Novel Biomarkers and Therapeutic Options to Address Neuronal Injury

Studies show that the aging population is at increased risk for folate deficiency, which may contribute to cognitive decline.[1] Data reveal that with increasing age, serum and cerebrospinal fluid (CSF) folate may drop below normal levels, while serum homocysteine (Hcy), a sensitive marker of functional folate deficiency, may rise to above normal levels. The association between elevated serum Hcy and Alzheimer's disease (AD) raises the possibility that vitamin therapy to optimize CSF folate[2,3] or lower Hcy levels may decrease the risk for AD or age-related cognitive decline.[4]

The Significance of One-carbon Metabolism

Abnormalities of one-carbon metabolism lead to elevated levels of Hcy. In one-carbon metabolism, methionine condenses to form S-adenosylmethionine (SAM), which is the substrate for many methyltransferase enzymes important in the synthesis of nucleic acids, phospholipids, proteins, neurotransmitters, and other molecules (see *Figure 1*). The methyltransferase enzymes convert SAM to S-adenosyl-L-homocysteine (SAH), which inhibits methylation reactions when it accumulates. SAH is converted to Hcy, and elevated levels of Hcy favor the accumulation of SAH. Therefore, the rapid removal of Hcy is essential in maintaining physiologically normal levels of SAH and methylation reactions. Homocysteine may accumulate due to defects in re-methylation, the primary pathway for Hcy metabolism. This results in increased SAH and decreased SAM. This primary pathway for Hcy metabolism, re-methylation, regenerates methionine by an enzymatic reaction requiring L-methylfolate and methylcobalamin. Suboptimal levels of either of these two co-factors for re-methylation will increase Hcy levels. Similarly, a genetic polymorphism in the enzyme 5,10 methyltetrahydrofolate reductase (MTHFR) enzyme (see *Figure 1a*) may compromise the ability to reduce dietary folate or synthetic folic acid to L methylfolate, also increasing Hcy levels.[5]

A secondary pathway of Hcy metabolism is reduction via transulfuration (see *Figure 1b*) to form cysteine. Elevated levels of Hcy may increase the use of the transulfuration pathway, which is of concern due to the vascular toxicity of cysteine. In brain tissue, the enzyme necessary for transulfuration, cystathione-beta synthase (CBS), is minimally expressed.[5] Due to the low expression of CBS, Hcy metabolism in the CNS is largely dependent on re-methylation (see *Figure 1c*). There are several acquired and genetic factors that can cause alterations in the metabolic pathways and lead to cognitive decline.

Acquired Factors

Hypomethylation related to hyperhomocysteinemia can result from a complex interaction of acquired and genetic factors. The most important acquired factor is a relative nutritional deficiency of methylfolate and methylcobalamin. Since 1998, the US Food and Drug Administration (FDA) has required that enriched grain products contain at least 140µg of folic acid per 100g. The effect of this low-level fortification on Hcy levels is not fully known.

Epidemiological studies[6,7] have found an association between low cobalamin levels and elevated plasma Hcy. Importantly, there was no association between high Hcy and low cobalamin intake, suggesting that, in contrast to folate, failure to absorb cobalamin—rather than inadequate dietary consumption—was the main culprit. Individuals above 60 years of age are of particular concern because of age-related declines in vitamin absorption and extraction of cobalamin from protein, and age-related increases in autoimmunity against intrinsic factor or the gastric parietal cells that produce intrinsic factor.[8]

Other factors that affect Hcy levels have received less attention. Drugs such as phenytoin, methotrexate, sulphasalazine, metformin, non-steroidal anti-inflammatory drugs (NSAIDS), niacin, and bile acid sequestrates (fenofibrates) cause elevations in Hcy levels by interfering with folate status. Other risk factors associated with decreased folate status and increased Hcy include coffee consumption of four or more cups daily, excessive alcohol intake, poor nutrition, atrophic gastritis, Crohn's disease, and a 20-year history of smoking.[9]

Genetic Factors

The methylenetetrahydrofolate reductase (MTHFR) 677 C→T genotype is a genetic factor controlling Hcy remethylation. This enzyme reduces 5,10-methylenetetrahydrofolate to L-methylfolate. L-methylfolate is needed to convert Hcy to methionine. Individuals with the C/T (heterozygous) or T/T (homozygous) polymorphism have higher concentrations of plasma Hcy. This is a common polymorphism, and may be present in as many of two-thirds of vascular dementia patients: 25.5% (T/T) homozygous, 40.6% (C/T) heterozygous.[10] The MTHFR mutation produces modestly elevated plasma Hcy levels and a reduction in CNS L-methylfolate.

