, sex, BMI, hypertension, diabetes, plasma LDL-c, HDL-c, triglycerides, blood glucose, and homocysteine concentration. In addition, we saw a positive relationship between plasma choline concentration and BMI. While we did not see any relationship between plasma betaine and self-reported cardiovascular disease risk or BMI, we did find those with *dietary* betaine intakes in the upper tertile had significantly lower odds of self-reported cardiovascular disease risk compared to those in the lower tertile. These conclusions are in contrast to those of a longitudinal study that found a significant negative relationship between *dietary* choline intake and volume of cerebral white matter hyperintensities, a measure of myelin integrity that has been associated with small vessel disease.[25] They did not report on the relationship with dietary betaine intake. It is also important to note that these studies only measured dietary or plasma choline, thus failing to fully account for both endogenous and exogenous pathways. It may also be possible that the relationship

between choline and vascular disease is different when considering the location of blood vessels, cerebrovascular or cardiovascular, as well as the size of the blood vessel, small vessel versus large vessel disease. This certainly seems to be the case in the NAME population, where we found the risk of a small vessel infarct was inversely related to plasma choline concentration while the risk of a large vessel infarct was positively related to plasma choline concentration.

While the pathologies of large and small vessel diseases have some similarities, there are also distinct differences. Both small and large vessels can become atherosclerotic, however, plaques found in small vessels do not usually contain cholesterol clefts and calcifications which are often present in plaques in large vessels.[53]

The previously mentioned studies examining relationships between choline and vascular disease did not measure other potentially significant choline-related compounds, such as phosphatidylcholine and sphingomyelin. There is evidence that the enzyme sphingomyelinase is involved in the aggregation of LDL-c in atherosclerosis by hydrolyzing LDL-sphingomyelin and that SM:(SM+PC) is an important factor in determining the susceptibility to sphingomyelinase.[31] Furthermore, SM:(SM+PC) has been found to be an independent predictor of coronary artery disease.[32] In the NAME population, we found that SM:(SM+PC) was positively related to LDL-c and homocysteine, but inversely related to triglyceride concentration. However, no study has looked at this ratio in relation to cerebrovascular disease or cognitive function.

In the NAME population, we found an inverse relationship between volume of WMHI and plasma SM:(SM+PC). This was contrary to our hypothesis, which was that higher SM:(SM+PC) would be predictive of markers of cerebrovascular disease, just as it was found previously to be a risk factor for coronary artery disease.[32] However, the results seem to be consistent with the idea that the relationship between choline and its related compounds on vascular disease is dependent on vessel location and size. Coronary artery disease is a large vessel disease, while volume of WMHI is associated with small vessel disease.

Regional Brain volume

In order to control for overall brain atrophy, and get a measurement that better represents regional specific atrophy, brain volume measurements were normalized to intracranial volume. Our findings were consistent with our hypothesis that higher dietary intakes or plasma choline or choline related compounds would be associated with larger regional brain volumes, indicating less atrophy. Dietary betaine intake was positively associated with hippocampal volume, an area of the brain involved in memory, and inversely associated with ventricular volume. The ventricles are the spaces within the brain where the cerebral spinal fluid flows, so atrophy of the brain would make the volume larger. Additionally, we found that the volume of the amygdala, a brain region involved in emotional aspects of memory, was positively associated with plasma choline concentration, while ventricular volume was inversely associated to plasma betaine concentration. These findings make sense in comparison to what others have found regarding the importance of choline and betaine for brain development[54] as well as the need for choline in the formation of new synapses in the adult brain.[55]

Limitations and Future Directions

The major limitation in this study is its cross-sectional nature. This prevents us from drawing any causal conclusions. In order to do this, future study designs should include randomized controlled trials, with the same outcome measurements. We were also limited by the plasma metabolites that we measured. Future studies should include measurements of plasma dimethylglycine (DMG) and trimethylamine oxide (TMAO) in order to get a more complete picture of choline metabolism.

Dimethylglycine is produced when betaine gives up a methyl group to form methionine from homocysteine. Since this is the only reaction that would produce plasma DMG, the ratio of plasma free choline plus betaine to dimethylglycine ((Cho+Bet)/DMG)) would be an appropriate measurement to represent methylation capacity from choline. Since changes in DNA methylation

have been reported to play a role in learning and memory,[56] this measurement may be important when trying to draw conclusions regarding the mechanism by which choline or its metabolites contribute to cognitive performance. In addition, due to relationships between cardiovascular disease risk and cognitive decline[29, 30] as well as cerebrovascular disease, [47] TMAO is an important metabolite to include in future studies.

Trimethylamine (TMA) is produced when bacteria in the large intestine digest dietary phosphatidylcholine. TMA is then oxidized to TMAO in the liver. TMAO has been associated with increased risk of cardiovascular disease in humans[57, 58] At this point, the evidence supporting TMAO from dietary choline intake is still emerging and it is not clear whether TMAO is related to increased risk of cerebrovascular disease in addition to cardiovascular disease. However, it may be an important covariate to consider when looking for relationships between dietary choline intake and cognitive function and cerebrovascular health.

Finally, single nucleotide polymorphisms (SNPs) in genes involved in choline metabolism may modify these relationships and should be taken into consideration in future studies. Already, previous studies have shown SNPs in some of these genes alter a person's susceptibility to choline deficiency,[59, 60] are related to cerebro-[61] or cardiovascular disease risk, [62] or are associated with memory in older adults. [63]

Conclusions

We evaluated a cross-sectional cohort for relationships between dietary intake and plasma concentrations of choline-related compounds, cardiometabolic risk factors, and MRI markers of cerebrovascular disease and cognition, and found different associations between the nutrients and outcome measures depending on whether dietary intake or plasma concentrations were used in the statistical model. While we did not find a relationship between dietary intake and cerebrovascular disease, we did find that those individuals with lower plasma concentrations

of choline had higher odds of radiological evidence of small vessel infarct on brain MRI, and those with higher plasma concentrations had higher odds of large vessel infarcts, indicating that the relationship between choline and vascular disease is different when considering the location of blood vessels, cerebrovascular or cardiovascular, as well as the size of the blood vessel, small vessel versus large vessel disease. We found similar discrepancies when assessing the relationship between cognitive function and dietary intake and plasma concentrations of choline and betaine. Dietary betaine intake was positively associated with executive function, while dietary choline intake was inversely related to memory; in contrast, plasma concentrations of choline were positively related to executive function and betaine plasma concentrations were positively associated with memory. These discrepancies may be due to variations in choline metabolism due to SNPs, variations in intestinal microflora that utilize phosphatidylcholine, or other unknown factors that influence the use of dietary choline and the production of endogenous choline. These associations, though different from what has been found in other cohorts, are further support of role for choline and choline-related compounds in brain function and cerebrovascular health in old age and help to generate hypotheses for future research.

Table 1: Population Characteristics

Variable	Mean ± SD or Frequency	Minimum	Maximum
Age (yrs)	75 ± 8.6	59	103
BMI	31.5 ± 8.6	15.4	76.9
Plasma HDL-c (mg/dl)	49.6 ± 14.8	9.0	153.8
Plasma LDL-c (mg/dl)	106.0 ± 35.9	13.6	302.2
Plasma triglycerides	142.9 + 94.6	37	1280
Plasma glucose	113.4 + 38.4	48	440
Plasma Folate (ng/ml)	15.1 ± 9.8	2.0	114.4
Plasma PLP (nm/l)	69.9 ± 78.2	4.3	857.8
Plasma B-12 (pg/ml)	586.5 ± 495.5	133.0	12095.0
Plasma total homocysteine (µmoles/l)	12.0 ± 5.5	3.6	71.2
Sex (%female)	75.3%		
Presence of hypertension	85%		
Presence of diabetes	37.4%		
Presence of high glucose	25.3%		
Self-reported cardiovascular disease	41.8%		
Taking cardiovascular medication	83.2%		
Education			
0-4th grade	1.6%		
5-8th grade	12.3%		
9-11th grade	20.5%		
12th grade/ high school graduate	33.2%		
Some college – bachelor's degree	28.0%		

Graduate school	4.4%	

Table 2: Choline and Choline-Related Compounds

Variable	Mean ± SD	Minimum	Maximum
Dietary Intake (mg/day)[a]			
Choline	305 ± 111*	45	296
Males	336 ± 124	98	786
Females	294 ± 105	45	926
Betaine	128 ± 55*	15	468
Males	136 ± 56	15	468
Females	126 ± 55	16	376
Plasma Concentration (µM)[b]			
Choline	9.7 ± 3.4	3.6	26.9
Males	10.3 ± 3.8	3.6	23.6
Females	9.6 ± 3.2	4.1	26.9
Betaine	40.8 ± 18.9*	13.7	239.4
Males	46.1 ± 25.2	18.9	239.4
Females	38.8 ± 15.3	13.7	179.0
Phosphatidylcholine	2237.1 ± 435.2*	1093.3	3856.5
Males	2030.0 ± 417.6	1093.3	3032.9
Females	2315.2 ± 416.6	1477.8	3856.5
Sphingomyelin	612.9 ± 127.1*	268.3	1057.3
Males	543.4 + 120.1	268.3	870.7
Females	639.0 + 119.8	367.3	1057.3

** Indicates significant difference between males and females ($p \leq 0.05$)*

a. N= 1084 (264 male, 820 female)

b. N= 296 (81 male, 215 female)

Table 3: Relationships between Dietary Choline Intake and Concentration of Plasma Choline-Related Compounds

Plasma Compound	Model 1		Model 2	
	β-coefficient	**p-value**	**β-coefficient**	**p-value**
(log) Choline	-0.003	0.97	0.006	0.91
(log) Betaine	**0.15**	**0.04**	**0.14**	**0.03**
Phosphatidylcholine (PC)	**-386.32**	**0.05**	**-349.32**	**0.04**
Sphingomyelin (SM)	76.00	0.21	57.75	0.27
SM:(SM+PC)	**0.99**	**0.0007**	**1.11**	**0.002**
(log) Betaine:Choline	**0.11**	**0.05**	**0.13**	**0.05**

Model 1: Covariates – age, sex, dietary vitamin B-12, dietary folate, dietary B-6; N=232

Model 2: Covariates – age, sex, plasma vitamin B-12, plasma folate, plasma PLP, plasma homocysteine; N=229

Table 4: Relationship between Dietary Choline or Betaine Intake and Cardiometabolic Risk Factors

Cardiometabolic Risk Factors	Dietary Choline		Dietary Betaine	
	β-coefficient	**p-value**	**β-coefficient**	**p-value**
(log) LDL-c[a]	-0.001	0.97	0.02	0.67
(log) HDL-c[b]	**0.14**	**0.0004**	**0.12**	**0.01**
(log) Triglycerides[b]	**-0.07**	**0.003**	0.03	0.24
(log) Glucose[b]	-0.02	0.58	-0.01	0.83
(log) Hcy[c]	**2.48**	**<.0001**	**-0.06**	**<.0001**
BMI[d]	0.0004	0.49	-0.001	0.13

covariates: age, sex

a. N = 990; b. N = 1000; c. N = 1029; d. 1023

LDL-c = low density lipoprotein cholesterol; HDL-c = high density lipoprotein cholesterol; TG = triglycerides; Hyc = homocysteine; BMI= body mass density

Table 5: Relationship between Plasma Choline-Related Compounds and Cardiometabolic Risk Factors

Plasma Compound	(log) LDL-c[a]		(log) HDL-c[b]		(log) TG[b]		(log) Glucose[b]		(log) Hcy[b]		BMI[c]	
	β	p	β	p	β	p	β	p	β	p	β	p
(log) Choline	-0.05	0.34	**-0.14**	**0.04**	0.03	0.5	-0.08	0.28	**0.09**	**<.0001**	**0.003**	**0.003**
(log) Betaine	**-0.15**	**0.01**	0.03	0.65	**-0.16**	**<0.0001**	-0.13	0.1	-0.02	0.37	-0.0003	0.80
PC	**1529.04**	**<0.0001**	**1512.47**	**<0.0001**	**824.69**	**<0.0001**	-285.78	0.21	-32.11	0.62	-14.06	<.0001
SM	**664.31**	**<0.0001**	**460.55**	**<0.0001**	-21.66	0.5	-82.78	0.2	20.57	0.27	-0.59	0.56
SM:(SM+PC)	**0.07**	**<0.0001**	0.01	0.43	**-0.07**	**<0.0001**	-0.003	0.84	**0.01**	**0.03**	**0.001**	**0.0001**
(log) Bet:Chol	-0.1	0.15	**0.18**	**0.03**	**-0.19**	**<0.0001**	-0.05	0.58	**-0.11**	**<.0001**	**-0.004**	**0.01**

Covariates: age, sex

a. N=291; b. N=295; c. N=280

LDL-c = low density lipoprotein cholesterol; HDL-c = high density lipoprotein cholesterol; TG = triglycerides; Hyc = homocysteine;

BMI= body mass density; β= beta coefficient; p = p-value

Table 6: Odds of Cardiovascular Disease and Tertiles of Dietary Choline or Betaine Intake

Dietary Compound	Model 1						Model 2					
	T2 vs T1			T3 vs T1			T2 vs T1			T3 vs T1		
	OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI	
Choline	1.00	0.75	1.35	0.79	0.58	1.07	0.99	0.70	1.40	0.85	0.60	1.21
Betaine	0.79	0.59	1.07	**0.72**	**0.53**	**0.97**	0.95	0.67	1.34	0.78	0.55	1.10

Model 1: covariates: age, sex; N=1062

Model 2: covariates: age, sex, BMI, HTN, diabetes, plasma LDL-c, HDL-c, Trig, Gluc, Hcy; N=901

T1= lowest tertile, T2= second tertile, T3= highest tertile

Table 7: Odds of Cardiovascular Disease and Tertiles of Plasma Choline-Related Compounds

Plasma Compound	Model 1						Model 2					
	T2 vs T1			T3 vs T1			T2 vs T1			T3 vs T1		
	OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI	
Choline	**2.34**	**1.21**	**4.54**	**3.20**	**1.66**	**6.15**	**2.24**	**1.08**	**4.67**	**2.91**	**1.41**	**6.01**
Betaine	1.09	0.59	2.02	1.28	0.68	2.39	1.21	0.60	2.40	1.33	0.66	2.72
PC	0.87	0.48	1.59	0.60	0.32	1.14	0.70	0.33	1.52	0.33	0.11	1.00
SM	0.72	0.39	1.34	0.53	0.28	1.02	0.71	0.32	1.59	0.59	0.21	1.68
SM:(SM+PC)	1.16	0.64	2.12	0.82	0.44	1.51	1.67	0.79	3.53	1.17	0.50	2.73
Bet:Chol	0.54	0.30	1.00	0.59	0.32	1.09	0.56	0.27	1.13	0.73	0.36	1.50

Model 1: covariates: age, sex; N=287

Model 2: covariates: age, sex, BMI, HTN, diabetes, LDL-c, HDL-c, Trig, Gluc, Hcy; N=261

T1= lowest tertile, T2= second tertile, T3= highest tertile

Table 8: Relationships between Cognitive Constructs and Dietary Choline or Betaine

Cognitive Construct	Dietary Measure	Model 1		Model 2	
		β-coefficient	**p-value**	**β-coefficient**	**p-value**
Executive Function	(log) Choline	-0.53	0.28	-0.11	0.82
	(log) Betaine	**0.73**	**0.001**	**0.61**	**0.01**
Memory	(log) Choline	**-1.38**	**0.01**	**-1.38**	**0.01**
	(log) Betaine	0.26	0.24	0.22	0.32
Attention	(log) Choline	0.83	0.10	0.59	0.25
	(log) Betaine	0.13	0.57	-0.03	0.90

N=849

Model 1: Covariates – calories, age, sex, education, body mass index, presence of hypertension, presence of diabetes, dietary saturated fat intake, dietary cholesterol intake

Model 2: Covariates– calories, age, sex, education, body mass index, presence of hypertension, presence of diabetes, dietary saturated fat intake, dietary cholesterol intake, dietary vitamin B-6, vitamin B-12, folate intake

Table 9: Relationships between Cognitive Constructs and Plasma Choline-Related Compounds

Cognitive Construct	Plasma Measure	Model 1		Model 2	
		β-coefficient	p-value	β-coefficient	p-value
Executive Function	(log) Choline	**1.20**	**0.004**	**1.23**	**0.01**
	(log) Betaine	0.16	0.68	-0.04	0.92
	PC	0.0002	0.14	0.0002	0.36
	SM	0.0009	0.07	0.0007	0.47
	SM:(SM+PC)	0.54	0.78	-0.69	0.77
	(log) Bet:Chol	**-0.76**	**0.03**	**-0.91**	**0.03**
Memory	(log) Choline	0.42	0.29	0.35	0.47
	(log) Betaine	**0.83**	**0.03**	0.59	0.17
	PC	**-0.0003**	**0.03**	**-0.0004**	**0.03**
	SM	-0.0007	0.88	-0.0003	0.73
	SM:(SM+PC)	3.38	0.07	3.10	0.18
	(log) Bet:Chol	0.35	0.31	0.27	0.50
Attention	(log) Choline	0.004	0.99	0.15	0.78
	(log) Betaine	0.11	0.78	-0.26	0.58
	PC	0.0002	0.22	0.0002	0.43
	SM	0.0005	0.31	0.0001	0.15
	SM:(SM+PC)	-0.12	0.95	1.37	0.58
	(log) Betaine:Choline	0.09	0.81	-0.34	0.45

Model 1: Covariates – age, sex, education; N=274

Model 2: Covariates – age, sex, education, body mass index, presence of hypertension, presence of diabetes, concentration of plasma high density lipoprotein cholesterol, low density lipoprotein cholesterol, vitamin B12, PLP, folate, and homocysteine; N=249

PC=phosphatidylcholine; SM=Sphingomyelin

Table 10: Relationships between Dietary Choline and Betaine Intake and Cerebrovascular Disease Markers

MRI Volumetric Measurement	Plasma Measure	Model 1		Model 2	
		β-coefficient	p-value	β-coefficient	p-value
Hippocampal Volume[a]	(log) Choline	266.06	0.72	256.73	0.74
	(log) Betaine	**743.44**	**0.04**	**841.39**	**0.02**
Amygdala Volume[a]	(log) Choline	-671.97	0.35	-538.78	0.46
	(log) Betaine	626.78	0.07	662.84	0.06
(log) Ventricular Volume[b]	(log) Choline	0.03	0.90	-0.001	1.00
	(log) Betaine	**-0.19**	**0.05**	**-0.20**	**0.05**
(log) White Matter Hyperintensities Volume[b]	(log) Choline	0.20	0.67	0.22	0.64
	(log) Betaine	-0.27	0.23	-0.22	0.33

MRI Measurement	Plasma Measure	T2 vs. T1		T3 vs. T1		T2 vs. T1		T3 vs. T1	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Small Vessel Infarct[c]	(log) Choline	1.28	0.52 - 3.15	0.77	0.21 - 2.74	1.48	0.58 - 3.78	0.90	0.24 - 3.41

	(log) Betaine	1.58	0.66 - 3.77	1.54	0.61 - 3.88	1.76	0.71 - 4.36	1.83	0.70 - 4.79
Large Vessel Infarct[c]	(log) Choline	1.14	0.26 - 5.06	3.29	0.51 - 21.12	1.19	0.26 - 5.49	3.55	0.50 -25.01
	(log) Betaine	0.47	0.12 - 1.87	1.31	0.34 - 5.09	0.46	0.11 - 1.89	1.28	0.32 - 5.17

