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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
Aβ	Amyloid beta
ADMA	Asymmetric dimethylargenin
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
APS	Ammonium persulfate
ATP	Adenosine-5-triphosphate
BACE	Beta secretase
BHMS	Betaine homocysteine methyl transferase
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CAMCOG	Cognitive and self-contained part of the Cambridge Examination for Mental Disorders of the Elderly
CAMDEX	Cambridge Examination for Mental Disorders of the Elderly
Cbl	Cobalamin
CβS	Cystathionine beta synthase
CERAD	Consortium to Establish a Registry for Alzheimer's disease
CHF	Cardiac heart failure
CI	Confidence intervals
CIND	Cognitive impairment non demented
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Cofficient of variation
CVD	Cerebrovascular disorder
CVLT	California Verbal Learning Test
Cys	Cystathionine
DMG	Dimethylglycine
DSM	Diagnostic and Statistical Manual of Mental Disorders
DRIs	Dietary reference intake
DS	Down Syndrome
DTT	Dithiothreitol
DZA	3-Deazaadenosine
EDTA	Ethylenediaminetetra acetic acid
FAD	Flavin adenine dinucleotide
FAD	Familial Alzheimer's disease
FBS	Fetal bovine serum
GCMS	Gas Chromatography Mass Spectrometry
GFR	Glomerular filtration rate
GSK	Glycogen synthase kinase
hICH	Hypertensive intracerebral hemorrhage
HDL	High density lipid
Hcy	Homocysteine
HHcy	Hyperhomocysteinemia
HMG CoA	Hydroxymethylglutaryl CoA
HPLC	High Performance Liquid Chromatography
ICD-10	International Statistical Classification of Diseases and Related Health Problems 10th Revision

IF	Intrinsic factor
IgG	Immunglobulin G
KD	Kilo dalton
LC MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LDL	Low density lipid
MAC	Membrane attack complex
Mattis DRS	Mattis Dementia Rating Scale
MAP	Microtubule associated protein
MAPK	Mitogen activated protein kinases
MCI	Mild cognitive impairment
MMA	Methylmalonic acid
MMSE	Mini-Mental State Examination
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MS	Methionine synthase
MTHF	Methylenetetrahydrofolate
NAC	N-acetyl-L-cystein
NINCDS-ADRDA	National Institute of Neurological and Communication Disorders and Stroke/Alzheimer's Disease and Related Disorders Association
NFTs	Neurofibriallary tangles
NMDA	N-methyl-D-aspartate
NO	Nitrogen species
OR	Odds ratio
P value	Probability
PARP	Poly ADP-ribose receptor
PD	Parkinson's Disease
PHF	Paired helical filament
PMLT	Post methionine loading test
PP2A	Protein phosphatase-2A
PS	Presenilin
PVDF	Polyvinylidinefluoride
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHM	Serine hydroxylmethyltransferase
SIDAM	Structured Interview for Diagnosis of Dementia of Alzheimer type, Multi-Infarct Dementia, and Dementia of other Etiology according to ICD-10
SPE	Solid phase extraction
TBS	Tris buffered saline
TEMED	Tetramethylethylendiamin
tHcy	Total homocysteine
TNF	Tumor necrosis factor
TG	Triglyceride
THF	Tetrahydrofolate
TC	Transcobalamin
UPDRS	Unified Parkinson's Disease Rating Scale
VaD	Vascular dementia

SUMMARY

Background: Hyperhomocysteinemia (HHcy) is a risk factor for neurodegenerative and psychiatric disorders, but causal relationship remains elusive. Vitamin B12 and folate play an important role in homocysteine (Hcy) metabolism. Although several studies have provided evidence for a significant inverse correlation between plasma total Hcy (tHcy) concentrations and plasma folate and vitamin B12 concentrations, the impact of B vitamins treatment on the cognitive performance remains controversial. The pathological hallmarks of Alzheimer's disease (AD) consist of amyloid plaques and neurofibrillary tangles in affected brain areas. Amyloid beta (Aβ) is formed from the amyloid precursor protein (APP) by a consequent splitting by means of β- and γ-secretases. Results from different studies suggest that alteration of the Hcy metabolism is related to increased accumulation of Aβ and may contribute to the amyloid pathology in normal aging and in AD. Our study aimed at investigating the relationship between markers of methylation and that of neurodegeneration and at investigating the effect of B vitamins treatment on cognitive performance in elderly subjects. In addition, we aimed at testing whether the methyl group metabolism affects APP level and/or its processing through the amyloidgenic pathway.