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Consequences of Hyperhomocysteinemia

Abnormal levels of substrates and byproducts of Hcy metabolism can damage neurons and other components that are necessary for normal cognitive abilities. Mechanisms of hyperhomocysteinemia-induced cognitive dysfunction include oxidative stress and excitotoxicity resulting in accelerated apoptosis, augmentation of the toxicity of beta-amyloid, interference with methylation reactions—potentially resulting in decreased synthesis of acetylcholine and increased phosphorylation of tau protein—and poly-ADP-ribose polymerase (PARP) activation, which results in a decreased ability to repair damaged DNA.

Oxidative Stress and Excitotoxicity

Toxic products of oxidative reactions[11] have been implicated in the pathogenesis of neurodegenerative diseases, including AD. In the extra-cellular space, Hcy is rapidly oxidized, producing reactive oxygen species.[12] Oxidative stress impairs the ability of the cobalamin-dependent enzyme methionine synthase to convert Hcy to methionine.[13] Furthermore, folate can also undergo irreversible oxidation, resulting in impaired conversion to L-methylfolate.[14]

> A potential mechanism of homocysteine's role in excitotoxicity is its ability to mimic glutamate, a neurotransmitter.

Excitotoxicity

It is important to note that homocysteine and its metabolites are potent agonists for N-methyl-D-aspartate (NMDA) receptors.[15] A potential mechanism of Hcy's role in excitotoxicity is its ability to mimic glutamate, a neurotransmitter. Elevated concentrations of glutamate, seen in individuals with cognitive impairment, induce overactivation of NMDA receptors and excitotoxicity-mediated cell death. Hcy may be attributed to the prolonged opening of NMDA ion channels, allowing excess calcium to enter the cell and resulting in a cascade of events culminating in apoptotic cell death.[16–18] There are multiple sites of Hcy binding—in addition to the glutamate NMDA receptor—that are responsible for adverse cell effects.[18] In fact, studies have shown that Hcy is toxic to cultured neurons at concentrations likely reached in the central nervous system (CNS) of hyperhomocysteinemic individuals after breakdown of the blood–brain barrier.[15,19]

Increased Toxicity of Beta Amyloid

Elevated Hcy levels may compound a primary excitotoxic insult, produced by either cerebral infarction or by the accumulation of β-amyloid peptide. Hcy and homocysteic acid can directly affect intracellular β-amyloid accumulation. Recent findings indicate that Hcy concentration in the CSF of AD patients was almost twice as high as in healthy controls (Hasegawa). They demonstrated that the exposure of neurons to homocysteic acid results in accumulation of amyloid beta subunit 42 (AB42), which plays a role in accelerating the neurodegenerative process in AD. Accelerated neuronal loss results in decreased cholinergic transmission

Figure 1: Homocysteine Metabolism
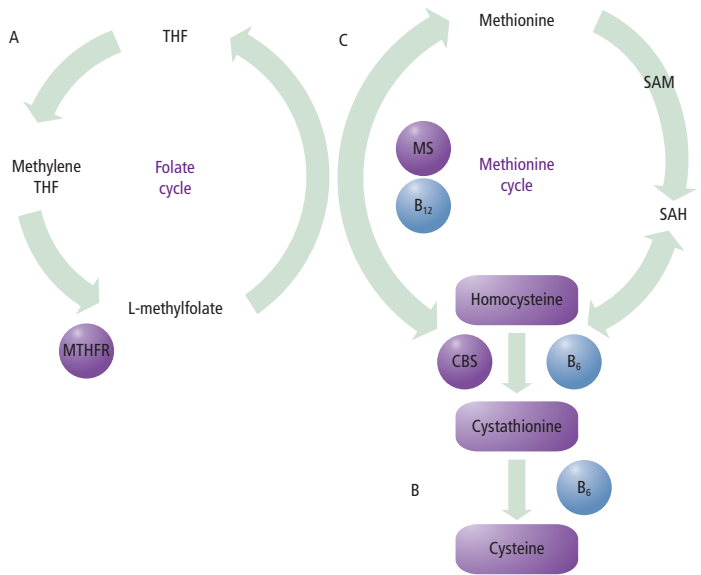
Figure 2: *De Novo* Synthesis of Acetylcholine
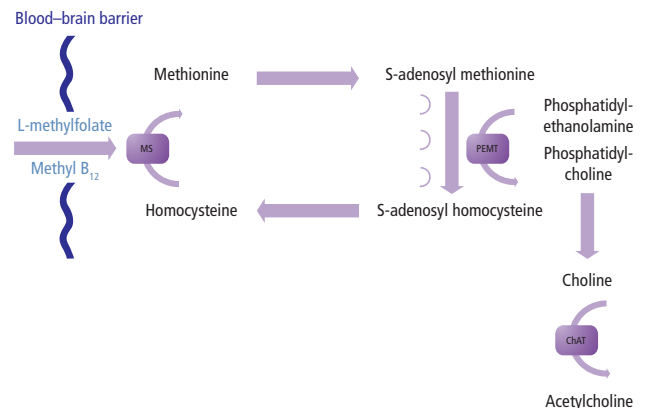
and thus cognitive decline. Eileen McGowan and Todd Golde of the Mayo Clinic College of Medicine report in the July 21, 2005 issue of *Neuron* definitive proof that Ab42 is, indeed, the culprit molecule.