Model 1: Covariates – calories, age, sex, education, BMI, HTN, DM, dietary saturated fat, cholesterol

Model 2: Covariates – calories, age, sex, education, BMI, HTN, DM, dietary saturated fat, cholesterol, vitamin B-6, vitamin B-12, folate

a. N=238, b. N=240, c. N=254

Table 11: Relationships between Plasma Choline-Related Compounds and Cerebrovascular Disease Markers

MRI Volumetric Measurement	Plasma Measure	Model 1		Model 2	
		β-coefficient	**p-value**	**β-coefficient**	**p-value**
Hippocampal Volume[a]	(log) Choline	529.07	0.10	439.98	0.27
	(log) Betaine	146.02	0.63	96.14	0.78
	PC	0.15	0.17	0.05	0.74
	SM	**0.79**	**0.03**	0.73	0.31
	SM:(SM+PC)	973.77	0.52	373.08	0.84
	(log)Betaine:Choline	-255.47	0.35	-215.22	0.51
Amygdala Volume[a]	(log) Choline	**1019.75**	**0.003**	**923.69**	**0.02**
	(log) Betaine	263.52	0.40	323.72	0.36
	PC	0.15	0.20	0.33	0.06
	SM	0.15	0.71	1.27	0.09
	SM:(SM+PC)	-1665.7	0.29	-1019.9	0.60
	(log)Betaine:Choline	-507.17	0.08	-339.33	0.32
	(log) Choline	-0.19	0.06	-0.10	0.40

(log) Ventricular Volume[b]	(log) Betaine	**-0.18**		**0.05**		**-0.25**		**0.02**	
	PC	0.00003		0.35		0.0001		0.10	
	SM	-0.0001		0.43		0.0002		0.43	
	SM:(SM+PC)	**-0.90**		**0.05**		-0.58		0.31	
	(log)Betaine:Choline	-0.02		0.83		-0.16		0.11	
(log) White Matter Hyperintensities Volume[b]	(log) Choline	0.08		0.70		0.37		0.14	
	(log) Betaine	0.05		0.82		0.03		0.88	
	PC	0.00004		0.52		0.0002		0.10	
	SM	-0.0003		0.27		-0.0004		0.35	
	SM:(SM+PC)	**-1.91**		**0.05**		**-2.36**		**0.04**	
	(log)Betaine:Choline	-0.02		0.91		-0.23		0.27	
MRI Measurement	**Plasma Measure**	**T2 vs. T1**		**T3 vs. T1**		**T2 vs. T1**		**T3 vs. T1**	
		OR	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**	**OR**	**95% CI**
Small Vessel Infarct[c]	(log) Choline	**0.43**	**0.21 - 0.89**	**0.47**	**0.24 - 0.95**	**0.43**	**0.19 - 0.98**	**0.36**	**0.16 - 0.82**
	(log) Betaine	0.78	0.39 - 1.54	**0.46**	**0.22 - 0.97**	0.70	0.33 - 1.49	**0.41**	**0.18 - 0.95**
	PC	0.61	0.30 - 1.26	0.94	0.47 - 1.91	0.70	0.31 - 1.58	1.15	0.44 - 2.99

	SM	0.85	0.41 - 1.78	0.87	0.42 - 1.80	0.81	0.32 - 2.07	0.57	0.18 - 1.87
	SM:(SM+PC)	1.08	0.55 - 2.13	0.77	0.37 - 1.57	0.80	0.37 - 1.72	0.53	0.23 - 1.23
	(log)Betaine:Choline	0.92	0.46 - 1.86	0.91	0.45 - 1.831	1.26	0.58 - 2.77	1.09	0.48 - 2.49
Large Vessel Infarct[c]	(log) Choline	**3.98**	**1.25 - 12.7**	**3.96**	**1.25 - 12.5**	**10.51**	**2.0 - 55.3**	**12.37**	**2.38 - 64.3**
	(log) Betaine	1.31	0.52 - 3.29	1.16	0.46 - 2.96	1.35	0.46 - 4.01	1.13	0.36 - 3.52
	PC	0.81	0.34 - 1.93	0.73	0.29 - 1.86	1.25	0.44 - 3.53	0.85	0.22 - 3.24
	SM	0.72	0.29 - 1.79	0.53	0.21 - 1.38	1.09	0.34 - 3.53	0.55	0.12 - 2.51
	SM:(SM+PC)	0.76	0.33 - 1.75	0.43	0.17 - 1.12	0.77	0.29 - 2.05	0.43	0.14 - 1.34
	(log)Betaine:Choline	1.33	0.55 - 3.24	1.00	0.40 - 2.52	1.03	0.37 - 2.84	0.63	0.21 - 1.91

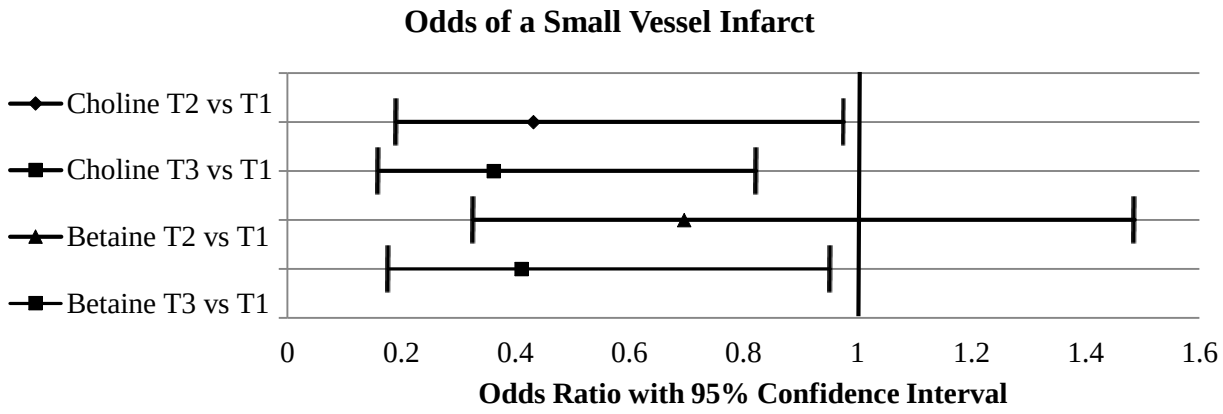
Model 1: Covariates – age, sex, education

Model 2: Covariates – age, sex, education, body mass index, presence of hypertension, presence of diabetes, concentration of plasma high density lipoprotein cholesterol, low density lipoprotein cholesterol, vitamin B-12, PLP, folate, and homocysteine

PC=phosphatidylcholine; SM=Sphingomyelin

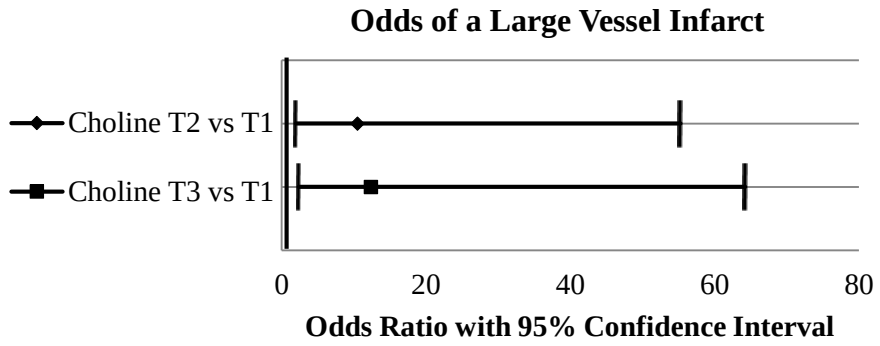
a. Model 1 N=292; Model 2 N=265, b. Model 1 N=294; Model 2 N=266, c. Model 1 N=289; Model 2 N=261

Figure 1



OR adjusted for age, sex, education, BMI, hypertension, diabetes, HDL-c, LDL-c, plasma PLP, vitamin B-12, folate, and homocysteine (T1= lowest tertile, T2= second tertile, T3= highest tertile)

Figure 2



OR adjusted for age, sex, education, BMI, hypertension, diabetes, LDL-c, HDL-c, plasma PLP, vitamin B-12, folate, and homocysteine (T1= lowest tertile, T2= second tertile, T3= highest tertile)

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Chapter 2: The relationship between dietary choline and cognitive performance is modified by SNPs in genes related to choline and folate metabolism

Introduction

Advancing age has been reported as the strongest risk factor for cognitive decline.[1] As Americans are living longer,[2] the rising economic and societal costs associated with age-related cognitive decline create a need for the development of novel and cost-effective public health strategies that will delay, slow, or even prevent this decline. Observational studies have provided evidence that the risk of cognitive decline can be modified by dietary factors[3-5] and thus further investigation into the role of specific nutrients in cognition is critical.

Choline is an essential nutrient with multiple links to brain health. As a component of phospholipids, choline-containing compounds are involved in cell signaling, lipid transport, and cell membrane structure. In addition, choline is a precursor to the neurotransmitter acetylcholine and the methyl donor betaine.[6] There is evidence that changes in DNA methylation play a role in early neuronal development[7] as well as in the pathogenesis of neurodegenerative diseases, such as Alzheimer's Disease and Parkinson's Disease, by altering gene expression.[8] In addition, plasma choline and betaine concentrations have been differentially associated with risk of cardiovascular disease, with higher concentrations of betaine predicting lower risk and higher concentrations of choline predicting higher risk.[9] Whether this relationship holds true for cerebrovascular disease risk has not been well investigated.

In a previous study (Nutrition, Aging and Memory in Elders; Chapter 1), we evaluated cross-sectional cohort relationships between dietary intake and plasma concentrations of choline and betaine and MRI markers of cerebrovascular disease and cognition. Different associations were identified between the nutrients and outcome measures depending on whether dietary intake or plasma concentrations were used in the statistical model. While we did not find a relationship

between dietary intake and cerebrovascular disease, we did find that those individuals with lower plasma concentrations of choline had higher odds of radiological evidence of small vessel infarct on brain MRI, and those with higher plasma concentrations had higher odds of large vessel infarcts. We found similar discrepancies when assessing the relationship between cognitive function and dietary intake and plasma concentrations of choline and betaine. Dietary betaine intake was positively associated with executive function, while dietary choline intake was inversely related to memory; in contrast, plasma concentrations of choline were positively related to executive function scores and betaine plasma concentrations were positively associated with memory.

It is possible that the discrepancy in findings between dietary and plasma relationships with cerebrovascular disease risk and cognitive function may be due to a modifying variable such as single nucleotide polymorphisms (SNPs). Although humans have the ability to produce choline endogenously, involving the activity of phosphatidylethanolamine N-methyltransferase (*PEMT*) in the liver, this production is generally not enough to meet daily needs and thus choline is required from the diet as well.[10] SNPs in *PEMT* may decrease its activity, suggesting that adequate intake (AI) values are too low for some people.[10-12] SNPs in other genes related to choline metabolism, methylene-tetrahydrofolate dehydrogenase (*MTHFD1*), choline dehydrogenase (*CHDH*). betaine: homocysteine methyltransferase (*BHMT*), and S-adenosylmethionine synthetase type 1 (*MAT1A*), may also modify an individual's dietary choline needs or have the potential to interact with dietary choline to influence cognitive performance.[11, 13-17] No previous study, however, has examined the interaction of dietary choline with SNPs in genes related to choline and folate metabolism on cognitive function.

The purpose of this study was to describe the relationships between dietary choline and cognitive outcomes and whether SNPs in choline and folate metabolism modify these relationships in the Boston Puerto Rican Health Study (BPRHS).[18] We hypothesized that total

dietary choline (free choline, glycerophosphocholine (GPC), phosphocholine (Pcho), phosphatidylcholine (PtdCho), sphingomyelin (SM)) would be positively related to cognitive performance and that this relationship would be modified by SNPs in genes related to choline and folate metabolism.

Methods

This study involved secondary analysis of existing data from the Boston Puerto Rican Heath Study (BPRHS).[18] The BPRHS is an ongoing longitudinal study designed to examine the role of psychosocial stress on health outcomes and potential modification by nutrients, genetic individuality, and social support in (age 45-75 yr) people of Puerto Rican decent living in the greater Boston, Massachusetts area.[18] Data from the BPRHS that were utilized in the current study include Food Frequency Questionnaire (FFQ) analysis for choline intake, SNP genotyping from a genome-wide chip, and cognitive measurements of memory and executive function, with composite scores derived from principal components analysis (PCA) of cognitive tests results. A detailed description of data collection from the BPRHS is described previously.[18]

Five genes related to choline and folate metabolism (*PEMT, MAT1A, BHMT, MTHFD1, CHDH*) were assessed. Each of these genes had published evidence of a relationship to susceptibility to a choline deficient diet (associated with increased markers of liver damage when on a choline deficient diet), cognitive function, neurodegenerative disease, or vascular disease risk. From the available genome-wide chip data, SNPs were excluded due to deviation from the Hardy-Weinberg equilibrium, and tagger pairwise tagging, using the program Haploview, along with setting a requirement for minor allele frequency of ≥ 0.05, narrowing the comparisons down to 64 SNPs of interest (see Table 1).

Statistical Analysis

Variables that were not normally distributed were log transformed. Multiple linear regression and logistic regression models were used to examine relationships between a) dietary

intake and cognitive outcomes, b) SNPs in choline and folate metabolism and cognitive outcomes, c) interaction of dietary intake and SNP status on cognitive outcomes. Covariates considered for inclusion into the model included age, sex, education, body mass index (BMI), presence of hypertension, presence of diabetes, calorie intake, and population ad-mix.

Results

Population Characteristics

Demographics and other cohort characteristics of the participants included in our analyses are presented in Table 2. There were 1011 participants with baseline cognitive data, 1001 participants with 2-year cognitive data, and 931 participants with complete cognitive testing at both baseline and follow-up. In the final models, participants were excluded for missing covariate data (dietary intake data -4, education data -2, BMI -13, sex -52, hypertension -8, and diabetes -16). The mean age at baseline was 57 ($\pm$8) years and participants were predominantly female (72%). The mean BMI was in the obese range (32 $\pm$ 7 kg/m^2) with a high prevalence of hypertension (68%) and diabetes (37%). Attained level of education was low, with 43% having a 9th grade education or below. Average dietary choline intake was below the AI in 74% of women and 72% of men.

Diet and Cognitive Outcomes

Dietary choline intake was positively associated with baseline and 2-year executive function scores when controlling for calorie intake, age, sex, education, BMI, presence of hypertension, and presence of diabetes (see Table 3). There were no statistically significant associations between choline intake and measures of memory.

SNPs and Cognitive Outcomes

Of the 64 SNPs evaluated, 25 were significantly associated with one or more cognitive outcomes and a Tukey-Kramer post hoc analysis was done to further explore this relationship (see Tables 4-9). Tukey Kramer results showed SNPs in *PEMT* were significantly related to baseline

executive function and memory, 2-year executive function and memory, and decline in memory. SNPs in *MTHFD1* were also associated with baseline memory, while *BHMT* was related to 2-year executive function and *CHDH* to 2-year memory scores.

SNPs, in the 5 genes assessed in this study, have previously been shown to be associated with cognition related measures and cardiovascular pathology. Of thee previously published SNPs, two were evaluated in the BPRHS cohort (rs2236225, rs3733890). There was no significant relationship between rs2236225 and cognitive outcomes. rs3733890 was significantly associated with baseline memory scores. However, least squared means were not different among the three genotypes (AA, AG, GG) when Tukey-Kramer post hoc analysis was performed (Table 5). In addition, we identified 24 other SNPs that were associated with cognitive outcomes and had significant Tukey-Kramer results (see Tables 4-9); 9 of which also modified the relationship between dietary choline intake and cognitive outcomes as discussed below.

Interaction between Dietary Intake and SNPs: Relationship with Cognitive Outcomes

Of the 64 SNPs evaluated, 22 interacted with dietary choline intake to significantly relate to one or more cognitive outcomes and were further stratified by alleles for additional analysis by multiple linear regression (see Tables 10-15). SNPs in *PEMT* modified the relationship between dietary choline intake and executive function, baseline memory, 2-year executive function, and decline in memory. SNPs in *MAT1A* also modified the relationship with baseline and 2-year executive function, while SNPs in *MTHFD1* were only modified the relationship with 2-year executive function. SNPs in *BHMT* modified the relationship between dietary choline intake and baseline and 2-year memory as well as decline in executive function.

Of previously published SNPs reported to influence susceptibility to choline deficiency, and thus have the potential to modify the relationship between dietary choline intake and cognitive function, one was evaluated in the BPRHS cohort. The interaction between dietary choline intake and rs2236225 was significantly related to 2-year executive function. Stratifying

by genotype revealed significant positive relationships between dietary choline intake and 2-year executive function scores in those with the AG or GG genotype (see Table 11). As mentioned above, 9 SNPS that were significantly related to cognitive outcomes also modified the relationship between dietary choline intake and one or more cognitive outcomes. rs1869771 and rs34075240 were both related to baseline executive function (Table 4), but only rs34075240 showed a significant relationship by genotypes after Tukey-Kramer post hoc analysis, with those with the AA genotype scoring significantly higher than those with the AG genotype (Figure 1). rs1869771 also modified the relationship between dietary choline intake and baseline executive function (Table 10), baseline memory (Table 11) and executive function decline (Table 14). rs34075240 was also significantly associated with 2-year executive function (Table 6), with those with the AA genotype scoring significantly higher than those with the AG or GG genotypes (Figure 2). In addition, when stratifying by genotype, only those with the AG genotype showed a significant positive relationship between dietary choline intake and baseline memory (Table 11).

Two-year executive function was associated with rs12941217, rs3785499, rs4646340, rs4646342, and rs492842 (Figures 3-7). When stratifying by genotype, those with the AA or AG rs1291217 genotype or the rs492842 TT genotype, showed a positive relationship between dietary choline intake and 2-year memory. Those with the rs3785499 CC or CT genotype or the rs6502603 GG or GT genotype showed a positive relationship between dietary choline intake and 2-year executive function (Table 12). Those with the rs3785499 CC, rs4646340 TT, rs4646342 AA, or rs9905728 CC genotypes showed positive relationships between dietary choline intake and odds of memory decline (Table 15). rs9905728 was also positively related to 2-year memory scores (Table 7), with those with the CC genotype scoring significantly higher than those with the TT genotype (Figure 8). rs6502603 was related to decline in memory; the odds of memory decline in those with the GG genotype was over 2 times that of those with the GT genotype (Table 9). In addition, there was a significant interaction between dietary choline intake and

rs6502603 on executive function decline. However, when stratified by genotype, no relationships met the p-value for significance (Table 14).

Discussion

Diet and Cognitive Outcomes

The Dietary Reference Intake (DRI) recommendations for choline were set by the Institute of Medicine in 1998.[6] Due to insufficient data to set an Estimated Average Requirement (EAR), and thus calculate a Recommended Daily Allowance (RDA), an Adequate Intake (AI) was developed for choline based largely on the results of a single depletion/repletion study[19] using maintenance of serum alanine amino transferase levels as a marker of liver damage due to a choline deficient diet.[20] The AI for adults age 19 and older is 550mg/day for men and 425mg/day for women. Dietary choline intakes in the BPRHS population were slightly higher than other cohorts (NHANES 2003-2004 (less than 10% met or exceeded the AI),[21] Framingham Offspring Study (313 $\pm$ 61 mg/day),[22] Atherosclerosis Risk in Communities Study (332 $\pm$ 125 mg/day for men, 294 $\pm$ 112 mg/day for women),[23] Nutrition Aging and Memory in Elders (NAME) cohort (336 $\pm$ 124 mg/day for men, 294 $\pm$ 105 mg/day for women; Chapter 1)), but still below the AI.