Materials and methods: The first part of this study included 182 patients with different neurological disorders. Concentration of Aβ (1-42) was measured in cerebrospinal fluid (CSF) samples. The second part was a double blind placebo controlled study on 69 patients who were randomly allocated to receive a vitamin or a placebo. Cognitive function was investigated by using the Mini-Mental State Examination test (MMSE) and the Structured Interview for Diagnosis of Dementia of Alzheimer Type, Multi-infarct Dementia and Dementia of other ethiology according to ICD-10 (SIDAM) test. Concentrations of S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM) were measured using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS).

The third part of the study is addressed to Down syndrome fibroblasts used as a culture model with enhanced risk for neurodegeneration. Cells were cultured either in a vitamin-rich or in a vitamin-free medium. Cells were treated with different concentrations of Hcy, SAH, SAM, lovastatin, and 3–deazaadenosine (DZA) and the expression of APP and its subunit (C99) were tested by westernblotting in the presence or absence of β- and γ-secretase inhibitors.

Results: The first study showed that aging was associated with higher concentrations of tHcy and SAH in the CSF, in addition to lower concentrations of CSF-folate and SAM/SAH ratio. No significant association between Aβ (1-42) concentration and quartiles of SAM, SAH, and folate was found in the total study subjects.

The second study showed that, at base line, higher methylmalonic acid (MMA) concentrations were associated with lower scores of intellectual abilities and higher SAM concentrations were associated with higher orientation scores. Both of the treatment arms showed improvement in total SIDAM scores at the end of the therapy.

The third study showed that while treating with SAM caused decreased APP expression, SAH caused increased APP expression and decreased C99 expression in vitamin free conditions. Hcy in a vitamin-free medium caused increased C99 protein expression. Inhibitors of β- and γ-secretases reversed the effect of SAH and that of SAM on protein expression of APP and C99 and the effect of Hcy on C99 protein. Lovastatin inhibited APP expression especially in a vitamin-rich medium and DZA reduced APP only in cells grown in a vitamin-rich medium.

***Disscusion*:** Our first study has shown that concentrations of folate decreased and that of tHcy increased in CSF with age. Therefore, elevation of tHcy in the blood may indicate its elevation in the brain where tHcy can have many neurotoxic effects. Concentrations of Aβ (1-42) are lower in CSF from patients with dementia when compared to non-demented patients. The accumulation of Aβ is a long term process that is thought to start at a young age. We found no association between Aβ (1-42) and methylation markers, vitamins, or tHcy in the total group or in patients with dementia.

A relationship between the status or intake of B vitamins and dementia is not consistent. We found that, at baseline, MMA and SAM concentrations were related to some cognitive tests and, at the end of the study, both of the tested arms showed similar improvement in most of cognitive function tests. We suggest that sufficient B vitamins intake might be more effective in disease prevention rather than in disease treatment.

The effects of the one-carbon metabolites on APP processing and Aβ accumulation have been reported in many cell culture and animal model studies. Our findings showed that the presence of β- or γ-secretase inhibitors reverse the effects of SAM and SAH on APP expression suggesting that while SAH increased Aβ accumulation via enhancing the enzymatic activity of the secretases affecting the amyloidgenic pathway, SAM may decrease Aβ accumulation via inactivating these secretases. In addition, the contradicting effect of Hcy on the expression of C99 in the absence and the presence of β- or γ-secretase inhibitors, suggest that Hcy stimulates APP processing to C99 by enhancing both β- and γ-secretases. Moreover, our observations suggest that DZA may affect APP generation and/or processing via mechanisms not related to its role as an inhibitor of SAH hydrolase and the proposed protective effect of lovastatin against dementia seems to depend on methylation status. In-vivo studies are warranted.

ZUSAMMENFASSUNG

Hintergrund: Hyperhomocysteinämie (HHCY) ist ein Risikofaktor für neurodegenerative und psychische Erkrankungen, aber ein kausaler Zusammenhang bleibt bislang ungeklärt. Vitamin B12 und Folat spielen eine wichtige Rolle im Homocystein- (Hcy) Metabolismus. Obwohl mehrere Studien Hinweise für eine signifikant inverse Korrelation zwischen Plasma-Gesamt-Hcy-Konzentrationen und Plasma-Folat- und Vitamin B12-Konzentrationen gezeigt haben, bleibt die Bedeutung einer Vitamin B12-Behandlung auf die kognitive Leistung kontrovers. Die pathologischen Kennzeichen der Alzheimer-Krankheit (AD) bestehen aus Amyloidplaques und neurofibrillären Tangles in betroffenen Hirnregionen. Amyloid beta (Aβ) wird aus dem Amyloid-Precursor-Protein (APP) durch konsequentes Splitting durch β- und γ-Sekretasen gebildet. Ergebnisse von verschiedenen Studien deuten darauf hin, dass Veränderungen des Hcy-Kreislaufs mit einer erhöhten Akkumulierung von Aβ im Zusammenhang stehen und zur Amyloid-Symptomatik bei normalem Altern und bei AD beitragen können. Das Ziel unserer Studie ist die Untersuchung des Zusammenhangs zwischen Methylierungsmarkern und Markern der Neurodegeneration und die Untersuchung der Auswirkung einer B-Vitaminbehandlung auf die kognitive Leistung in älteren Menschen. Zusätzlich untersuchen wir, ob der Methylgruppenmetabolismus das APP-Level und/oder seine Prozessierung durch den amyloidogenen Kreislauf beeinflusst.