Interference with Methylation Reactions

Synthesis of Acetylcholine

SAM is the universal source of one-carbon units for the synthesis of nucleic acids, phospholipids, proteins, polysaccharides, and biogenic amines.[19,20] The generation of one-carbon units is dependent on Hcy metabolism. Inadequate L-methylfolate and B_{12} levels can impair methylation of phospholipids, necessary for the production of phosphtidylcholine and ultimately, the synthesis of acetylcholine.[21–23]

The Formation of Neurofibrillary Tangles

Deposited amyloid plaques and oxidized Hcy result in the generation of reactive oxygen species. The increased production of reactive oxygen

species can activate several protein kinases that stimulate production of phosphorylated forms of tau protein. Phospho tau can aggregate to form neurofibrillary tangles inside neurons, leading to the destabilization of microtubules, an important structural component. This results in impaired axonal transport and cell death.

Low levels of SAM due to inadequate L-methylfolate and B_{12} also leads to a reduction in the methylation of protein phosphate 2A (PP2A). PP2A aids in the removal of phosphate. When SAM levels are decreased and/or SAH levels increased, PP2A activity is reduced, resulting in elevated levels of phospho tau. Adequate levels of L-methylfolate and methylcobalamin ensures that Hcy and SAH levels are kept low and SAM levels are high, promoting optimum activity of PP2A.[24]

Poly-ADP-ribose Polymerase Activation
Gene expression is partly controlled by methylation of short stretches of DNA. In fact, hypomethylation (due to mechanisms discussed) can induce gene transcription and DNA strand breakage. Studies have shown that Hcy itself also induces DNA breakages in cultured neurons, probably due to free-radical induced damage. The maintenance and repair of DNA is critical to normal physiology. Poly-ADP-ribose polymerase (PARP) recognizes such damaged DNA and prepares it for repair. However, in cells with excessive DNA damage, such as may occur with disrupted one-carbon metabolism, PARP triggers a cascade of events that leads to neuronal cell death.

PARP-mediated neuronal cell death is a major pathway for the death of neurons, and activation of this cascade may therefore be another important consequence of disturbed one-carbon metabolism.[25] If exacerbation of oxidative stress is the mechanism of Hcy-associated neurodegeneration, therapy with antioxidant compounds may also be necessary in addition to vitamin therapy to maximally protect neurons.

> Recent findings indicate that homocysteine concentration in the cerebrospinal fluid of Alzheimer's disease patients was almost twice as high as in healthy controls.

N-acetylcysteine (NAC) has been shown to significantly reduce the toxic oxidative effects of Hcy and beta amyloid.[26] NAC is also the precursor of glutathione, the most important antioxidant in the brain.

There is compelling evidence of an association between high serum levels of Hcy and AD. A deficiency of either vitamin B_{12} or folate may cause elevated Hcy levels. Epidemiological studies found that AD is associated with relative deficiencies of vitamin B_{12} and folate.[27–29] In addition, independent case-control studies have established an association between elevated serum Hcy levels and AD.[30–33]

The underlying metabolic pathways may be exploited to treat cognitive decline. The association of elevated levels of serum Hcy and AD raises the possibility that unique therapeutic doses of pharmacological vitamin therapy can optimize CSF folate[2,8] and/or lower Hcy levels to decrease the risk for age-related cognitive decline[4,5,34] and the incidence of AD and/or reduce the rate of disease progression.[33,35]

Most treatment studies are flawed because they have used folic acid, the synthetic form that is converted to L-methylfolate in the body, but folic acid is not capable of crossing the blood–brain barrier. Endogenous L-methylfolate has been shown to be seven times more bioavailable than