The importance of choline in human brain development has been the focus of several studies,[24] while the importance of choline for cognitive function later in life has not been as extensively studied. Intervention studies have shown choline-containing supplements enhanced learning and memory[25-27] and prevented or reversed cognitive decline in patients with cerebrovascular disease.[28-30] In addition, cross-sectional studies demonstrate associations between choline intake or foods high in dietary choline and global cognition or memory.[31, 32] Previously we found that dietary choline intake was inversely associated with memory scores in cross-sectional analysis of an elderly (age $\geq$60yr) cohort (NAME; Chapter 1). Conversely, in a group of elderly persons in Madrid, those who made no errors on a test of general cognitive capacity consumed more eggs, a food high in choline, than those who made errors and after

adjusting for energy intake and education level, there was a negative association between the number of errors and choline intake.[32] Similarly, choline intake, assessed by food frequency questionnaire, at the same time as cognitive tests were administered, in a healthy cohort of older adults (Framingham Offspring Study) was positively associated with scores on tests of verbal memory and visual memory, but not with verbal learning or executive function.[31]

In the BPRHS cohort, we did not find any relationship between memory measurements and dietary choline intake, however, there was a positive association between dietary choline intake and baseline and 2-year executive function scores (see Table 3). Executive function is a cognitive construct that can be described as mental flexibility, the ability to multi-task, plan and organize. It is a higher order cognitive process, dependent on connections between subcortical structures and the prefrontal cortex.

The inconsistent findings among these studies may be due to differences in population characteristics. One such difference may lie in genetic variants which have the potential to modify the relationship between dietary choline and cognitive function due to their influence on choline and folate metabolism, vascular pathology, or a direct relationship with cognitive function.

SNPs and Cognitive Outcomes

SNPs in three of the 5 genes assessed in this study have previously been shown to be associated with cognition related measures, and two associated with cardiovascular pathology. The rs7946 SNP in *PEMT* is associated with Alzheimer's disease in Han Chinese women,[12] while the rs2236225 SNP in *MTHFD1* might be a weak risk factor for early onset Alzheimer's disease.[13] The rs3851059 and rs4933327 SNPs in *MAT1A* were associated with poorer memory scores at age 11 and 70 years in a longitudinal study (Lothian Birth Cohort 1936), but not with general cognition, general speed of processing, or scores on a test of verbal reasoning.[17] The rs3851059 SNP in *MAT1A*, a gene which is involved in the production of the universal methyl-

donor s-adenosylmethionine, has also been associated with risk of stroke while the rs7087728 SNP is associated with risk of hypertension.[16] Furthermore, the rs3733890 SNP in *BHMT*, a gene involved in the remethylation of homocysteine from betaine, was associated with decreased risk of coronary artery disease in people over age 60.[15] Of these previously published SNPs, only two were evaluated in the BPRHS cohort (rs2236225, rs3733890) and only one was significantly related to cognitive measures. rs3733890 was significantly associated with baseline memory scores. However, least squared means were not different between the three genotypes (AA, AG, GG) when Tukey-Kramer post hoc analysis was performed.

Interaction between Dietary Intake and SNPs: Relationship with Cognitive Outcomes

Several SNPs have been reported to influence susceptibility to choline deficiency, and thus have the potential to modify the relationship between dietary choline intake and cognitive function. In *PEMT*, rs12325817 and rs7946 are associated with increased susceptibility to choline deficiency in women and nonalcoholic fatty liver disease, respectively.[10, 11] SNPs in *CHDH*, involved in the conversion of choline to betaine, have been shown to have variable effects (rs9001 protective effect and rs12676 increased susceptibility to choline deficiency) that will modify an individual's dietary choline needs.[14] The rs2236225 SNP in *MTHFD1*, a gene involved in DNA synthesis from 5,10-methyene-tetrahydrofolate, increases susceptibility to organ damage when fed a choline deficient diet.[11] Of these previously published SNPs, only one was evaluated in the BPRHS cohort. There was a positive association between dietary intake and 2-year executive function in those with the rs2236255 AG or GG genotype.

Limitations

The genetic make-up of the Boston Puerto Rican cohort is unique, and thus results of this study are not generalizable to other populations. In addition, our sample size was relatively small for identifying significant gene-diet interactions. Furthermore, we did not have data on

concentrations of choline-related compounds in the plasma, which is a better marker of total choline than dietary intake.

Conclusion

In this longitudinal cohort, the BPRHS, we have found significant associations between certain SNPs in choline and folate metabolism and cognitive outcomes, as well as interactions between dietary choline intake and certain SNPs on cognitive outcomes. These relationships should be assessed in similar populations in order to draw significant conclusions regarding dietary choline recommendations based on genotype.

Tables and Figures

Table 1: Single Nucleotide Polymorphisms Analyzed in the Boston Puerto Rican Cohort

Chromosome	Associated Gene	Position	dbSNP RS ID	Reference Alleles
3	*CHDH*	53851503	rs17053530	[T/C]
		53852835	rs2289209	[T/C]
		53854068	rs7625247	[T/G]
		53856050	rs6445606	[T/C]
		53870840	rs13317328	[A/C]
		53878452	rs2289206	[T/C]
		53861491	rs2015498	[A/C]
		53862515	rs7620929	[T/C]
		53873578	rs930367	[T/C]
5	*BHMT*	78411426	rs16876527	[T/C]
		78412044	rs16876528	[T/C]
		78421959	rs3733890	[A/G]
		78413504	rs3797546	[T/C]
		78409987	rs492842	[T/C]
		78411324	rs7700970	[T/C]
10	*MAT1A*	82035560	rs4934027	[T/C]
		82037734	rs76776628	[T/G]
		82044100	rs28539197	[T/G]
		82045876	rs11202421	[T/C]
		82042782	rs12411742	[A/G]

		82049603	rs17677908	[A/G]
		82032240	rs1985908	[A/G]
		82056667	rs2185427	[A/C]
14	*MTHFD1*	64849415	rs2983733	[A/G]
		64852905	rs1956545	[T/C]
		64849864	rs78043435	[A/G]
		64858679	rs73271893	[T/C]
		64884076	rs56811449	[T/C]
		64911627	rs1256143	[T/C]
		64887711	rs17824591	[A/G]
		64888055	rs1885031	[T/C]
		64882380	rs1950902	[A/G]
		64915182	rs2236222	[A/G]
		64909151	rs2236224	[A/G]
		64902116	rs2295639	[T/C]
		64876545	rs34110441	[T/G]
		64868671	rs8006686	[T/C]
17	*PEMT*	17410053	rs4646409	[A/G]
		17415217	rs3785499	[T/C]
		17432456	rs16961845	[T/C]
		17445680	rs6502603	[T/G]
		17469455	rs4646360	[T/C]
		17480195	rs2124344	[A/G]
		17484654	rs7224725	[T/C]

		17484934	rs9944423	[A/G]
		17503206	rs12941217	[A/G]
		17504609	rs11867208	[T/C]
		17547236	rs12602348	[A/G]
		17561094	rs1869771	[A/C]
		17493272	rs4646342	[A/G]
		17417808	rs66637059	[T/C]
		17427492	rs75529714	[A/G]
		17464077	rs34075240	[A/G]
		17475350	rs897450	[T/G]
		17505383	rs117588728	[T/C]
		17567943	rs79020652	[T/C]
		17559605	rs10401011	[T/C]
		17525476	rs11869492	[T/C]
		17503544	rs2350631	[T/C]
		17494015	rs4646340	[T/C]
		17492077	rs4646343	[T/G]
		17530554	rs4925048	[A/G]
		17506892	rs8071007	[T/C]
		17564523	rs9905728	[T/C]

CHDH = choline dehydrogenase; *BHMT*= betaine: homocysteine methyltransferase; *MAT1A* = S-adenosylmethionine synthetase type 1; *MTHFD1*= methylene-tetrahydrofolate dehydrogenase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

Table 2: Descriptive Characteristics

Variable	**Mean ± SD or Frequency**	**Minimum**	**Maximum**
Age (yrs)	57 ± 8	45	76
Body Mass Index	32 ± 6.5	17	63
Sex (%female)	70		
Presence of hypertension (%)	68		
Presence of diabetes (%)	37		
Hemoglobin A1C (% glycosylated hemoglobin)	7.0 ± 1.7	3.7	15.1
Glucose (mg/dL)	119.7 ± 49.4	51.0	526.0
LDL-c (mg/dL)	107.8 ± 35.0	16.0	235.0
VLDL-c (mg/dL)	29.9 ± 15.8	7.0	173.0
HDL-c (mg/dL)	44.9 ± 12.5	8.0	114.0
Triglycerides (mg/dL)	163.3 ± 116.9	38.0	1760.0
Vitamin B-6 (nm/L)	58.0 ± 62.2	5.5	737.4
Vitamin B-12 (pg/mL)	544.6 ± 277.4	78.0	2320.0
Folate (ng/ml)	19.1 ± 8.8	3.2	87.6
Homocysteine (μmoles/L)	9.0 ± 4.7	3.9	69.4
Education (%)			

No schooling or less than 5th grade	18		
5th - 9th grade	25		
9th -12th grade or GED	41		
Some college or bachelor's degree	14		
At least some graduate school	2		
Dietary choline intake (mg/day)	383.9 ± 208.3	36.5	1659.7
Males	470.2 ± 249.8	122.2	1618.7
Females	349.7 ± 179.8	36.5	1659.7

Table 3: Relationships between Cognitive Constructs and Dietary Choline Intake

Cognitive Outcome	**β-coefficient***	**p-value**
Baseline Executive Function[a]	**0.74**	**0.01**
Baseline Memory[a]	0.37	0.25
2-yr Executive Function[b]	**1.17**	**0.0001**
2-yr Memory[b]	-0.26	0.40
Cognitive Outcome	**Odds Ratio Point Estimate**	**p-value**
Decline in Executive Function[c]	0.23	0.19
Decline in Memory[c]	3.45	0.29

a N=893; b N=883; c N=829

*Covariates – calorie intake, age, sex, education, body mass index, presence of hypertension, presence of diabetes

Table 4: Relationships between SNPs and Baseline Executive Function

Associated Gene	dbSNP RS ID	p-value for main effect	Alleles	LS Mean	StdErr	N
MTHFD1	rs78043435	0.04	AA	0.01	0.04	848
			AG	0.13	0.09	85
			GG	-1.84	0.85	1
PEMT	rs12602348	0.008	AA	-0.17[a]	0.10	80
			AG	-0.03[a,b]	0.05	419
			GG	0.10[b]	0.05	434
	rs1869771	0.02	AA	-0.10	0.08	112
			AC	-0.03	0.05	440
			CC	0.10	0.05	383
	rs34075240	0.01	AA	0.07[a]	0.04	637
			AG	-0.08[b]	0.06	273
			GG	-0.26[a,b]	0.18	23

LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

MTHFD1= methylene-tetrahydrofolate dehydrogenase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

A=adenine; G=guanine

LS = least square; StdErr = standard error; N= sample size

Table 5: Relationships between SNPs and Baseline Memory

Associated Gene	dbSNP RS ID	p-value for main effect	Alleles	LS Mean	StdErr	N
CHDH	rs17053530	0.04	CC	0.55	0.35	7
			CT	-0.29	0.11	81
			TT	-0.11	0.04	846
BHMT	rs3733890	0.03	AA	0.01	0.09	110
			AG	-0.21	0.05	414
			GG	-0.09	0.05	411
MTHFD1	rs56811449	0.02	CC	-0.13[a]	0.04	636
			CT	-0.08[a]	0.06	272
			TT	-0.59[b]	0.18	27
PEMT	rs4646360	0.005	CC	-0.16[a]	0.04	784
			CT	-0.03[a]	0.08	143
			TT	0.83[b]	0.32	8
	rs66637059	0.02	CC	-0.11[a]	0.04	729
			CT	-0.16[a]	0.07	194
			TT	-0.86[b]	0.27	12
	rs117588728	0.05	CC	-0.12	0.04	837
			CT	-0.20	0.10	91
			TT	-1.20	0.46	4

LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

CHDH = choline dehydrogenase; *BHMT*= betaine: homocysteine methyltransferase; *MTHFD1*= methylene-tetrahydrofolate dehydrogenase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

LS = least square; StdErr = standard error; N= sample size

Table 6: Relationship between SNPs and 2-YR Executive Function

Associated Gene	dbSNP RS ID	p-value for main effect	Alleles	LS Mean	StdErr	N
BHMT	rs492842	0.01	CC	0.15[a]	0.07	171
			CT	0.05[a,b]	0.05	462
			TT	-0.09[b]	0.06	291
PEMT	rs3785499	0.02	CC	-0.03[a,b]	0.08	112
			CT	-0.04[a]	0.05	438
			TT	0.13[b]	0.05	375
	rs12941217	0.03	AA	-0.21[a]	0.11	69
			AG	0.00007[a,b]	0.05	349
			GG	0.08[b]	0.04	507
	rs11867208	0.02	CC	-0.06[a]	0.05	301
			CT	0.08[a,b]	0.05	467
			TT	0.18[b]	0.08	125
	rs12602348	0.02	AA	-0.11[a,b]	0.10	78
			AG	-0.04[a]	0.05	420
			GG	0.11[b]	0.05	426
	rs4646342	0.007	AA	-0.13[a]	0.07	196
			AG	0.04[b]	0.05	429
			GG	0.11[b,c]	0.05	499
	rs66637059	0.004	CC	0.07[a]	0.04	721
			CT	-0.11[b,c]	0.07	192
			TT	-0.48[a,c]	0.24	13
	rs34075240	0.003	AA	0.09[a]	0.04	628
			AG	-0.08[b]	0.06	270

			GG	-0.32[b]	0.17	26
	rs897450	0.02	GG	-0.14[a]	0.07	154
			GT	0.04[a,b]	0.04	476
			TT	0.10[b]	0.06	293
	rs11869492	0.02	CC	-0.06	0.05	364
			CT	0.07	0.05	433
			TT	0.14	0.08	129
	rs2350631	0.01	CC	-0.10[a]	0.06	191
			CT	0.02[a,b]	0.04	501
			TT	0.15[b]	0.06	229
	rs4646340	0.02	CC	0.14[a,b]	0.09	107
			CT	0.08[a]	0.05	451
			TT	-0.06[b]	0.05	366
	rs4646343	0.02	GG	0.10[a]	0.05	406
			GT	0.01[a,b]	0.05	411
			TT	-0.16[b]	0.09	107

LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

BHMT= betaine: homocysteine methyltransferase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

LS = least square; StdErr = standard error; N= sample size

Table 7: Relationship between SNPs and 2-YR Memory

Associated Gene	dbSNP RS ID	p-value for main effect	Alleles	LS Mean	StdErr	N
CHDH	rs17053530	0.02	CC	0.41[a,b]	0.30	9
			CT	-0.15[a]	0.10	89
			TT	0.09[b]	0.04	827
PEMT	rs4925048	0.04	AA	0.03[a]	0.04	565
			AG	0.19[b]	0.06	301
			GG	0.09[a,b]	0.13	49
	rs9905728	0.01	CC	0.20[a]	0.06	265
			CT	0.07[a,b]	0.05	450
			TT	-0.05[b]	0.06	211

LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

CHDH = choline dehydrogenase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

LS = least square; StdErr = standard error; N= sample size

Table 8: Relationship between SNPs and Decline in Executive Function

Associated Gene	dbSNP RS ID	p-value for main effect	Alleles	Odds Ratio	95% CL		N		
			CC vs. CT	2.98	0.17	51.05	CC	CT	TT
BHMT	rs3797546	0.01	CC vs. TT	6.16	0.37	101.97	2	96	767
			CT vs. TT	**2.07**	**1.21**	**3.52**			
			AA vs. AG	1.56	0.50	4.88	AA	AG	GG
PEMT	rs4646409	0.03	AA vs. GG	1.28	0.42	3.88	23	283	559
			AG vs. GG	0.82	0.53	1.27			

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

BHMT= betaine: homocysteine methyltransferase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

LS = least square; StdErr = standard error; N= sample size

Table 9: Relationship between SNPs and Decline in Memory

Associated Gene	dbSNP RS ID	p-value for main effect	Alleles	Odds Ratio	95% CL		N		
PEMT	rs6502603	0.001	**GG vs. GT**	**2.36**	**1.45**	**3.84**	GG	GT	TT
			GG vs. TT	1.17	0.68	2.02	229	452	183
			GT vs. TT	0.50	0.29	0.85			
	rs897450	0.005	**GG vs. GT**	**1.98**	**1.10**	**3.57**	GG	GT	TT
			GG vs. TT	0.96	0.53	1.72	142	451	271
			GT vs. TT	0.48	0.30	0.77			
	rs4646343	0.004	**GG vs. GT**	**1.58**	**1.00**	**2.52**	GG	GT	TT
			GG vs. TT	0.74	0.40	1.39	383	385	98
			GT vs. TT	0.47	0.24	0.90			

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

PEMT= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; G=guanine

CL= confidence limit; N= sample size

Table 10: Interaction between Dietary Choline and SNPs Related to Baseline Executive Function

Associated Gene	dbSNP RS ID	p-value for interaction	Alleles	β-coefficient	p-value	N
MAT1A	rs1985908	0.05	**AA**	**1.07**	**0.01**	**403**
			AG	0.46	0.33	397
			GG	-0.20	0.84	85
PEMT	rs1869771	0.05	**AA**	**2.64**	**0.01**	**104**
			AC	0.27	0.53	421
			CC	**1.04**	**0.03**	**368**

All values are adjusted for total calories, age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

MAT1A = S-adenosylmethionine synthetase type 1; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

C=cytosine; A=adenine; G=guanine

N= sample size

Table 11: Interaction between Dietary Choline and SNPs Related to Baseline Memory

Associated Gene	dbSNP RS ID	p-value for interaction	Alleles	β-coefficient	p-value	N
BHMT	rs16876527	0.01	CC	*	*	3
			CT	1.48	0.13	81
			TT	0.15	0.66	793
	rs492842	0.04	CC	-1.00	0.23	159
			CT	0.20	0.65	447
			TT	**1.13**	**0.05**	**285**
MAT1A	rs4934027	0.05	CC	0.11	0.78	570
			CT	0.71	0.23	285
			TT	0.86	0.59	35
MTHFD1	rs1950902	0.05	AA	4.08	0.45	16
			AG	0.95	0.16	199
			GG	0.13	0.72	678
PEMT	rs7224725	0.03	CC	0.57	0.13	672
			CT	-0.05	0.93	207
			TT	-2.59	0.55	13
	rs12941217	0.01	**AA**	**2.39**	**0.05**	**59**
			AG	**1.33**	**0.02**	**341**
			GG	-0.54	0.20	492
	rs1869771	0.02	AA	0.75	0.48	104
			AC	**1.07**	**0.02**	**421**
			CC	-0.65	0.20	368

	rs34075240	0.03	AA	-0.32	0.41	606
			AG	**1.67**	**0.01**	**263**
			GG	2.99	0.35	22

All values are adjusted for total calories, age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