Materialien und Methoden: Der erste Teil dieser Studie schließt 182 Patienten mit verschiedenen neurodegenerativen Erkrankungen ein. Die Konzentrationen von Aβ(1-42) wurden in CSF-Proben gemessen. Der zweite Teil beinhaltet eine doppelblinde placebokontrollierte Studie an 69 Patienten, die zufällig verteilt entweder Vitamine oder Placebo erhielten. Die kognitive Funktion wurde durch Mini-Mental-Status-Tests (MMSE) und durch Strukturierte Interviews für die Diagnose einer Demenz vom Alzheimer Typ, der Multiinfarkt-Demenz und Demenzen anderer Ätiologie nach ICD-10 (SIDAM) untersucht. Konzentrationen von SAH und SAM wurden mittels Flüssigkeitschromatography-Tandem-Massenspektrometrie (LC-MS/MS) bestimmt.

Der dritte Teil der Studie beschäftigt sich mit Down Syndrom Fibroblasten als Zellkulturmodell mit erhöhtem Risiko für Neurodegeneration. Die Zellen wurden entweder in vitaminreichem oder in vitaminfreiem Medium kultiviert. Die Zellen wurden mit verschiedenen Konzentrationen von Hcy, S-Adenosylhomocystein (SAH), S-Adenosylmethionin (SAM), Lovastatin und 3-Deazaadenosin (DZA) behandelt und die Expression von APP und seiner Untereinheit (C99) wurde mittels Westernblotting in An- oder Abwesenheit von β- und γ-Sekretaseinhibitoren untersucht.

Ergebnisse: Die Studie hat gezeigt, dass das Altern mit höheren Konzentrationen von Hcy und SAH im CSF und niedrigeren Konzentrationen von CSF-Folat und der SAM/SAH Ratio assoziiert war. In allen Studiensubjekten konnten keine signifikanten Korrelationen zwischen Aβ(1-42)-Konzentrationen und Quartilen von SAM, SAH und Folat nachgewiesen werden.
Die zweite Studie zeigte, dass vor Behandlungsbeginn höhere Methylmalonsäure- (MMA) Konzentrationen mit niedrigeren Punktzahlen bei intellektuellen Fähigkeiten und höhere SAM-Konzentrationen mit höheren Orientierungspunktzahlen assoziiert waren. Beide Behandlungsarme zeigten am Ende der Therapie eine Verbesserung in den Gesamt-SIDAM-Punktzahlen.
Die dritte Studie zeigte, dass eine Behandlung mit SAM eine erniedrigte APP-Expression verursachte, während SAH unter vitaminfreien Bedingungen eine erhöhte APP-Expression und eine erniedrigte C99-Expression verursachte. Hcy verursachte im vitaminfreien Medium eine erhöhte C99-Proteinexpression. Inhibitoren von β- und γ-Sekretasen kehrten den Effekt von SAH und SAM auf die Proteinexpression von APP und C99 und den Effekt von Hcy auf das C99-Protein um. Lovastatin inhibierte vor allem im vitaminreichen Medium die APP-Expression, wohingegen DZA APP nur bei Zellen, die im vitaminreichen Medium wuchsen, reduzierte.
Diskussion: Unsere erste Studie zeigte, dass sich im Alter die CSF-Konzentrationen von Folat erniedrigen und die von Hcy erhöhen. Aus diesem Grund können Erhöhungen von tHcy im Blut darauf hinweisen, dass tHcy im Gehirn, wo es viele neurotoxische Effekte haben kann, ebenfalls erhöht ist. Konzentrationen von Aβ(1-42) sind im CSF bei Patienten mit Demenz niedriger als bei nicht-dementen Patienten. Die Akkumulation von Aβ ist ein Langzeitprozess, von dem man annimmt, dass er bereits im jungen Alter beginnt. Wir fanden keine Assoziation zwischen Aβ(1-42) und Methylierungsmarkern, Vitaminen oder Hcy in der Gesamtgruppe oder bei Patienten mit Demenz.
Ein Zusammenhang zwischen dem Status oder der Einnahme von B-Vitaminen und Demenz ist nicht widerspruchsfrei. Wir fanden, dass vor Behandlungsbeginn die MMA- und SAM-Konzentrationen mit einigen kognitiven Tests im Zusammenhang standen und dass am Ende der Studie beide untersuchte Arme eine ähnliche Verbesserung in den meisten kognitiven Funktionstests zeigten. Wir sind der ansicht, dass eine ausreichende B-Vitamineinnahme effektiver in der Prävention von Erkrankungen ist als bei der Behandlung von bereits bestehenden Erkrankungen.
Die Auswirkung von Einkohlenstoffmetaboliten auf die APP-Prozessierung und die Aβ-Akkumulierung konnte bereits in vielen Zellkultur- und Tiermodellstudien gezeigt werden.