> The MTHFR enzyme pathway and the reduction of homocysteine appear to be one of the most promising targets for primordial and primary prevention of cognitive decline.

synthetic folic acid,[36] is unaffected by MTHFR polymorphisms, and is three times more effective in lowering serum Hcy than naturally occurring folic acid.[37,38] Therefore, compared with synthetic and naturally occurring folic acid, L-methylfolate may offer additional benefits.[36,39–46]

L-methylfolate is marketed in the US as a 'prescription medical food,' also called Cerefolin NAC, which contains 5.6mg L-methylfolate, 2mg methylcobalamin, and 600mg N-acetylcysteine. According to the FDA, a medical food is different both from a drug and from a food, and is defined as "a food that is formulated to be consumed orally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation." Medical foods are required when dietary management cannot achieve specific nutrient requirements. Further research is necessary to determine the exact priority this approach should be given in treatment algorithms for early memory loss.

Treatment of Cognitive Decline
The current paradigm of treatment for cognitive decline is such that intervention takes place relatively late in the course of disease. The use of medications currently approved by the FDA requires the establishment of a measurable cognitive deficit within strictly defined parameters. Progressive memory loss spans the spectrum from mild cognitive impairment as an entry point ranging to severe dementia at the extreme.

Thus, in today's paradigm, physicians are largely in the mindset of secondary prevention, in which the burden of existing disease is reduced by putting in place measures that reduce the impact of risk factors on the expression of that disease. This includes the institution of acetylcholinesterase inhibitors or NMDA receptor antagonists. These agents have been shown to effectively reduce the rate of cognitive decline or even transiently improve performance of activities of daily living.[47]

However, as with other disease states such as hypertension and cardiovascular disease, there is a growing recognition that an