*not enough data

BHMT= betaine: homocysteine methyltransferase; *MAT1A* = S-adenosylmethionine synthetase type 1; *MTHFD1*= methylene-tetrahydrofolate dehydrogenase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

N= sample size

Table 12: Interaction between Dietary Choline and SNPs Related to 2-Year Executive Function

Associated Gene	dbSNP RS ID	p-value for interaction	Alleles	β-coefficient	p-value	N
MAT1A	rs1985908	0.01	**AA**	**1.38**	**0.002**	**400**
			AG	**1.19**	**0.01**	**390**
			GG	0.39	0.70	85
	rs2236225	0.04	AA	0.29	0.72	147
			AG	**1.39**	**0.002**	**442**
			GG	**1.25**	**0.01**	**289**
	rs2983733	0.01	**AA**	**1.56**	**0.004**	**231**
			AG	**1.64**	**0.0004**	**423**
			GG	-0.42	0.53	219
MTHFD1	rs1956545	0.002	CC	*	*	8
			CT	**2.06**	**0.02**	**120**
			TT	**1.09**	**0.001**	**749**
	rs2236224	0.05	AA	-0.36	0.70	108
			AG	**1.34**	**0.002**	**445**
			GG	**1.49**	**0.002**	**330**
	rs34110441	0.01	**GG**	**1.64**	**<.0001**	**471**
			GT	**1.17**	**0.02**	**337**
			TT	**-2.41**	**0.05**	**74**
PEMT	rs3785499	0.004	**CC**	**2.51**	**0.01**	**108**
			CT	0.13	0.77	413

			TT	**2.18**	**<.0001**	**361**
	rs6502603	0.03	**GG**	**1.95**	**0.001**	**238**
			GT	**0.82**	**0.05**	**453**
			TT	1.24	0.08	189

All values are adjusted for total calories, age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

*not enough data

MAT1A = S-adenosylmethionine synthetase type 1; *MTHFD1*= methylene-tetrahydrofolate dehydrogenase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

N= sample size

Table 13: Interaction between Dietary Choline and SNPs Related to 2-Year Memory

Associated Gene	dbSNP RS ID	p-value for interaction	Alleles	β-coefficient	p-value	N
BHMT	rs16876528	0.01	CC	*	*	4
			CT	**2.82**	**0.002**	**114**
			TT	**-0.78**	**0.02**	**764**
PEMT	rs16961845	0.03	CC	-0.45	0.21	666
			CT	0.57	0.40	201
			TT	-4.49	0.08	16
	rs7224725	0.01	CC	-0.30	0.40	670
			CT	-0.01	0.99	199
			TT	-3.95	0.37	13

All values are adjusted for total calories, age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

* not enough data

BHMT= betaine: homocysteine methyltransferase; *PEMT*= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine

N= sample size

Table 14: Interaction between Dietary Choline and SNPs Related to Executive Function Decline

Associated Gene	dbSNP RS ID	p-value for interaction	Alleles	Odds Ratio Point Estimate	95% Confidence Interval		p-value	N
PEMT	rs6502603	0.01	GG	0.24	0.001	45.20	0.59	221
			GT	0.44	0.03	7.18	0.56	433
			TT	0.10	<0.001	40.83	0.45	172
	rs1869771	0.04	AA	0.01	<0.001	114.19	0.35	98
			AC	0.31	0.02	6.30	0.44	396
			CC	0.50	0.01	18.63	0.70	335

All values are adjusted for total calories, age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

PEMT= phosphatidylethanolamine n-methyl transferase

dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

N= sample size

Table 15: Interaction between Dietary Choline and SNPs Related to Memory Decline

Associated Gene	dbSNP RS ID	p-value for interaction	Alleles	Odds Ratio Point Estimate	95% Confidence Interval		p-value	N
PEMT	rs3785499	0.03	**CC**	**>999.99**	**6.27**	**>999.99**	**0.02**	**102**
			CT	1.52	0.04	60.12	0.82	389
			TT	1.77	0.07	48.31	0.74	337
	rs2124344	0.03	AA	26.45	0.01	>999.99	0.43	99
			AG	0.13	0.003	4.87	0.27	410
			GG	**30.04**	**1.06**	**852.89**	**0.05**	**318**
	rs4646342	0.04	**AA**	**>999.99**	**11.12**	**>999.99**	**0.01**	**170**
			AG	0.55	0.02	20.42	0.75	384
			GG	0.20	0.003	12.11	0.45	274
	rs2350631	0.04	CC	86.44	0.63	>999.99	0.08	167
			CT	0.53	0.02	14.62	0.71	453
			TT	13.44	0.11	>999.99	0.29	206
	rs4646340	0.02	CC	21.51	0.004	>999.99	0.49	96

			CT	0.16	0.01	5.57	0.31	406
			TT	**39.83**	**1.32**	**>999.99**	**0.03**	**325**
	rs9905728	0.01	**CC**	**149.58**	**1.78**	**>999.99**	**0.03**	**230**
			CT	1.38	0.05	39.02	0.85	402
			TT	0.23	0.001	40.05	0.58	197

All values are adjusted for total calories, age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

PEMT= phosphatidylethanolamine n-methyl transferase

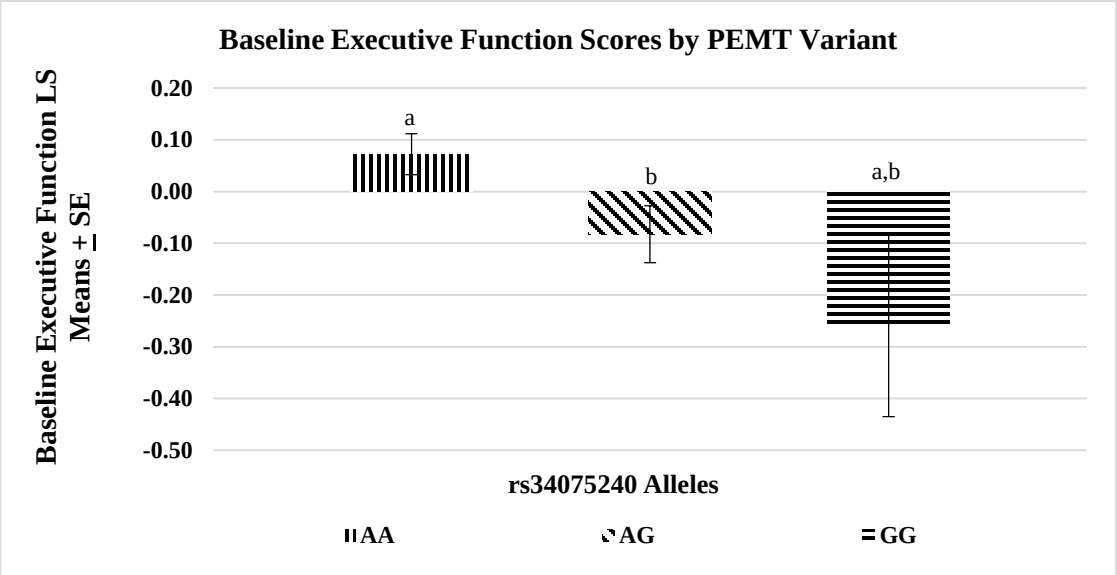
dbSNP= Single Nucleotide Polymorphism Database

rsID= reference SNP identification number

T=thymine; C=cytosine; A=adenine; G=guanine

N= sample size

Figure 1

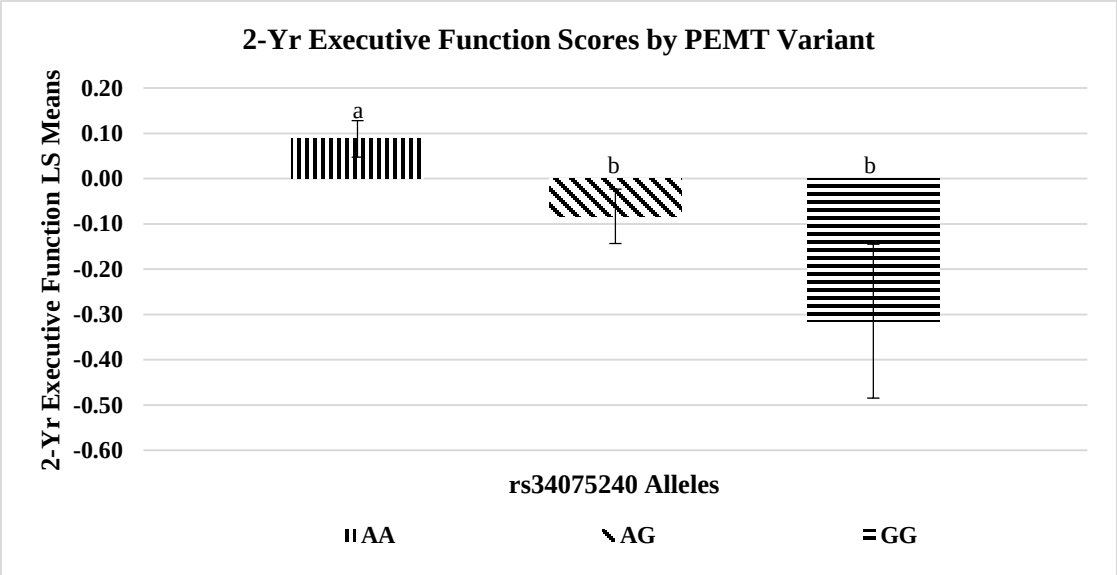


LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

AA: N=637; AG: N=273; GG: N=23

Figure 2

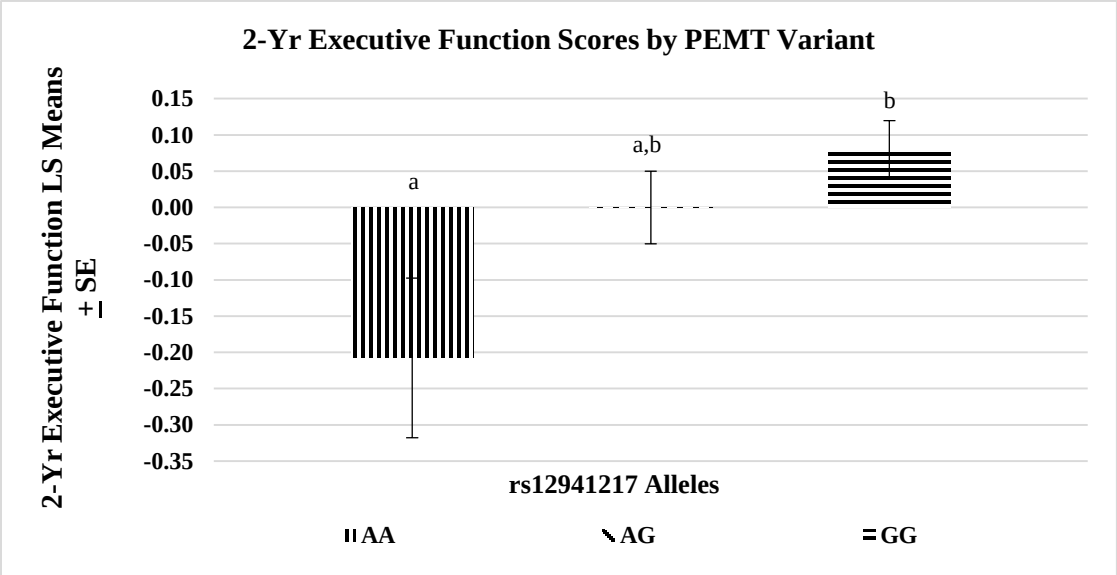


LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

AA: N=628; AG: N=270; GG: N=26

Figure 3

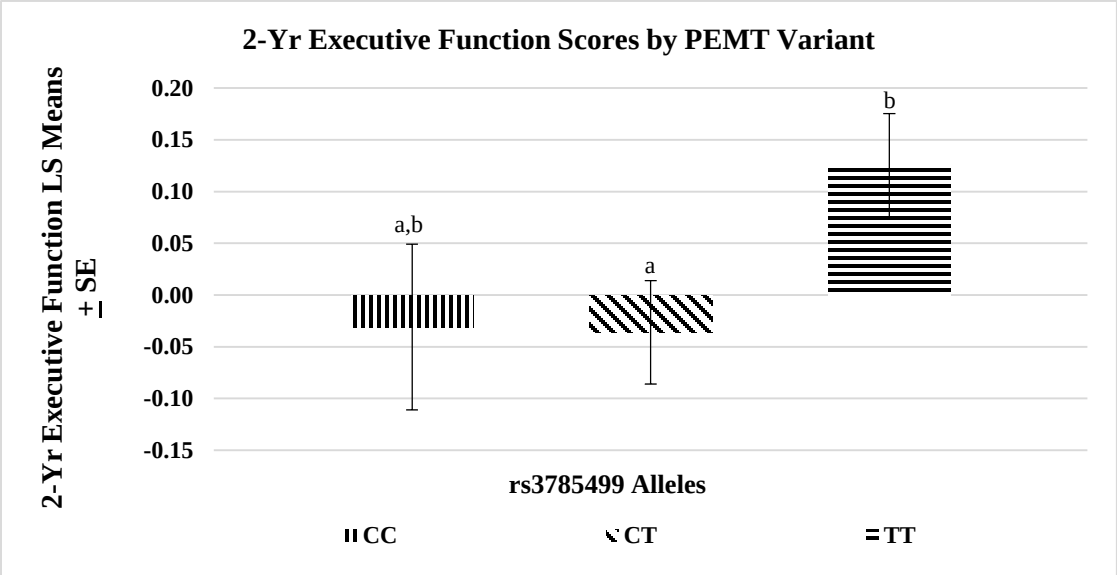


LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

AA: N=69; AG: N=349; GG: N=507

Figure 4

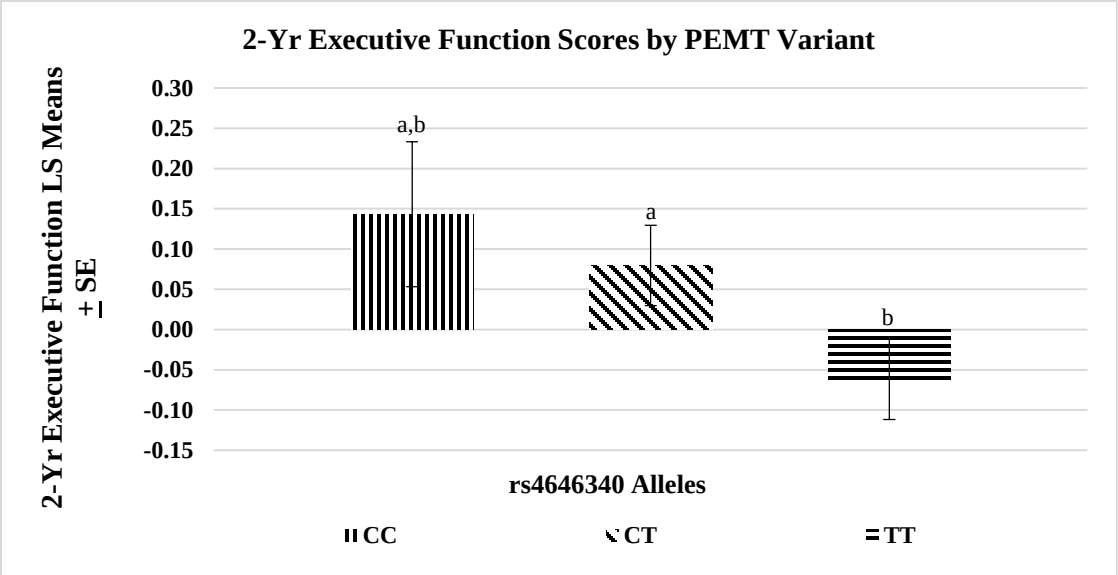


LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

CC: N=112; CT: N=438; TT: N=375

Figure 5

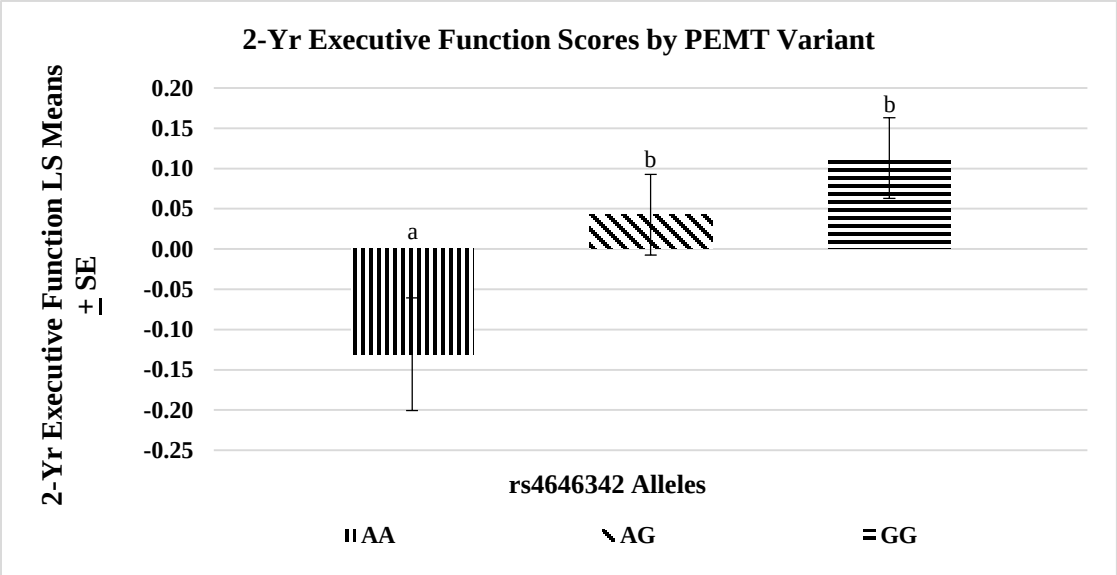


LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

CC: N=107; CT: N=451; TT: N=366

Figure 6

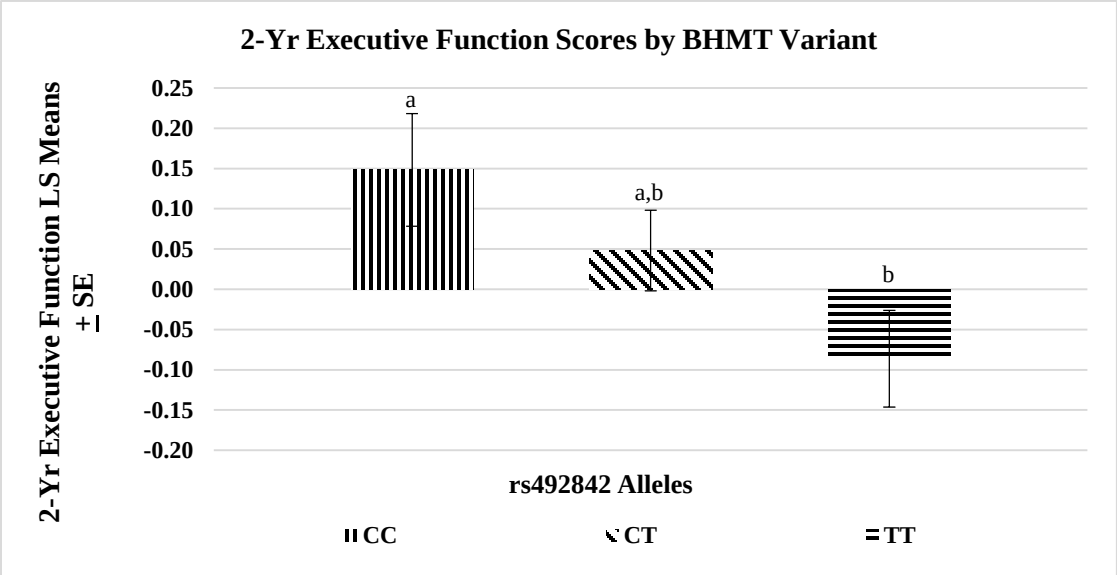


LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

AA: N=196; AG: N=429; GG: N=499

Figure 7

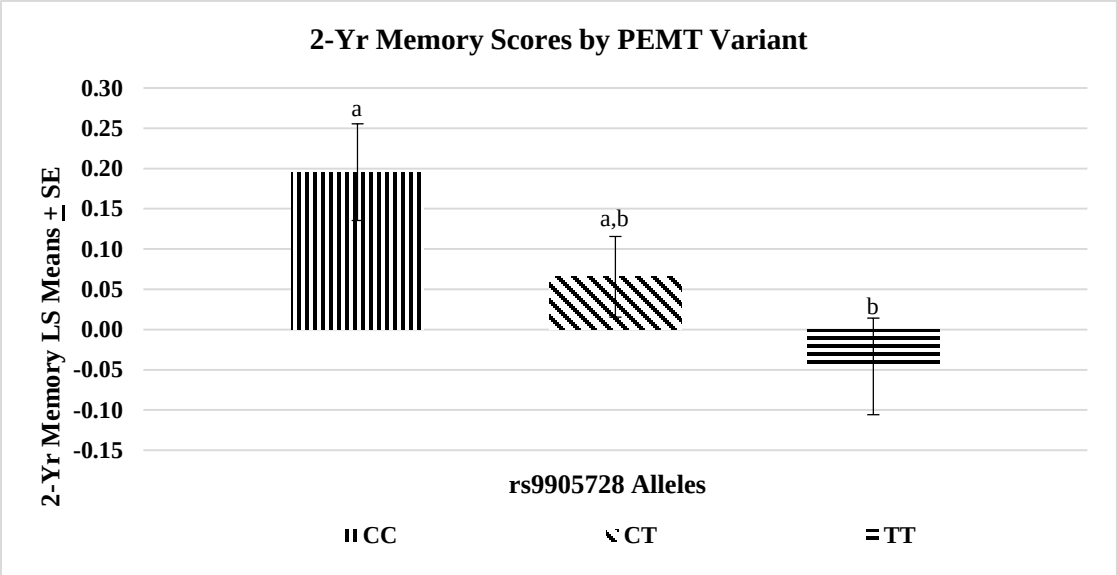


LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

CC: N=171; CT: N=462; TT: N=291

Figure 8



LSMeans with unique superscripts within a SNP are significantly different ($p \leq 0.05$, Tukey-Kramer adjustment).

All values are adjusted for age, sex, education, body mass index, presence of hypertension, presence of diabetes, and population ad mixture.

CC: N=265; CT: N=450; TT: N=211

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Chapter 3: Methylation capacity due to choline is associated with global cognitive function and *ERG-1* gene expression in the frontal cortex of centenarians

Introduction

Advancing age has been reported as the strongest risk factor for cognitive decline.[1] As Americans are living longer,[2] the rising economic and societal costs associated with age-related cognitive decline create a need for the development of novel and cost-effective public health strategies that will delay, slow, or even prevent this decline. Observational studies have provided evidence that the risk of cognitive decline can be modified by dietary factors[3-5] and thus further investigation into the role of specific nutrients in cognition is critical.

Choline is an essential nutrient with multiple links to brain health. As a component of phospholipids, choline-containing compounds are involved in cell signaling, lipid transport, and cell membrane structure. In addition, choline is a precursor to the neurotransmitter acetylcholine and the methyl donor betaine.[6] There is evidence that changes in DNA methylation play a role in early neuronal development[7] as well as in the pathogenesis of neurodegenerative diseases, such as Alzheimer's Disease and Parkinson's Disease, by altering gene expression.[8]

Much of the work exploring the role of methylation in memory has focused on the hippocampus, which is necessary for memory formation. However, it is theorized that once formed, memories are only dependent on the hippocampus for a short time, and for long-term storage, persistent changes need to occur in the cortex.[9] Sui and colleagues (2012) demonstrated this phenomenon in an animal model, showing that methylation patterns of plasticity genes (*brain derived neurotropic factor* and *reelin*) expressed in the medial-prefrontal cortex were necessary for long-term memory.[10] Further evidence supporting the role of methylation in cognitive function comes from studies of Bading and colleagues showing that expression of genes involved in methyl group transfer (e.g. *dnmt3a*) are decreased in mice with age-related cognitive impairment, and that over-expression of these genes resulted in changes in global methylation

patterns as well as improved long-term memory.[11] Additional genes of interest that have shown to be necessary for formation of long-term memories in rodent models, but warrant further study in humans include activity-regulated cytoskeletal gene (*ARC*),[12] early growth response-1 (*EGR-1*),[13] and growth arrest and DNA damage-inducible β (*GADD45β*).[14]

S-adenosylmethionine is a major methyl donor for a number of methylation reactions, including DNA methylation. It can be formed from the remethylation of homocysteine via folate or betaine. The betaine pathway produces the unique byproduct dimethylglycine, making the ratio of serum free choline and betaine to dimethylglycine [(CHO+BET)/DMG] a marker of methylation potential from choline.

The purpose of this study was to determine relationships between methylation capacity from choline (assessed by serum (CHO+BET)/DMG) and a) cognitive test outcomes and b) expression of genes related to long-term memory. This aim involved secondary analysis of brain and serum samples as well as cross-sectional pre-mortem cognitive data from the Georgia Centenarian Study. [15] We hypothesized that methylation capacity from choline as assessed by the serum (CHO+BET)/DMG would be positively associated with measures of global cognitive function as well as expression of genes related to long-term memory (*ARC, BDNF, REELIN, EGR1, GADD45β*) in the frontal cortex.

Methods

Cognitive data and biological samples from decedents of the Georgia Centenarian Study (GCS)[15] were analyzed. This was a longitudinal study conducted between 1988 and 2006 to identify adaptational characteristics, longevity genes, neuropathology, and functional capacity of a population-base sample of centenarians, octogenarians, and sexagenarians in Georgia.[15] In addition to stored serum samples and cognitive assessment less than one year prior to death, 48 participants in this study donated their brain upon death. Descriptions of cognitive tests can be found in Table 1. Due to availability, concentrations of choline, dimethylglycine, and betaine

were measured in serum from 38 decedents using liquid chromatography-stable isotope dilution-multiple reaction monitoring-mass spectrometry (LC-SID-MRM-MS)[16] (Appendix A) in order to assess the methylation capacity due to choline.

Frontal cortex brain tissue was available from 27 of the decedents with serum choline analysis. From these 27 tissues, quality RNA was extracted from 22 samples to measure expression of genes related to long-term memory[17, 18] (*ARC, BDNF, REELIN, EGR-1, GADD45β*) using real time- polymerase chain reaction (RT-PCR) (see Appendix B for details).

Statistical Analysis

Pearson's correlations were used to assess relationships between methylation capacity from choline and global cognitive function. Student's t-tests were used to compare expression of genes related to long-term memory (*ARC, BDNF, REELIN, EGR-1, GADD45β*) in those with high or low methylation capacity from choline. Significance was set at $p \leq 0.05$.

Results

Subject Characteristics

Descriptive characteristics of the study population are presented in Table 2. On average, subjects were 100 years of age, with a high school education, female, with healthy body weight. Comparing demographic and health characteristics between those with methylation capacity above and below the median did not result in any significant differences.

Methylation Capacity and Cognitive Outcomes

Correlations between cognitive test scores and methylation capacity from choline are presented in Table 3. Methylation capacity from choline was positively related to Fuld Object Memory Evaluation delayed recall scores and inversely related to rating on the Global Deterioration Rating Scale. These relationships remained significant when controlling for level of education.

Methylation Capacity and Gene Expression

Expression of genes related to long-term memory in those with methylation capacity above the median is expressed relative to gene expression in those with methylation capacity below the median in this population (Table 4). Expression of *EGR1* was significantly greater in those with methylation capacity above the median compared to expression in those with methylation capacity below the median.

Discussion

Choline from animal products such as eggs, milk, and beef, is a major source of methyl groups in the U.S. diet.[19] Animal models provide evidence that dietary choline deficiency during fetal development alters global and gene-specific DNA methylation in the hippocampus[20] and leads to compromised cognitive function and increased decline later in life.[21] Several animal studies have shown beneficial effects on memory of offspring when maternal diet is supplemented with choline.[19] There is limited research, however, on how the consumption of choline or choline-rich foods affects cognition and brain health in humans, especially later in life.

Previous studies provide some evidence of the importance of choline for cognitive function later in life. Intervention studies have shown choline-containing supplements enhanced learning and memory[22-24] and prevented or reversed cognitive decline in patients with cerebrovascular disease.[25-27] In addition, cross-sectional studies demonstrate positive associations between choline intake or foods high in dietary choline and global cognition or memory.[28, 29] In another cohort (Boston Puerto Rican Health Study - BPRHS), we did not find any relationship between memory measurements and dietary choline intake, however, there was a positive association between dietary choline intake and baseline and 2-year executive function scores (Chapter 2). Others have evaluated plasma concentrations and found that compared with low concentrations, high plasma choline was associated with better test scores assessing perceptual speed, executive function, and global cognition.[30] In a previous study, we evaluated a cross-

sectional cohort (Nutrition, Aging, and Memory in Elders – NAME) for relationships between dietary intake and plasma concentrations of choline and betaine and cognition, and found different associations between the nutrients and outcome measures depending on whether dietary intake or plasma concentrations were used in the statistical model (Chapter 1). Dietary betaine intake was positively associated with executive function, while dietary choline intake was inversely related to memory; in contrast, plasma concentrations of choline were positively related to executive function scores and betaine plasma concentrations were positively associated with memory. Differences between dietary and plasma associations with cognitive function may be due to the fact that choline can be produced endogenously and thus plasma concentrations better represent total available choline or choline-related compounds. In addition, in the BPRHS cohort, we found significant associations among cognitive outcomes and gene-diet interactions between dietary choline intake and single nucleotide polymorphisms in genes involved in choline and folate metabolism (Chapter 2). These polymorphisms may be important to take into consideration when comparing associations with diet and plasma.

These previous studies identify associations, but do little to examine mechanistic relationships between choline measure and cognitive function. Choline is a precursor to the methyl donor betaine.[6] A recent review by Yu et al. (2011) summarizes the current state of knowledge regarding the role of changes in DNA methylation on learning and memory [31]. Briefly, DNA methylation in the brain may be transient or stable depending on the function of the gene as well as the location in the brain. For example, DNA in hippocampal cells, coding for plasticity genes has been shown to become demethylated following a learning task, allowing for the translation of new proteins necessary for inter-neuronal connections. In addition, the methylation pattern of memory suppressor genes has been shown to increase following learning, thus decreasing their expression. These methylation patterns, however, return to baseline after a

short time. In order for memories to persist, there is evidence that stable changes in methylation patterns on DNA coding for other proteins occurs.[31]

In the GCS population we found methylation capacity from choline was inversely related to Global Deterioration Rating Scale scores, indicating that higher methylation capacity was associated with less cognitive impairment. In addition, we found a positive relationship between methylation capacity and number of objects recalled after a 20 minute delay (FOME – delayed recall scores), indicating a positive association with memory. Furthermore, we found that the expression of *EGR-1*, a gene shown to be necessary for long-term memory in rodent models, was greater in those with methylation capacity in the upper 50% compared to those with methylation capacity in the lower 50%. This was the first study to look at gene expression related to methylation capacity in humans. While these relationships are correlational, they do provide some support of the hypothesis that choline is related to cognitive function via its role in methylation.

Conclusion

Greater methylation capacity from choline was associated with less cognitive impairment and better memory as well as greater expression of the long-term memory related gene, *EGR-1* in a centenarian cohort. Future research should assess gene-specific methylation in order to determine whether the increased expression is due to changes in methylation.

Tables

Table 1: Cognitive Tests

Test	**Abbreviation**	**Cognitive Construct**	**Description**
Mini Mental State Exam	MMSE	Global Cognition	Brief assessment of orientation, memory, attention, and ability to follow verbal and written commands[32]
Global Deterioration Rating Scale	GDRS	Dementia Severity	Rating scale of dementia stages[33]
Severe Impairment Battery	SIB	Global Cognition	Exam divided into 9 domains for neuropsychological evaluation of severly demented patients[34]
Fuld Object Memory Evaluation – Delayed Recall	FOME – Recall	Memory	Evaluates the episodic memory functions of encoding, storage, and recall across five recall trials and a delayed recall trial[35]
Fuld Object Memory Evaluation – Delayed Recognition	FOME – Recognition	Memory	
Fuld Object Memory Evaluation – Delayed Retention	FOME – Retention	Memory	
Controlled Oral Word Association Test	COWAT	Verbal Fluency	Participants are given a letter of the alphabet and asked to

			name as many words as they can that begin with that letter[36]
Wechsler Adult Intelligence Scale-III Similarities subtest	WAIS-III Similarities	Concept Formation, Abstraction	Verbal test that requires participants to describe how two given things are alike[37]
Behavioral Dyscontrol Scale	BDS	Executive Function	Clinical measure related to frontal lobe integrity and executive function[38]
Geriatric Depression Scale – Short Form	GDSSFA	Depressive Symptoms	Self-report instrument to screen for clinical depression in elderly[39]
Consortium to Establish a Registry for Alzheimer's Disease	CERAD	Clinical Evidence for Dementia	Standardized, validated measures for assessment of Alzheimer's disease[40]

Table 2: Descriptive Characteristics

Variable	Methylation Capacity Below Median	Methylation Capacity Above Median	p-value (chi-square or t-test)
BMI (mean ± SD)	21± 4	24± 7	0.10
Age (years ± SD)	101± 2	100± 2	0.14
Education (mean ± SD)	9± 4	12± 3	0.08
Sex (% Female)	89	84	0.63
Diabetes (%)	63	37	0.10
Stroke (%)	21	16	0.68
Kidney Disease (%)	0	0	-
Hypertension (%)	58	63	0.74
Depression (%)	11	16	0.63
Cardiovascular Disease (%)	79	89	0.37
Anxiety (%)	5	0	0.31
Methylation Capacity [(Choline + Betaine)/DMG]	12.70± 2.50	21.39 ±4.47	<0.0001

SD=standard deviation

DMG= dimethylglycine

Methylation capacity was measured in serum.

N= 19 per group

Table 3: Relationship between Cognitive Function Measurements and Methylation Capacity from Choline [(Choline + Betaine): Dimethylglycine] in Serum

Cognitive Test	N	Model 1		Model 2*	
		Pearson's Correlation	P-Value	Pearson's Partial Correlation	P-value
Mini Mental State Exam	38	0.30	0.06	0.28	0.11
Global Deterioration Rating Scale	**38**	**-0.42**	**0.01**	**-0.40**	**0.02**
Severe Impairment Battery	38	0.28	0.09	0.27	0.12
FOME -delayed recall	**38**	**0.31**	**0.05**	**0.31**	**0.05**
FOME -delayed recognition	38	0.04	0.80	0.02	0.93
FOME -delayed retention	38	0.29	0.08	0.26	0.12
Controlled Oral Word Association Test	38	0.14	0.39	0.08	0.64
WAIS-III Similarities	38	0.07	0.66	0.01	0.96
Behavioral Dyscontrol Scale	38	0.21	0.19	0.21	0.19
Geriatric Depression Scale- Short Form	38	0.05	0.80	0.07	0.71
Consortium to Establish a Registry for Alzheimer's Disease	16	0.07	0.81	-0.08	0.78

* Adjusted for education

Table 4: Expression of Memory-Related Genes in the Frontal Cortex

Gene	Relative Expression	p-value (t-test)
ARC	1.76	0.11
BDNF	0.58	0.36
EGR-1	**1.72**	**0.03**
GADD45β	1.28	0.50
RELN	0.98	0.96

Relative Expression= expression in those with methylation capacity above the median relative to those with methylation capacity below the median

Methylation Capacity = serum [(choline+betaine)/dimethylglycine)

N=8 (methylation capacity below the median); N=14 (methylation capacity above the median)

ARC= activity regulated cytoskeletal-associated protein; *BDNF*= brain-derived neurotrophic factor; *EGR-1*= early growth response 1; *GADD45β*= growth arrest and DNA-damage-inducible, beta; *RELN*=reelin

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Chapter 4: Progress Report: A six-month intervention to increase dietary choline intake in adults effects concentration of plasma choline-related metabolites, cardiometabolic risk factors, and cognitive outcomes

Introduction

Advancing age has been reported as the strongest risk factor for cognitive decline.[1] As Americans are living longer,[2] the rising economic and societal costs associated with age-related cognitive decline create a need for the development of novel and cost-effective public health strategies that will delay, slow, or even prevent this decline. Observational studies have provided evidence that the risk of cognitive decline can be modified by dietary factors[3-5] and thus further investigation into the role of specific nutrients in cognition is critical.

Choline is an essential nutrient with multiple links to brain health. Choline is a precursor to the neurotransmitter acetylcholine and is needed to form phosphatidylcholine and sphingomyelin, which are essential to cell membrane structure and integrity and are precursors to cell signaling molecules. Choline can also be oxidized to form betaine which can donate methyl groups to homocysteine to form the body's primary methyl donor s-adenosylmethionine.[6] S-adenosylmethione then provides methyl groups for several reactions, including the endogenous synthesis of choline and DNA methylation which can influence gene expression and may play a role in cognitive function.

Although humans have the ability to synthesize choline in the liver, through methylation of phosphatidylethanolamine to form phosphatidylcholine, catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT), this production is inadequate to meet daily requirements, thus making choline an essential nutrient.[7] Analyses of several cohorts in the US (NHANES 2003-2004[8], Framingham Offspring Study[9], Atherosclerosis Risk in Communities Study[10]) demonstrate choline intakes far below the Adequate Intake (AI) recommendations (550mg/ day for men $\geq$ 19y; 425 mg/day for women $\geq$19y). Since the AI is based on the intake

required to prevent liver damage,[6] it is unknown if this is optimal for cognitive function, or if higher or lower intakes should be recommended.