Table 1: Efficacy Studies of High-dose Vitamin B Therapy in Cognitive Impairment

Study Design and Objective (Author, Year)	Patient Population	Results	Conclusion
Double-blind, placebo-controlled study with random assignment of 800mcg folic acid supplementation; three-year follow-up to compare cognitive performance. (Durga, 2007[48])	818 participants aged 50–70 years from the FACIT trial with Hcy levels >13µmol/l. Baseline MMSE = 29. All participants were B_{12}-replete.	Improvements in global cognitive function (p=0.033), memory (p=0.010), and information processing speed (p=0.016). No effect on complex speed and word fluency.	Folate therapy improved functions that tend to decline with age.
Double-blind, placebo-controlled pilot study to assess random assignment for six months of 1mg of folic acid supplementation and ChI, and whether outcome measures were affected by changes in Hcy levels. (Connelly, 2007[49])	57 consecutive outpatients with AD. Mean age 76.27±6.23 years. Baseline MMSE = 23.49±3.53.	The improvement in IADL scores and the combined IADL/SB scores was statistically significant (both p=0.03) and not related to changes in Hcy; no change in MMSE scores.	The effect of folate supplementation on ChI was positive on secondary outcome measures and improvement suggests that folate may act synergistically with ChI.
Prospective, observational study to examine whether diet plus intake of vitamins is associated with a reduced risk for AD. (Corrada, 2005[50])	579 non-demented participants (age 49–93 years) from the Baltimore Longitudinal Study of Aging. Age at first intake report: 69.6 years (range 49–93); age at last follow-up: 78.9 years (range 61–102).	57 participants developed AD after a mean follow-up of 9.3 years. Cox regression analysis of all variables found that only total intake of folate (diet plus vitamins) at or above the RDA was significant for reduced risk of AD.	After adjusting for age, gender, education, and caloric intake, folate was associated with a reduced risk (RR=0.45) for AD.
Double-blind, placebo-controlled study to assess the effects on cognition and mood from random assignment of 750mcg folic acid, 15mcg B_{12}, 75mg B_6, or placebo for 35 days. (Bryan, 2002[51])	211 women without dementia in three age groups: 25.20±3.19 years (n=56), 49.19±2.6 years (n=80), 74.08±5.75 years (n=75).	Supplements had a significant positive effect on various standardized tests of cognitive processing resources, memory, executive function, and verbal ability, but no effect on self-reported mood measures.	Folate intake was associated with improved performance on a variety of measures of cognition (speed, memory, and fluency); vitamin B_{12} and vitamin B_6 intake was associated with improved memory performance.
Patients in the treatment arm received an oral prescription of 10mg folic acid, 2mg B_{12}, and 80mg B_6 for an average of 270 days. (Lehmann, 2003[52])	The treatment arm consisted of 30 consecutive ambulatory patients with mild cognitive impairment, elevated serum Hcy levels, and a mean age of 72 years; the control arm consisted of 35 healthy age-matched controls with a mean age of 68 years.	Hcy levels returned to normal values of 9.5µmol/l (p<0.0001); albumin ratio decreased from baseline, indicating an improvement in blood–brain barrier function (p<0.0002); MMSE scores remained unchanged; CSF-tau was expected to increase, but an unexpected downward trend was observed.	No MCI patient progressed to dementia. Treatment with a high-dose combination of vitamin B_{12}–B_6–folate appears to improve blood–brain barrier function in patients with mild cognitive impairment and hyperhomocysteinemia.
Open-label trial to determine the effect of two months' supplementation with 5mg folic acid and 1mg cyanocobalamin for two months on cognitive function in elderly patients with dementia. (Nilsson, 2001[53])	33 consecutive patients with mild to moderate dementia. Mean age 78.4±8.1 years and plasma Hcy levels >19.9µmol/l.	Following vitamin therapy, Hcy levels were reduced to 11µmol/l (p <0.001) and MMSE and SKT scores were significantly improved (p<0.01).	An elevated plasma Hcy concentration is a valuable marker for detecting psychogeriatric patients with treatable cobalamin/folate deficiency.
Ad hoc, double-blind, placebo-controlled pilot study in which patients were randomly assigned 15mg folic acid or placebo for 60 days. (Fioravanti, 1997[54])	30 volunteers (mean age 80.23±5.53 years) with serum folate levels <3ng/ml. Very mild to moderate cognitive decline as assessed by GDS. MMSE scores 16–24.	Greater folate deficiency at the beginning of treatment was related to greater cognitive improvement over two months of treatment as measured by RMT.	Patients treated with folate showed a significant improvement in both memory and attention efficiency. Degree of memory improvement was positively correlated with initial severity of folate deficiency. Severity of initial cognitive decline was unrelated to the degree of folate deficiency.
Open-label trial using folinic acid supplementation to study the relationship between folate deficiencies and neuropsychiatric disturbances. (Rapin, 1988[55])	38 patients with conditions that included depression, asthenia, irritability, forgetfulness, lack of attention, and erythrocyte folate levels <300ng/ml were matched with 100 age-matched controls (62±5 years).	Patients treated with folinic acid experienced an increase in folate levels (192 versus 347ng/ml) and improvements in Zung depression and BRC scores.	A relationship was observed between folate deficiency and decreases in learning, memory, concentration, and vigilance; deterioration of associative memory and disruption of spatio-temporal organization had the greatest association with folate deficiency. After treatment patients reported that they 'feel better' and 'have more interest in life.'
Open-label trial to determine the effect of 50mg folinic acid given parenterally (on days one, 11, and 21) to patients with folate deficiency and depression and/or dementia. (Brocker, 1986[56])	50 patients (age 81±7 years) were enrolled from a frequency study in which 75% of 1,000 elderly hospitalized patients were folate-deficient (serum folate <5ng/ml).	Depression symptoms were resolved in four patients; self-sufficiency was recovered in three dementia patients; and 32 patients showed improvement in either geriatric or psychiatric scores.	

AD = Alzheimer's disease; FACIT = folic acid and carotid intima-media thickness; ChI = cholinesterase inhibitor; MMSE = Mini-Mental State Examination; MCI = minimal cognitive impairment; IADL = instrumental activities of daily living; SKT = Syndrom-Kurztest short cognitive performance test; SB = social behavior subscale; RDA = recommended daily allowance; GDS = global deterioration scale; RMT = Randt memory test; BRC = a battery of rating scales.

opportunity to intervene has already been missed by the time frank disease has emerged. Therefore, primary prevention is geared more toward reducing the likelihood that existing risk factors lead to the expression of disease.

Moving into an even earlier stage in the life-cycle, primordial prevention is aimed at interfering with the emergence of risk factors in the first place. The appeal of early intervention in the primordial or primary stage is the preservation of existing function rather than simply the slowing of an inevitable decline. The MTHFR enzyme pathway and the reduction of Hcy appear to be one of the most promising targets for primordial and primary prevention of cognitive decline, not to mention rescue or salvage therapy for patients already suffering from diminished cognitive abilities. Folate supplementation has been shown to be effective in treating age-related cognitive decline or dementia, as demonstrated in the nine studies that are summarized in *Table 1*.[48–56] ■

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