Previous studies provide some evidence of the importance of choline for cognitive function later in life. Intervention studies have shown choline-containing supplements enhanced learning and memory[11-13] and prevented or reversed cognitive decline in patients with cerebrovascular disease.[14-16] In addition, cross-sectional studies demonstrate positive associations between choline intake or foods high in dietary choline and global cognition or memory.[17, 18] Previously (Nutrition, Aging and Memory in Elders Study - NAME; Chapter 1) we found that dietary choline intake was inversely associated with memory scores in a cross-sectional cohort, while in another (Boston Puerto Rican Health Study – BPRHS; Chapter 2) we did not find any relationship between memory measurements and dietary choline intake, however, there was a positive association between dietary choline intake and baseline and 2-year executive function scores. Plasma choline may be a better marker of total choline (from exogenous and endogenous sources), and has also been found to be positively related to scores of tests assessing perceptual speed, executive function, and global cognition.[19] We also found that plasma choline concentration was positively related to executive function and amygdala volume (NAME; Ch. 1). In addition, we found that the ratio of plasma betaine to choline was inversely associated with executive function (NAME; Ch. 1). This ratio provides information on the activity of the endogenous pathway involving PEMT. An increase in PEMT activity results in a lower betaine to choline ratio (Bet:Chol), as betaine would be used as a methyl donor in the production of phosphatidylcholine from phosphatidylethanolamine.[20]

In addition to direct relationships with cognitive function, plasma choline and betaine concentrations have been differentially associated with risk of cardiovascular disease and may also be related to cognitive performance via cerebrovascular health. Higher concentrations of betaine predicted lower risk and higher concentrations of choline predicted higher risk.[21] Some

evidence suggests that sphingomyelinase is involved in the aggregation of low density lipoproteins (LDL) in atherosclerosis by hydrolyzing LDL-sphingomyelin and that the ratio of plasma sphingomyelin to total choline-containing phospholipids (sphingomyelin plus phosphatidylcholine) [SM:(SM+PC)] is an important factor in determining whether sphingomyelinase will hydrolyze LDL-sphingomyelin.[22] Furthermore, there is some evidence that plasma SM:(SM+PC) has been found to be an independent predictor of coronary artery disease.[23] Previously, we found that SM:(SM+PC) was inversely related to the volume of white matter hyperintensities (WMHI), which have been associated with small vessel disease, and is the opposite of the relationship with large vessel disease (NAME; Ch. 1). Another connection between choline and vascular disease involves the choline containing phospholipid phosphatidylcholine. Phosphatidylcholine is digested by intestinal bacteria and produces trimethylamine (TMA), which is then oxidized to trimethylamine oxide (TMAO) in the liver. TMAO has been associated with increased risk of cardiovascular disease in humans.[24, 25] At this point, the research on TMAO from dietary choline intake is still emerging and it is not clear whether TMAO, in addition to cardiovascular disease, is related to increased risk of cerebrovascular disease. Plasma TMAO concentration may be an important covariate when assessing the relationships between dietary choline and cognitive function, cerebrovascular health, and cardiometabolic risk factors.

Eggs are a major source of choline in the diet, the majority in the form of phosphatidylcholine,[26] with just two large scrambled eggs providing half of the AI recommended for men and over 60% of the AI for women.[6, 27] Consumption of choline rich foods increases plasma choline concentrations.[28] Choline is transported bidirectionally across the blood brain barrier, entering the brain when plasma concentrations are high following a choline-rich meal, and leaving the brain to enter the plasma when plasma concentrations are low.[29] Proton magnetic resonance spectroscopy has shown that there is a significant increase in choline-containing

compounds in human brain after ingestion of choline bitartrate,[30] demonstrating that dietary interventions can be effective and have the potential to influence brain function.

Thus, the purpose of this study was to describe the effect of an intervention to increase dietary choline intake on the concentration of plasma choline-related compounds, cardiometabolic risk factors, and cognitive outcomes over a 6-month period.

Methods

Subjects and Study Design

This study was ancillary to a randomized controlled trial designed to evaluate the effect of an egg intervention on cognitive function. The trial is ongoing, with a planned sample size of forty healthy men and women age 50 years and older. After screening for eligibility and exclusion criteria (see Table 1), Subjects were randomly assigned to one of two groups. The EGG group consumes 2 eggs per day for 6 months, while the CONTROL group consumes 4 oz. of egg whites per day for 6 months. Compliance was determined by dietitian interview, self-report and returning lids from empty EGG/CONTROL containers.

Cognitive Performance

The computerized cognitive assessment battery CANTAB (Cambridge Cognition Ltd, Tunbridge Court, Turnbridge Lane, Bottisham, Cambridge, UK) is being used to assess cognitive function at baseline, 3months, and 6 months.[31] A cognitive test battery was chosen to include tests of attention, visual memory, and executive function and working memory as shown in Table 2.

Dietary Intake

The Willett Food Frequency Questionnaire is being used to assess baseline dietary intake for the 12-months prior to study enrollment and analyzed for choline and betaine intake using the USDA database for the choline content of common foods, release 2 (https://catalog.data.gov/dataset/usda-database-for-the-choline-content-of-common-foods).

Plasma Biomarkers

Fasting blood samples are collected at baseline, 3 months, and 6 months. Samples are drawn into ethylenediaminetetraacetic acid (EDTA) vacutainers and centrifuged within 30 minutes (15 min, 1000g, 4°C) to separate plasma. Aliquots are stored at -70°C until analysis. Plasma samples are analyzed for concentrations of free choline (Cho), betaine (Bet), phosphatidylcholine (PtdCho), sphingomyelin (SM), and trimethylamine oxide (TMAO) using liquid chromatography/electrospray ionization-isotope dilution mass spectrometry LC/ESI-IDMS.[32, 24] See Appendix A for further methodological details.

Total cholesterol, triglycerides, and high density lipoprotein cholesterol (HDL-c) will be directly measured by enzymatic reaction. Low density lipoprotein cholesterol (LDL-c) and very low density lipoprotein cholesterol (VLDL-c) will be calculated from triglyceride HDL-c and cholesterol measurements. See Appendix D for further methodological details.

Statistical Methods

At the time of this dissertation, subjects were still being recruited and enrolled in the study. Thus, data presented here are for 22 subjects who completed the study and an additional 4 subjects who completed baseline and 3-month testing. The change in concentration of plasma choline and choline metabolites was compared between groups using repeated measures ANOVA. Multiple linear regression models were used to identify 1) cross-sectional relationships between plasma choline-related compounds and cognitive outcomes at baseline 2) relationships between change in plasma choline-related compounds and change in cognitive outcomes from baseline to 3-months and baseline to 6-months.

Covariates considered for inclusion in the models included baseline cognitive scores and plasma concentrations, age, sex, and education. Analyses were completed using SAS 9.2 and results were considered significant at $p \leq 0.05$.

Preliminary Data

Baseline Characteristics

Baseline characteristics of the study population are presented in Table 3. The only significant difference between groups was in baseline dietary betaine intake, which was higher in the CONTROL group compared to the EGG group.

Effect of Dietary Choline Intervention on Plasma Choline-Related Compounds

In our preliminary analysis, there was no difference in compliance, measured as a percent of eggs or whites given that were eaten, between groups (95% EGG; 93% CONTROL). The concentrations of plasma choline-related compounds at each time point, separated by group, are presented in Table 4. There was a significant group by time interaction on plasma choline concentrations, with those in the EGG group showing significantly higher concentrations at 3-months compared to baseline. In addition, those in the EGG group had higher choline concentrations at 3-months compared to those in the CONTROL group. There was also a significant main effect of group on plasma betaine concentrations, with those in the EGG group showing higher concentrations at all time points assessed than those in the CONTROL group. There were no significant between or within group differences for plasma sphingomyelin, phosphatidylcholine, or trimethylamine oxide concentrations.

Cross-Sectional Relationship between Choline-Related Compounds and Cognitive Measures at Baseline

Preliminary analysis of relationships between plasma choline-related compounds and cognitive test scores at baseline are shown in Tables 5-7. Baseline plasma betaine concentration was inversely related to signal detection score in the Rapid Visual Information Processing (RVP) test (Table 5). Plasma TMAO concentration was significantly related to two outcome measurements of the Paired Associates Learning (PAL) test (Table 6). Higher TMAO was associated with fewer errors and more stages completed. These same relationships were seen

with Bet:Chol. Stockings of Cambridge (SOC) scores were positively associated with plasma phosphatidylcholine and sphingomyelin concentrations and inversely associated with Bet:Chol (Table 7).

Relationship between Change in Plasma Choline-Related Compounds and Change in Cognitive Outcomes at 3-months

Preliminary analysis of change in plasma betaine concentration from baseline to 3-months was associated with all attention test outcomes (Table 8). A greater change in plasma betaine concentration was associated with slower reaction times on the Choice Reaction Time (CRT) test, and lower signal detection scores (RVP). The change in plasma betaine concentration was also inversely related to the number of stages completed in PAL (Table 9) and positively related to the number of errors on the Spatial Working Memory (SWM) test (Table 10).

Relationship between Change in Plasma Choline-Related Compounds and Change in Cognitive Outcomes at 6-months

Greater change in plasma choline concentration from baseline to 6-months was associated with greater change in reaction times on the CRT test, indicating slower reaction time (Table 11), while change in choline concentration was positively related to change in total error score on the PAL test (Table 12). Change in sphingomyelin concentration and change in SM:(SM+PC) were both positively associated with change in signal detection scores (RVP) (Table 11), while change in span length on the Spatial Span Reverse (SSR) test was inversely related to change in SM:(SM+PC) (Table 13). Conversely, change in span length on the Spatial Span Reverse (SSR) test was positively related to change in plasma TMAO concentration (Table 13). Change in plasma betaine concentration was positively related to the change in mean total thinking time on 4-move problems in the SOC test (Table 13).

Discussion of Preliminary Results

The Dietary Reference Intake (DRI) recommendations for choline were set by the Institute of Medicine in 1998.[6] Due to insufficient data to set an Estimated Average Requirement (EAR), and thus calculate a Recommended Daily Allowance (RDA), an Adequate Intake (AI) was developed. These values were based largely on the results of a single depletion/repletion study[33] using maintenance of serum alanine amino transferase levels as a marker of liver damage due to a choline deficient diet.[34] In the current study, average dietary choline intake was below the AI, which is similar to what has been shown in other US cohorts.[8-10] The AI for males (age 19≥ yr) is 550mg/day and 425mg/day for females (age 19≥ yr). There were only 2 of the 26 subjects whose initial dietary choline intake met or exceeded the AI, making this population ideal for an intervention to increase dietary choline intake. By adding 2 eggs per day (250 mg choline), subjects in this study would raise average dietary intakes from 335mg/day to intakes above the AI.

In our preliminary analysis, the increase in dietary choline did not significantly change plasma concentrations of betaine, sphingomyelin, or phosphatidylcholine across time, and the rise seen in plasma choline at 3-months decreased toward baseline at 6-months. In order to better understand relationships between dietary choline intake and plasma concentrations, it may be helpful to assess changes in plasma concentration of folate, vitamin B-12, and vitamin B-6. The metabolic pathways of choline, folate, vitamin B-12, and B-6 intersect at the point of homocysteine in one-carbon metabolism. Both betaine, from the oxidation of choline, and 5-methyl-tetrahydrofolate can be used to remethylate homocysteine, while vitamin B-6 is involved in the transmethylation of homocysteine.[35] Thus, a fluctuation in concentration of any of these nutrients has the potential to alter utilization and concentration of the others. Changes in plasma concentrations of choline and related metabolites in response to dietary restriction or supplementation with B-vitamins have been demonstrated in both animal and human studies.

Folate restriction in a group of pre-menopausal women resulted in decreased plasma phosphatidylcholine concentrations.[36] Rats fed a diet low in vitamins B-6, B-12, and folate then supplemented to repletion, had significantly higher concentrations of plasma free choline and lower homocysteine than rats maintained on the B-vitamin poor diet, despite adequate choline intake in both groups.[37] Similarly, plasma betaine concentration was increased and homocysteine decreased when elderly people consumed a vitamin B-12 and folate supplement.[38] Knowing the B-vitamin status of participants in the current study may help when interpreting changes in plasma concentrations of choline.

In addition to choline and choline containing compounds, we also assessed plasma TMAO concentrations. Observational studies demonstrate a positive relationship between cardiovascular risk in mid-life and age-related cognitive decline.[39, 40] Recent studies have highlighted possible relationships between dietary choline and vascular disease, not by a direct relationship, but rather by a relationship with TMAO. In 1989, Zeisel and colleagues[41] reported that regular dietary intakes from food sources were not likely to be large enough to overload choline absorption processes in the small intestine and significantly increase TMA production by colon micro flora. However, recent interest in the microbiome has prompted new research in this area. Wang et al. (2011) demonstrated a mechanistic link between the oxidation of TMA produced by microbial metabolism of dietary phosphatidylcholine and atherosclerosis in an animal model, as well as a significant relationship with increased risk of cardiovascular disease in humans.[24] Most recently, Tang et al. (2013) demonstrate a significant bacteria-dependent increase in plasma TMAO when humans are fed two-eggs and that fasting TMAO levels predict risk of major cardiovascular disease events.[25] This work certainly points to a potential clinical significance of TMAO. In the current preliminary analysis, we did not find a significant sustained increase in TMAO in subjects who consumed 2 eggs per day for three or six months. However, we did find relationships between plasma TMAO concentration and certain cognitive

test outcomes. Baseline plasma TMAO was inversely related to the total errors and positively related to the stages completed in the PAL test. This indicates that greater concentrations of TMAO were associated with better visual memory and new learning performance. Similarly, change in TMAO concentration at 6-months was positively associated with change in span length on the SSR test, indicating that a greater increase in TMAO was associated with improved performance on a test of working memory. These results are in contrast to the hypothesis that increases in TMAO would decrease cognitive performance via influence on vascular health. However, it is important to note that the metabolite produced by the gut bacteria is TMA and it is the oxidized form of this metabolite that is associated with cardiovascular risk. Bennett et al. (2013) identified several factors that regulate the oxidation of TMA in the liver of both animals and humans, indicating that the relationship between dietary phosphatidylcholine and plasma TMAO is not a direct one.[42] We tried to control for this by including baseline plasma choline or change in plasma choline as covariates in the cross-sectional and longitudinal analysis, respectively. At this point, the evidence supporting TMAO from dietary choline intake is still emerging and it is not clear whether TMAO is related to increased risk of cerebrovascular disease in addition to cardiovascular disease. However, it may be an important covariate to consider when looking for relationships between dietary choline intake and cognitive function and cerebrovascular health.

Previous studies provide some evidence of the importance of choline for cognitive function later in life. Intervention studies have shown choline-containing supplements enhanced learning and memory[11-13] and prevented or reversed cognitive decline in patients with cerebrovascular disease.[14-16] In addition, cross-sectional studies demonstrate positive associations between choline intake or foods high in dietary choline global cognition or memory.[17, 18] Conversely, we previously found that dietary choline intake was inversely associated with memory scores in cross-sectional analysis of an elderly (age $\geq$60yr) cohort (NAME; Ch. 1). In

another cohort of individuals of Puerto Rican descent, living in the Boston, Massachusetts area (BPRHS; Ch. 2), we did not find any relationship between memory measurements and dietary choline intake, however, there was a positive association between dietary choline intake and baseline and 2-year executive function scores. In addition to dietary intake, humans acquire choline from endogenous production in the liver, through methylation of phosphatidylethanolamine to form phosphatidylcholine, catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT).[7] In order to fully understand the relationships between choline and cognitive outcomes, it may be necessary to measure plasma choline in order to capture both endogenous and exogenous sources. Nurk and colleagues[19] investigated the relationship between plasma choline and cognitive function of elderly subjects (Hordaland Health Study) and found that compared with low concentrations, high plasma choline (>8.4μmol/l) was associated with better scores of tests assessing perceptual speed, executive function, and global cognition. However, we know that choline is metabolized or used in the formation of phospholipids and thus relationships with other choline related compounds may be more important than relationships with choline directly.

In the NAME cohort (Ch. 1), we measured plasma free choline, betaine, sphingomyelin, and phosphatidylcholine and found that plasma choline concentration was positively related to executive function. Furthermore, plasma phosphatidylcholine concentration was positively related to memory scores, while plasma betaine concentration was inversely related. The relationship with betaine, however, was attenuated when controlling for presence of hypertension and diabetes, and plasma concentration of high density lipoprotein cholesterol, low density lipoprotein cholesterol, and vitamins B-12, B-6, and folate, and homocysteine. In the current preliminary analysis, baseline plasma betaine was inversely related to a test of attention while a higher Bet:Chol ratio was associated with fewer errors on a test of visual memory. Similarly, a higher Bet:Cho ratio was associated with faster thinking time on a test of spatial planning and

spatial working memory. While the change in Bet:Chol was not associated with change in cognitive test outcomes, there were associations with change in betaine alone. Greater increase in plasma betaine concentration from baseline to 3-months was associated with poorer scores on tests of attention and one test of visual memory and one of spatial working memory. The relationships seen at 3-months were not seen at 6-months, however, changes in choline or SM:(SM+PC) were related to changes in cognitive test outcomes at 6-months. Greater increases in plasma choline concentrations were related to slower reaction times on a test of attention and more errors on a test of visual memory. Greater increases in 6-mth SM:(SM+PC) were related to greater increases in signal detection on a test of attention but lower increases in performance on a test of working memory.

Analysis of Complete Dataset

The change in plasma choline-related compounds and cardiometabolic risk factor biomarkers (total cholesterol, HDL-c, LDL-c, triglycerides) will be compared between groups using ANCOVA. The change in scores on individual cognitive tests will also be compared between groups using ANCOVA. In addition, multiple linear regression models will be used to identify relationships between mean change in individual cognitive tests and mean change in plasma choline-related compounds or cardiometabolic risk risk factor biomarkers. Covariates considered for inclusion in the models include a) age, sex, education and b) plasma homocysteine, vitamin B-12, vitamin B-6, folate, and lutein. When analyzing relationships with choline-related compounds, concentrations of cardiometabolic risk factor biomarkers and TMAO will be added to a final model.

Conclusion

Since the results presented only represent a little over 50% of the necessary sample size, we cannot draw any conclusions just yet. For the final analysis, it will also be important to control for other health related variables such as plasma lipids and plasma vitamin B-12, B-6, and

folate. In addition, rather than analyzing the data by linear regression, once the study is complete, we can be unblinded to assess the change in cognitive function outcomes by group. This will allow us to look at causal relationships rather than simple associations.

Tables

Table 1: Eligibility and Exclusion Criteria for Study Enrollment

Eligibility Criteria	**Exclusion Criteria**
Lutein intake of <3 mg/d	A history of active small bowel disease or resection, atrophic gastritis, hyperlipidemia, insulin-requiring diabetes, alcoholism (>2 drinks/d or 14 drinks/week), pancreatic disease, anemia, and bleeding disorders (as determined by screening interview)
DHA intake <250 mg/d (including supplements)	
Mini mental state exam (MMSE) score ≥24	
Macular pigment density <0.4 at 0.5 degrees	
Beck Depression Inventory <20	
Free of known disease	Egg allergy
Body Mass Index 19-29 kg/m^2	Pregnancy or lactation
Must be able to give written informed consent	Diseases or medications that interfere with fat absorption (as determined by screening interview)
Have normal hematologic parameters, plasma albumin, liver and kidney function, as determined by laboratory normal values	Past or present smokers or nicotine patches or gum within the past 6 months
No use of carotenoid, n3 fatty acid, choline, or multivitamin/mineral supplements (>2 month)	Use of drugs suspected of interfering with metabolism of blood clotting, e.g. warfarin (as determined by screening interview)
	Subjects having extremely high dietary intakes of carotenoids as indicated by screening serum values > NHANES 95^{th} percentile for lutein/zeaxanthin, β-carotene, cryptoxanthin, or lycopene

Table 2: Cognitive Test Battery

Cognitive Test	Description	Outcome Measures
Attention Tests		
Choice Reaction Time (CRT)	2-choice reaction time test with two possible stimuli and two possible responses	Mean correct latency (response speed)
Rapid Visual Information Processing (RVP)	Test of sustained attention	A' - Signal detection measure of sensitivity to the target B" - Signal detection measure of the strength of trace required to elicit a response
Visual Memory Tests		
Delayed Match to Sample (DMS)	Test of simultaneous and delayed matching to sample	Percent correct (all delays)
Paired Associates Learning (PAL)	Assesses visual memory and new learning	Total errors (adjusted) Stages completed
Executive Function, Working Memory and Planning Tests		
Spatial Span (SSP)	Assesses working memory	Span length
Spatial Span Reverse (SSP-R)	Assesses working memory	Span length
Spatial Working Memory (SWM)	Tests ability to retain spatial information and manipulate items in working memory	Between errors Strategy
Stockings of Cambridge (SOC)	Test of spatial planning and spatial working memory	Problems solved in minimum moves Mean total thinking time 4 moves

Table 3: Baseline Characteristics

Baseline Characteristic	Descriptive Statistic	EGG	CONTROL
Age (yrs)	Mean ± Std Dev	63.78 ± 10.14	61.24 ± 9.62
Sex (frequency)	Male	18	15
	Female	18	23
Education	0-8th grade	0	0
	high school	0	0
	high school graduate or GED	3	3
	some college (includes Associates Degree, Technical Degree, Apprenticeship)	9	14
	college graduate (4 year degree)	13	15
	graduate degree	11	6
Handedness (frequency)	Right	30	35
	Left	6	3
Dietary Betaine (mg/day)	Mean ± Std Dev	122 ± 35*	192 ± 96*
Dietary Choline (mg/day)	Mean ± Std Dev	320 ± 76	3499 ± 132

* Significantly different between groups ($p \leq 0.05$)

N=13 per group

Table 4: Plasma Compound Concentrations by Time and Group

Plasma Compound (µM)	EGG (mean ± SD)			CONTROL (mean ± SD)		
	Baseline	3mth	6mth	Baseline	3mth	6mth
Choline	10.0 ± 1.7	11.5±2.4ǂ*	10.6 ± 2.3	9.2 ±2.6	8.8 ±2.2*	9.0 ± 1.9
Betaine	49.5 ±11.7*	52.3 ±12.5*	51.8 ± 15.2*	38.1 ±10.3*	37.4± 10.0*	39.3 ± 11.9*
SM	522.1 ±78.4	551.5±91.5	560.3 ± 99.8	591.0±129.1	571.0 ±97.5	590.3 ± 116.5
PC	2001.9 ±540. 8	2099.2 ±583.1	2042.3 ± 565.7	2147.4 ±499.9	2114.3 ±356.0	2094.4 ± 336.6
TMAO	3.6±1.3	6.9±5.9	3.5 ± 1.3	6.4±6.8	3.9 ±2.2	5.0 ± 3.6

At baseline and 3mth, N=13 per group; At 6mth, N=10 EGG, N=12 CONTROL

*Significantly different between groups ($p<0.05$)

ǂSignificantly different from baseline ($p<0.05$)

SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide

Table 5: Cross-sectional relationship between plasma choline-related compounds and attention test outcomes at baseline

Plasma Compound	Attention Tests Outcomes					
	Cholice Reaction Time - Mean Correct Latency		**Rapid Visual Information Processing - A**		**Rapid Visual Information Processing - B**	
	β - Estimate	P-Value	β - Estimate	P-Value	β - Estimate	P-Value
Choline*	-4.76	0.40	-0.002	0.82	-0.002	0.86
Betaine*	1.60	0.19	**-0.004**	**0.03**	-0.004	0.23
PC*	-0.004	0.85	0.000003	0.94	-0.0000002	1.00
SM*	0.02	0.86	0.0001	0.64	0.0002	0.54
TMAO**	3.90	0.20	-0.003	0.54	0.01	0.50
SM:(SM+PC) *	4.37	0.99	0.19	0.76	0.65	0.52
Bet:Chol*	24.58	0.04	-0.03	0.11	-0.02	0.44

*Adjusted for age, sex, education, baseline TMAO

**Adjusted for age, sex, education, baseline choline

N=26

SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

Table 6: Cross-sectional relationship between plasma choline-related compounds and visual memory test outcomes at baseline

Plasma Compound	Visual Memory Tests Outcomes					
	Delayed Match to Sample - Percent Correct		**Paired Associates Learning - Total Errors**		**Paired Associates Learning - Stages Completed**	
	β - Estimate	P-Value	β - Estimate	P-Value	β - Estimate	P-Value
Choline*	0.09	0.95	1.26	0.33	-0.09	0.11
Betaine*	-0.12	0.70	-0.47	0.08	0.02	0.18
PC*	0.001	0.88	-0.003	0.58	-0.00002	0.93
SM*	-0.01	0.81	0.003	0.92	0.001	0.65
TMAO**	0.47	0.54	**-1.38**	**0.05**	**0.06**	**0.05**
SM:(SM+PC) *	-28.37	0.78	102.79	0.26	0.84	0.85
Bet:Chol*	-1.08	0.73	**-6.55**	**0.01**	**0.36**	**0.002**

*Adjusted for age, sex, education, baseline TMAO

**Adjusted for age, sex, education, baseline choline

N=26

SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

Table 7: Cross-sectional relationship between plasma choline-related compounds and executive function, working memory and planning test outcomes at baseline

Plasma Compound	Executive Function, Working Memory and Planning Tests Outcomes											
	Spatial Span - Span length		Spatial Span Reverse - Span length		Spatial Working Memory - Between Errors		Spatial Working Memory - Strategy		Stockings of Cambridge - Problems Solved in Minimum Moves		Stockings of Cambridge - Mean Total Thinking Time - 4 Move Problem	
	β	P	β	P	β	P	β	P	β	P	β	P
Choline	0.02	0.83	0.18	0.07	0.29	0.89	0.49	0.41	0.22	0.41	2158.98	0.08
Betaine	-0.004	0.85	0.03	0.24	0.28	0.53	0.06	0.65	0.01	0.89	-272.51	0.33
PC	0.001	0.11	0.0003	0.42	-0.01	0.15	-0.004	0.08	0.0003	0.74	**10.18**	**0.04**
SM	0.002	0.25	0.000	1.00	-0.05	0.20	-0.01	0.58	0.004	0.46	**47.15**	**0.04**
TMAO	-0.03	0.57	-0.01	0.88	1.57	0.16	0.16	0.61	-0.17	0.23	165.57	0.79
SM:(SM+PC)	-5.44	0.40	-5.64	0.44	45.35	0.76	58.20	0.17	12.62	0.50	-33174.08	0.72
Bet:Chol	-0.08	0.69	-0.09	0.69	1.58	0.73	-0.37	0.78	-0.19	0.74	**-7089.62**	**0.01**

*Adjusted for age, sex, education, baseline TMAO

**Adjusted for age, sex, education, baseline choline

N=26; β = Beta Estimate; P = P-value**;** SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

Table 8: Relationship between 3-mth change in plasma choline-related compounds and 3-mth change in attention tests outcomes

Plasma Compound	Attention Tests Outcomes					
	Cholice Reaction Time - Mean Correct Latency		**Rapid Visual Information Processing - A**		**Rapid Visual Information Processing - B**	
	β - Estimate	P-Value	β - Estimate	P-Value	β - Estimate	P-Value
Choline	2.24	0.81	0.23	0.55	0.26	0.47
Betaine	**3.61**	**0.03**	**-0.14**	**0.03**	**-0.12**	**0.03**
PC	-0.07	0.36	0.0002	0.92	0.0003	0.90
SM	-0.03	0.94	-0.003	0.82	-0.002	0.86
TMAO	2.64	0.54	-0.11	0.49	-0.11	0.45
SM:(SM+PC)	1144.06	0.17	-9.46	0.75	-8.23	0.76
Bet:Chol	-0.02	0.83	-0.0005	0.87	-0.001	0.80

*Adjusted for baseline cognitive and plasma values, age, sex, education, change in TMAO

**Adjusted for baseline cognitive and plasma values, age, sex, education, change in choline

N=26

SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

Table 9: Relationship between 3-mth change in plasma choline-related compounds and 3-mth change in visual memory tests outcomes

Plasma Compound	Visual Memory Tests Outcomes					
	Delayed Match to Sample - Percent Correct		**Paired Associates Learning - Total Errors**		**Paired Associates Learning - Stages Completed**	
	β - Estimate	P-Value	β - Estimate	P-Value	β - Estimate	P-Value
Choline	-1.74	0.28	0.74	0.71	0.97	0.54
Betaine	-0.45	0.13	0.46	0.21	**-0.62**	**0.03**
PC	-0.01	0.31	-0.01	0.51	0.002	0.88
SM	-0.04	0.50	-0.10	0.10	-0.002	0.96
TMAO	0.42	0.59	1.16	0.16	-0.54	0.44
SM:(SM+PC)	12.58	0.94	-125.13	0.51	-35.22	0.79
Bet:Chol	0.01	0.68	0.01	0.60	-0.003	0.81

*Adjusted for baseline cognitive and plasma values, age, sex, education, change in TMAO

**Adjusted for baseline cognitive and plasma values, age, sex, education, change in choline

N=26

SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

Table 10: Relationship between 3-mth change in plasma choline-related compounds and 3-mth change in executive function, working memory and planning tests outcomes

Plasma Compound	**Executive Function, Working Memory and Planning Tests Outcomes**											
	Spatial Span - Span length		**Spatial Span Reverse - Span length**		**Spatial Working Memory - Between Errors**		**Spatial Working Memory - Strategy**		**Stockings of Cambridge - Problems Solved in Minimum Moves**		**Stockings of Cambridge - Mean Total Thinking Time - 4 Move Problem**	
	β	P	β	P	β	P	β	P	β	P	β	P
Choline	0.30	0.46	-0.05	0.79	1.93	0.35	-0.89	0.29	-0.33	0.33	893.50	0.23
Betaine	-0.13	0.07	0.06	0.08	**0.88**	**0.01**	-0.30	0.29	0.04	0.55	153.41	0.26
PC	-0.0005	0.86	0.0002	0.86	-0.01	0.31	0.002	0.74	0.002	0.33	-2.68	0.62
SM	-0.01	0.41	-0.005	0.41	-0.05	0.43	-0.0002	0.99	0.01	0.37	26.37	0.29
TMAO	-0.07	0.70	-0.06	0.46	-0.84	0.37	0.08	0.83	0.18	0.24	587.73	0.06
SM:(SM+PC)	-21.69	0.53	-15.76	0.34	178.36	0.35	-0.55	0.99	-14.02	0.67	128022.09	0.10
Bet:Chol	0.001	0.81	0.001	0.45	0.01	0.49	0.005	0.49	0.004	0.13	-4.42	0.49

*Adjusted for baseline cognitive and plasma values, age, sex, education, change in TMAO

**Adjusted for baseline cognitive and plasma values, age, sex, education, change in choline

N=26; β = Beta Estimate; P = P-value; SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

Table 11: Relationship between 6-mth change in plasma choline-related compounds and 6-mth change in attention tests outcomes

Plasma Compound	Attention Tests Outcomes					
	Cholice Reaction Time - Mean Correct Latency		**Rapid Visual Information Processing - A**		**Rapid Visual Information Processing - B**	
	β - Estimate	P-Value	β - Estimate	P-Value	β - Estimate	P-Value
Choline	**7.85**	**0.04**	-0.01	0.14	-0.004	0.72
Betaine	0.25	0.70	0.001	0.43	0.001	0.63
PC	0.02	0.39	0.00004	0.55	0.0001	0.06
SM	-0.10	0.36	**0.0005**	**0.05**	**0.001**	**0.04**
TMAO	1.20	0.63	0.01	0.31	-0.002	0.77
SM:(SM+PC)	-841.59	0.09	**1.83**	**0.04**	0.19	0.86
Bet:Chol	-0.11	0.83	-0.0002	0.86	0.001	0.50

*Adjusted for baseline cognitive and plasma values, age, sex, education, change in TMAO

**Adjusted for baseline cognitive and plasma values, age, sex, education, change in choline

N=22

SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

Table 12: Relationship between 6-mth change in plasma choline-related compounds and 6-mth change in visual memory tests outcomes

Plasma Compound	Visual Memory Tests Outcomes					
	Delayed Match to Sample - Percent Correct		**Paired Associates Learning - Total Errors**		**Paired Associates Learning - Stages Completed**	
	β - Estimate	P-Value	β - Estimate	P-Value	β - Estimate	P-Value
Choline	-0.87	0.49	**3.45**	**0.04**	-0.08	0.35
Betaine	-0.14	0.48	0.47	0.08	-0.01	0.27
PC	-0.002	0.76	0.02	0.19	-0.0004	0.48
SM	0.02	0.51	0.07	0.22	-0.002	0.39
TMAO	-0.01	0.99	-1.17	0.22	0.06	0.21
SM:(SM+PC)	59.12	0.65	-61.65	0.77	0.29	0.98
Bet:Chol	0.13	0.29	0.15	0.59	0.002	0.85

*Adjusted for baseline cognitive and plasma values, age, sex, education, change in TMAO

**Adjusted for baseline cognitive and plasma values, age, sex, education, change in choline

N=22

SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

Table 13: Relationship between 6-mth change in plasma choline-related compounds and 6-mth change in executive function, working memory and planning tests outcomes

Plasma Compound	Executive Function, Working Memory and Planning Tests Outcomes											
	Spatial Span - Span length		Spatial Span Reverse - Span length		Spatial Working Memory - Between Errors		Spatial Working Memory - Strategy		Stockings of Cambridge - Problems Solved in Minimum Moves		Stockings of Cambridge - Mean Total Thinking Time - 4 Move Problem	
	β	P	β	P	β	P	β	P	β	P	β	P
Choline	-0.17	0.20	0.11	0.50	0.87	0.68	-0.03	0.96	-0.12	0.61	2180.65	0.06
Betaine	-0.01	0.53	0.02	0.52	0.15	0.57	0.06	0.44	-0.06	0.13	**373.07**	**0.03**
PC	0.0005	0.54	0.00004	0.97	0.003	0.82	0.002	0.56	0.002	0.24	-0.0001	1.00
SM	0.01	0.06	-0.003	0.41	-0.01	0.87	0.02	0.16	-0.002	0.80	-2.33	0.95
TMAO	0.15	0.09	**0.15**	**0.05**	-0.21	0.86	0.04	0.90	-0.11	0.44	-1023.27	0.14
SM:(SM+PC)	6.02	0.73	**-37.56**	**0.05**	-35.29	0.89	74.43	0.21	-56.10	0.06	24744.24	0.87
Bet:Chol	0.01	0.37	0.01	0.58	-0.11	0.54	-0.07	0.17	-0.01	0.82	-174.87	0.17

*Adjusted for baseline cognitive and plasma values, age, sex, education, change in TMAO

**Adjusted for baseline cognitive and plasma values, age, sex, education, change in choline

N=22; β = Beta Estimate; P = P-value; SM= sphingomyelin; PC= phosphatidylcholine; TMAO= trimethylamine oxide; Bet= betaine; Chol= choline

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Summary and Discussion

Choline is an essential nutrient with many links to brain function that relate to cognition as demonstrated by animal models [1-3] and observational studies.[4-6] As a component of phospholipids, choline is involved in cell signaling, lipid transport, and cell membrane structure. In addition, choline is a precursor to the neurotransmitter acetylcholine and is oxidized to form the methyl donor betaine.[7] There is evidence that changes in DNA methylation play a role in early neuronal development[8] as well as in the pathogenesis of neurodegenerative diseases, such as Alzheimer's Disease and Parkinson's Disease, by altering gene expression.[9] In addition, plasma choline and betaine concentrations have been differentially associated with risk of cardiovascular disease[10] and may also be related to cognitive performance via cerebrovascular health. Higher plasma choline concentration has been found to be positively associated with higher serum triglycerides and glucose concentrations, body mass index, percent body fat, and waist circumference, which would predict higher risk of cardiovascular disease. The opposite was found for plasma betaine concentrations. Whether this same relationship holds true for cerebrovascular disease risk has yet to be evaluated. Furthermore, no study in humans has evaluated both dietary intake and plasma concentration of choline and choline metabolites in relation to cognitive function, cardiometabolic risk and cerebrovascular pathology. By looking at a range of biochemical, cognitive, anatomical, and genetic parameters, our approach for exploring the role of cognitive function in four different adult populations will help to fill this critical knowledge gap.

Choline from animal products such as eggs, milk, and beef, is a major source of methyl groups in the diet;[2] however, analyses of several cohorts in the U.S. demonstrate choline intakes far below the Adequate Intake (AI).[11-13] Given that the AI for choline is currently based on one study evaluating the amount of choline necessary to prevent liver damage,[7] it is unknown if this amount, or more or less, is needed for optimal cognitive function. It is possible that too much

choline is just as problematic as too little choline. Choline in the form of phosphatidylcholine is digested by intestinal bacteria and produces trimethylamine (TMA), which is then oxidized to trimethylamine oxide (TMAO) in the liver. TMAO has been associated with increased risk of cardiovascular disease in humans[14, 15] At this point, the research on TMAO from dietary choline intake is still emerging and it is not clear whether TMAO, in addition to cardiovascular disease, is related to increased risk of cerebrovascular disease. Better understanding the role of choline in cognition and cerebrovascular health will be important factors in improving dietary intake recommendations for this nutrient and age group.

The role of choline in cognitive performance and cerebrovascular health in the older adult was explored through four separate studies: 1) A cross-sectional study evaluated relationships between dietary and plasma choline and related compounds, and cardiometabolic risk factors, cognitive performance and cerebrovascular pathology. 2) A longitudinal study evaluated the relationship between dietary choline and cognitive outcomes and whether SNPs in choline and folate metabolism modify this relationship. 3) An analytical study involved analysis of stored brain tissue (with associated pre-mortem cognitive measures) and serum for relationships between measures of methylation capacity from choline in serum and gene expression of genes related to memory in the frontal cortex, as well as global cognitive performance. 4) An intervention study, designed to increase dietary choline intake in the older adult, evaluated relationships between change in plasma choline and related compounds relative to change in cognitive outcomes.

We evaluated a cross-sectional cohort (Nutrition, Aging, and Memory in Elders Study – NAME; Chapter 1) for relationships between dietary intake and plasma concentrations of choline-related compounds and cardiometabolic risk factors, MRI markers of cerebrovascular disease, and cognitive outcomes. Dietary choline was positively associated with plasma betaine, the ratio of plasma sphingomyelin to total choline containing phospholipids (SM:(SM+PC)), and the ratio

of plasma betaine to choline (Bet:Chol), and inversely associated with plasma phosphatidylcholine concentration. We found different associations between the nutrients and outcome measures depending on whether dietary intake or plasma concentrations were used in the statistical model. While we did not find a relationship between dietary intake and radiological evidence of cerebrovascular disease or self-reported cardiovascular disease, we did find that those individuals with lower plasma concentrations of choline had higher odds of radiological evidence of small vessel infarct on brain MRI, and those with higher plasma concentrations had higher odds of large vessel infarcts and self-reported cardiovascular disease, indicating that the relationship between choline and vascular disease is different when considering the location of blood vessels, cerebrovascular or cardiovascular, as well as the size of the blood vessel, small vessel versus large vessel disease. We found similar discrepancies when assessing the relationship between cognitive function and dietary intake and plasma concentrations of choline and betaine. Dietary betaine intake was positively associated with executive function, while dietary choline intake was inversely related to memory. In contrast, plasma concentrations of choline were positively related to executive function and betaine plasma concentrations were positively associated with memory. These discrepancies may be due to variations in choline metabolism due to single nucleotide polymorphisms (SNPs), variations in intestinal microflora that utilize phosphatidylcholine, or other unknown factors that influence the use of dietary choline and the production of endogenous choline.

In a longitudinal cohort (Boston Puerto Rican Health Study – BPRHS; Chapter 2), we have found significant associations between 25 SNPs in choline and folate metabolism and cognitive outcomes, as well as interactions between dietary choline intake and 22 SNPs on cognitive outcomes. In addition, 9 SNPs with main effects also had significant SNP x dietary choline interactions and thus modified the relationships between dietary choline intake and

cognitive outcomes. These relationships should be assessed in similar populations in order to draw significant conclusions regarding dietary choline recommendations based on genotype.

In another population (Georgia Centenarian Study – GSC; Chapter 3) we found methylation capacity from choline was inversely related to Global Deterioration Rating Scale scores, indicating that higher methylation capacity was associated with less cognitive impairment. In addition, we found a positive relationship between methylation capacity and number of objects recalled after a 20 minute delay (Fuld Object Memory Evaluation – delayed recall scores), indicating a positive association with memory. Furthermore, we found that the expression of *EGR-1*, a gene shown to be necessary for long-term memory in rodent models, was greater in those with methylation capacity in the upper 50% compared to those with methylation capacity in the lower 50%. This was the first study to look at gene expression related to methylation capacity in humans. While these relationships are correlational, they do provide some support of the hypothesis that choline is related to cognitive function via its role in methylation. Future studies should look at gene specific DNA methylation and determine whether differences in methylation are related to differences in gene expression.

Finally, we have preliminary results from a randomized controlled trial assessing the effects of a 6-month dietary intervention to increase dietary choline intake in adults on concentration of plasma choline-related compounds, cardiometabolic risk factors, and cognitive outcomes. In these preliminary results, we have not yet analyzed blood samples for high density lipoprotein cholesterol, low density lipoprotein cholesterol, or triglycerides in order to assess how the dietary intervention affects these cardiometabolic risk factors. In addition, we were only able to analyze preliminary results by multiple linear regression in order to remain blinded to the group effect on cognition. While we cannot draw any significant conclusions yet, our preliminary analysis has yielded significant associations between changes in concentration of plasma choline-related compounds and changes in scores on cognitive tests.

The role of choline in cognition and cerebrovascular health in the older adult was explored through four specific aims. From the first two aims, a cross-sectional cohort and a longitudinal cohort, we were able to identify the best biomarker of dietary choline intake in blood and directly relate this parameter to measures of cognitive function and cerebrovascular pathology as well as identify single nucleotide polymorphisms that may modify these relationships. Aim 3 provided insight into the possible mechanism by which choline affects cognitive function by assessing methylation capacity and gene expression, and helped to generate hypotheses for future studies. From the fourth aim, we were able to test our hypothesis that increasing daily dietary choline intake will improve cognitive function. This intervention study is on-going and a progress report was presented in this dissertation.

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Appendix A

Choline Assay

Chemicals:

HPLC grade solvent methanol (MeOH) and chloroform ($CHCl_3$) were purchased from Fisher Scientific (Pittsburg, PA). Ammonium formate, choline (Cho), betaine (Bet), and phosphatidylcholine (PtdCho) were purchased from Sigma-Aldrich (St. Louis, MO). Cho-d9 and Bet-d9 were purchased from Cambridge Isotopes (Tewksbury, MA). Sphingomyelin (SM) and PCho-d9 was purchased from Avanti Polar Lipids (Alabaster, AL). Sphingomyelin-d3^{13}C was purchased from Ricerca (Concord, OH).

Method:

Plasma free choline, betaine, phosphatidylcholine, and sphingomyelin were quantified using liquid chromatography-stable isotope dilution-multiple reaction monitoring mass spectrometry (LC-SID-MRM/MS). All samples were stored at −80 °C until the time of analysis. Briefly 50 µL plasma aliquots were extracted based on a previously published method[1] with fixed amounts of stable isotope standards added to the samples prior to sample extraction. A mixture of 187.5 µL MeOH/$CHCl_3$ (2:1) was used for the first extraction. Each protein pellet was further extracted with 125 µL of MeOH/$CHCl_3$/H_2O (2:1:0.8). Supernatants from both extractions were combined. Phase separation was achieved by adding 50 µl $CHCl_3$ and 50 µl H_2O to the combined extract: the aqueous phase (top) contains Cho and Bet and the organic phase (bottom) contains PtdCho and SM. The aqueous phase and organic phase were analyzed with two separate runs on a Waters ACQUITY UPLC system coupled to a triple quadrupole detector (TQD) (Waters Corp, Milford, USA).

Chromatographic separations were performed on an Atlantis Silica HILIC 3µm 4.6×50mm column (Waters Corp, Milford, USA). The column was heated to 40°C, and the flow rate was maintained at 1 mL/min. Mobile phases: A was 10% acetonitrile/90%water with 10 mM

ammonium formate and 0.125% formic acid and B was 90% acetonitrile/10% water with 10 mM ammonium formate and 0.125% formic acid. The gradient for aqueous phase (Cho and Bet): 0-0.05 min, 5% A; 0.05-0.40 min, 5%-15% A; 0.40-1.00 min, 15-20% A; 1.00-2.00 min, 20-30% A; 2.00-2.55 min, 30-45% A; 2.55-2.60 min, 45-55% A; 2.60-3.50 min, 55% A, 3.50-3.55 min, 55-5% A, 3.55-5.00 min, 5% A. The gradient for organic phase (PtdCho and SM): 0-0.05 min, 5% A; 0.05-3.00 min, 5-20% A; 3.00-3.05 min, 20-55% A; 3.05-4.00 min, 55% A; 4.00-4.05 min, 55-5% A; 4.05-7.00 min, 5% A.

Mass spectrometry was performed with electrospray ionization probe operated in positive ion mode, with capillary voltage at 1.2kV. The temperatures of source and desolvation gas were set at 130°C and 350°C, respectively. Nitrogen was used as the cone and desolvation gases with flow rates at 1 L/Hr and 600 L/Hr, respectively. Argon was used as collision gas with a flow rate of 0.1 mL/min. The metabolites and their corresponding isotopes were monitored using characteristic precursor-product ion transitions: 104→45 for Cho, 113→45 for Cho-d9, 118→59 for Bet, 127→68 for Bet-d9, 184→184 for PtdCho and SM-d3^{13}C, 188→188 for SM, 193→193 for PtdCho-d9. Concentration of each metabolite in samples was determined from a calibration curve using peak area ratio of the metabolite to its isotope.

Reference:

1. Koc H, Mar MH, Ranasinghe A, Swenberg JA, Zeisel SH. Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. *Anal Che*m. 2002;74(18):4734-4740.

Appendix B

Gene Expression Protocol

A. RNA Extraction

Trizol® Plus RNA Purification Kit was purchased from Life Technologies (catalogue #12183-555). Kit instructions were performed as follows:

Lysase Preparation with TRizol® reagent:

1. Homogenize tissue samples in 1mL TRIzol® Reagent per 50-100mg tissue using a tissue homogenizer or rotor-stator. The sample volume should not exceed 10% of the volume of TRizol® Reagent used for homogenization.

Phase Separation:

2. Incubate the lysate with TRIzol® Reagent at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes.
3. Add 0.2mL chloroform per 1mL TRIzol® Reagent used. Shake the tube vigorously by hand for 15 seconds.

   NOTE: Vortexing may increase DNA contamination of your RNA sample. Avoid vortexing if your downstream application is sensitive to the presence of DNA or perform a DNase-digestion step during RNA purification or after purification.
4. Incubate at room temperature for 2-3 minutes.
5. Centrifuge the sample at 12,000 x g for 15 min at 4˚C.

   NOTE: After centrifugation, the meixture separates into a lower, red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase which contains the RNA. The volume of the aqueous upper phase is ~600µL.
6. Transfer ~600µL of the colorless, upper phase containing the RNA to a fresh RNase-free tube.

7. Add an equal volume of 70% ethanol to obtain a final ethanol concentration of 35%. Mix well by vortexing.
8. Invert the tube to dispense any visible precipitate that may form after adding ethanol.

Binding, Washing, and Elution

9. Transfer up to 700µL of sample to a Spin Cartridge (with a Collection Tube).
10. Centrifuge at 12,000 x g for 15 seconds at room temperature. Discard the flow-through and reinsert the Spin Cartridge into the same Collection Tube.
11. Repeat Steps 1-2 until the entire sample has been processed.
12. Add 700µL Wash Buffer I to the Spin Cartridge. Centrifuge at 12,000 x g for 15 seconds at room temperature. Discard the flow-through and the Collection Tube. Insert the Spin Cartridge into a new Collection Tube.
13. Add 400µL Wash Buffer II with ethanol to the Spin Cartridge.
14. Centrifuge at 12,000 x g for 15 seconds at room temperature. Discard the flow-through and reinsert the Spin Cartridge into the same collection Tube.
15. Repeat Steps 5-6 once.
16. Centrifuge the Spin Cartridge and Collection Tube at 12,000 x g for 1 minute at room temperature to dry the membrane with attached RNA. Discard the Collection Tube and insert the Spin Cartridge into a Recovery Tube.
17. Add 25µL RNase-Free Water to the Spin Cartridge.
18. Incubate at room temperature for 1 minute.
19. Centrifuge the Spin Cartridge with the Recovery Tube for 2 minutes at > 12,000 x g at room temperature.
20. Repeat steps 9-11 3 times, collecting the elute in the same Collecting Tube.
21. Discard the Spin Cartridge. The Recovery Tube contains the purified total RNA.
22. Check purity and quality by absorbance at 260nm, 280nm, and 230nm using NanoDrop .

23. Store at -80˚C.

B. cDNA Synthesis

SuperScript II™ Reverse Transcriptase was purchased from Invitrogen by Life Technologies (catalogue #18064-022) and cDNA created per manufacturer instructions as follows:

1. Add the following components to a nuclease-free microcentrifuge tube:

   Oligo(dT)12-18 (500 μg/mL) *or* 1 μL

   50–250 ng random primers *or*

   2 pmole gene-specific primer (GSP)

   1 ng to 5 μg total RNA *or* x μL

   1–500 ng of mRNA

   1 μL dNTP Mix (10 mM each) 1 μL

   Sterile, distilled water to 12 μL
2. Heat mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add:

   5X First-Strand Buffer 4 μL

   0.1 M DTT 2 μL
3. Mix contents of the tube gently. If you are using oligo(dT)12-18 or GSP, incubate at 42°C for 2 min. If you are using random primers, incubate at 25°C for 2 min.
4. Add 1 μL (200 units) of SuperScript™ II RT and mix by pipetting gently up and down.
5. If you are using less than 1 ng of RNA, reduce the amount of SuperScript™ II RT to 0.25 μL (50 units) and add sterile, distilled water to a 20 μL final volume.
6. If you are using random primers, incubate tube at 25°C for 10 min.
7. Incubate at 42°C for 50 min.
8. Inactivate the reaction by heating at 70°C for 15 min.
9. Test quality by Western Blot bands.

10. Store at -80˚C.

C. Primer Optimization

1. Primers were purchased from Invitrogen.
2. Add diethylpyrocarbonate (DPEC) treated water to dry primers to make 100 µM solution.
3. Add 50µL forward primer and 50µL reverse primer to 900µL DPEC-water to make 5µM solution.
4. Add 1 µL of each sample of cDNA to a microcentrifuge tube to create a pooled sample (Sample 1).
5. Calculate the volume of each dilution needed (2 µl per gene)
6. Add 5 µl of pooled sample to a new microcentrifuge tube + 15 µl DPEC water (Sample 2)
7. Add 5 µl of Sample 2 to a new microcentrifuge tube + 15 µl DPEC water (Sample 3)
8. Add 5 µl of Sample 3 to a new microcentrifuge tube + 15 µl DPEC water (Sample 4)
9. Add 20 µl of DPEC water to a new microcentrifuge tube (Sample 5)
10. Make Master Mix: For each Dilution Sample, run in duplicate + 10% extra for loss
    a. 10 µl SYBR green
    b. 1.2 µl primers
    c. 7.8 µl DPEC water
11. Load plates: Each gene will have 2 lanes. Add 19 µl master mix to each well. Add 1 µl of Dilution Sample to well.

GENE A	Sample 1+MM	Sample 2 + MM	Sample 3 + MM	Sample 4 + MM	Sample 5 + MM
GENE A	Sample 1+MM	Sample 2 + MM	Sample 3 + MM	Sample 4 + MM	Sample 5 + MM

GENE B	Sample 1+MM	Sample 2 + MM	Sample 3 + MM	Sample 4 + MM	Sample 5 + MM
GENE B	Sample 1+MM	Sample 2 + MM	Sample 3 + MM	Sample 4 + MM	Sample 5 + MM

12. Cover plates
13. Vortex
14. Spin

RT-PCR Conditions

The optimum temperature determined from melting point analysis was then used for quantitative PCR using the following thermal cycling programme: stage 1, 50°C for 2 min stage 2, 95°C for 10 min. Stage 3 consisted of; 95°C for 15 s with 40 repeated cycles. A dissociation stage was added to the program with 15 second ramp time to 60°C for 1 min, stage 4, 95°C for 15 sec, stage 5, 60°C for 30 seconds and finally to 95C for 15 sec. SYBR Green is used for the real-time PCR product detection.

Primers

Gene	**Forward**	**Reverse**
ARC	CCAGCACAGCAGCAAAGACT	AGGGGCTGAGTCCTCAAATC
BDNF	CTCCGCCATGCAATTTCCAC	CGTGTACAAGTCTGCGTCCT
GADD454β	GATGTCATCCTCCTCCTCCTC	ACAGTGGGGGTGTACGAGTC
RELN	TCACGTGAGAGGCTACCACA	TTGGAGGTTCCAGTGCTTTC
GAPDH	AATGAAGGGGTCATTGATGG	AAGGTGAAGGTCGGAGTCAA
EGR1	GTTTGGCTGGGGTAACTGGT	AGCCCTACGAGCACCTGAC
ACTβ	GTTGTCGACGACGAGCG	GCACAGAGCCTCGCCTT

Appendix C

Aim 1: Sample Size Calculation

Based on relationships between dietary intake or plasma choline and cognitive outcomes in the previously mentioned Madrid cohort [1] and Hordaland Health Study [2] (Table 1), a sample size of 180 in NAME gave us greater than 80% power to detect a significant relationship at the 0.05 significance level, even after controlling for 11 covariates (www.danielsoper.com/statcalc3).

Table 1: Sample Size Calculation for Aim 1

Reference Cohort	Madrid Cohort	Hordaland Health Study
Dependent Variable	Global cognitive function	Executive function
Independent Variable	Egg consumption	Plasma free choline
Effect Size	0.20	0.16
Sample Size (4 predictors)	65	79
Adjusted Sample Size (12 predictors)	98	119
Adjusted Sample Size ($p \leq 0.01$)	131	161

Aim 2: Sample Size Calculation

In order to identify significant gene-diet interactions, we estimated a sample size of 720 would be needed, based on the general guideline of four times the sample size needed to see a main effect. The sample size of the BPRHS cohort with dietary choline data and change in cognitive function (N=895) was sufficient.

Aim 3: Sample Size

The sample size was chosen based on availability of tissue and serum as well as time and cost feasibility for this pilot aim.

Aim 4: Sample Size Calculation

The parent trial sample size calculation estimated that a sample size of 20 participants per group would give an 80% chance of finding a significant intervention group difference in macular pigment at the 0.05 level. This was based on previous studies that identified significant changed in macular pigment density following egg consumption [3, 4] and changes in serum lutein concentration [4] that were similar to between group differences associated with significant differences in global cognitive function (preliminary data, unpublished).

This sample size is also appropriate for the analysis in this proposal as well. Based on the between group difference in change in betaine (6.3 μmol/L, within group standard deviation of 6.25) seen when older adults were given a B-12 and folic acid supplement in a Dutch cohort in which participants with the highest change in betaine also showed the highest increase in memory performance [5], a sample size of 17 per group would give 80% power of seeing this type of change at a 0.05 level of significance (http://www.jerrydallal.com/LHSP/SIZECALC.HTM).

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Appendix D

Cardiometabolic Risk Factor Biomarkers

Lipidprotein profiles

Cholesterol Test (CHOL) - Direct Measurement

Triglyceride Test (TRIG) - Direct Measurement

High Density Lipoprotein Test (HDL) - Direct Measurement

Low Density Lipoprotein (LDL) - Calculated (See Below)

Very Low Density Lipoprotein (VLDL) - Calculated (See Below)

VLDL = TRIG/5

LDL = CHOL - (VLDL + HDL)

Cholesterol

Matrix:	Serum (non-hemolyzed); Plasma (heparinized or EDTA, non-hemolyzed)
Reaction Type:	Enzymatic,colorimetric,endpoint (Aminoantipyrene/Phenol/Peroxidase)
POA:	Beckman Coulter AU400
Reagent:	Beckman Coulter OSR6116
Calibrator:	Beckman Coulter Calibrator (Catalog No. DR0070)
Controls:	Bio-Rad Lyphochek Assayed Chemistry Control, Levels 1 & 2
Normal Range:	Male - 130-250 mg/dL
	Female - 130-250 mg/dL
CV%:	Intra Assay 1.6
	Inter Assay 2.8

References:

1. Flegg, H.M., Ann Clin Biochem, 10: 79, 1973.

2. Richmond, W.,, Scand J. Clin Invest, 29, Suppl 126: 3.25, 1972.

3. Hernandez, H.H. and Chaikoff, I.L., J Biol Chem, 228: 447, 1957.

4. Hyun, J., Kothari, E., Herm, E., Treadwell, C.R. and Vahouny, G.V., J Biol Chem, 224: 1937, 1969.

5. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W. and Fu, P.C., Clin Chem, 20: 470, 1974.

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Triglycerides

Matrix: Serum (recommended, fasting, (≥ 12 hour), non-hemolyzed); Plasma (heparinized or EDTA, non-hemolyzed)

Reaction Type: Enzymatic, colorimetric, endpoint (Glycerol Phosphate Oxidase)

POA: Beckman Coulter AU400

Reagent: Beckman Coulter OSR6133

Calibrator: Beckman Coulter Calibrator (Catalog No. DR0070)

Controls: Bio-Rad Lyphochek Assayed Chemistry Control, Levels 1 & 2

Normal Range: Male - 50-212 mg/dL

Female - 41-204 mg/dL

CV%: Intra Assay 2.0

Inter Assay 3.4

References:

1. Trinder, P., Ann Clin Biochem, 6: 24, 1969.

2. Bucolo, G. and David. H., Clin Chem 19: 476, 1973.

High Density Lipoprotein (HDL)

Matrix: Serum; Plasma (heparinized or EDTA, non-hemolyzed)

Trigylceride values of >900 mg/dL may interfere with the procedure.

Reaction type: Enzymatic, colorimetric (Enzymatic Accelerator Selective Detergent)

POA: Beckman Coulter AU400

Reagent: Beckman Coulter OSR6195

Calibrator: Beckman Coulter HDL Cholesterol Calibrator (Catalog No. ODC0023)

Controls: BioRad Tri-Level Lipid Controls

Normal Range: 30-85 mg/dL

CV%	Intra Assay	3.0
	Inter Assay	7.0

Reference: Protocol as specified in Beckman Coulter AU400 standard operating procedure insert for HDL (Beckman Coulter America Inc., 2 Corporate Center Drive, Melville, NY, 11747-3157).