Unsere Ergebnisse zeigten, dass die Präsenz von β- oder γ-Sekretaseinhibitoren die Wirkung von SAM und SAH auf die APP-Expression umkehrt. Dies suggeriert, dass SAH die Aβ-Akkumulierung über die Verstärkung der enzymatischen Aktivität der Sekretasen erhöht und somit den amyloidogenen Pathway beeinflusst. SAM hingegen kann die Aβ-Akkumulierung über eine Inaktivierung der Sekretasen vermindern. Zusätzlich weist die widersprüchliche Wirkung von Hcy auf die Expression von C99 in An- oder Abwesenheit von β- oder γ-Sekretaseinhibitoren darauf hin, dass Hcy die Prozessierung von APP zu C99 durch die Verstärkung der β- oder γ-Sekretasen stimuliert. Darüber hinaus deuten unsere Beobachtungen darauf hin, dass DZA die Bildung von APP und/oder die Prozessierung über Mechanismen beeinflusst, die nicht mit dessen Rolle als Inhibitor der SAH-Hydrolase in Zusammenhang steht. Der beabsichtigte protektive Effekt von Lovastatin gegen Demenz scheint vom Methylierungsstatus abhängig zu sein. In vivo-Studien sind gerechtfertigt.

1. INTRODUCTION

1-1. Dementia, cognitive dysfunction, and hyperhomocysteinemia:

Dementia is a clinical syndrome characterized by a progressive deterioration of cognitive skills that are severe enough to interfere with daily activities, including social and professional functioning. The World Health Organization estimated that in 2005, 0.379% of people worldwide had dementia, and that the prevalence would increase to 0.441% in 2015 and to 0.556% in 2030 (www.who.int/mental_health/neurology/neurodiso/en/index.html), mainly because of large increase in the old segment of the population. Two categories of dementia: Alzheimer's disease (AD) and vascular dementia (VaD) account for the vast majority of dementia cases and the coexistence of both categories may be the most common cause (Erkinjuntii and Sulkava, 1991). Alzheimer's disease, a degenerative disease and terminal illness, was first described by the German psychiatrist Alois Alzheimer in 1906. Generally it is diagnosed in people over 65 years of age and accounts for 50-70% of the total dementia prevalence (Small et al., 1997). Vascular dementia, the second most common type of dementia after AD, accounting for 15-30% of all dementia cases, is usually defined as an acquired intellectual deficit resulting from brain injury due to a cerebrovascular disorder (CVD) (Tatemichi et al., 1994). Mild cognitive impairment (MCI) is clinically defined as impairment in one or more cognitive domains (typically memory) and represents a transitional state between normal aging and mild dementia. Results from longitudinal studies indicate that subjects with MCI are likely to develop AD at an accelerated rate (Morris et al., 2001).

Age and years of education are among the most relevant risk factors for dementia, but in recent years the role of homocysteine (Hcy) as a risk factor for cognitive dysfunction, including AD and VaD has also been investigated. The link between hyperhomocysteinemia (HHcy) and neurological disorders was first described in patients suffering from mental retardation and cognitive dysfunction in addition to severely elevated plasma total Hcy (tHcy) (Mudd et al., 1985). Over the past two decades, numerous epidemiologic studies have confirmed the correlation between HHcy and dementia. In 2007, Obeid and colleagues reviewed evidence from 4 prospective follow-up studies and 7 retrospective cross sectional studies and concluded that there is good evidence to suggest that HHcy is positively related to cognitive dysfunction (Obeid et al., 2007b).

A causal role of tHcy in dementia is contro versial. On one hand, a number of studies have demonstrated a marked correlation between tHcy concentration and severity of cognitive decline. In a prospective follow-up study of 180 participants, over a mean of 2.3 years, results

indicated that at baseline participants with elevated tHcy concentrations had lower stroop scores than did participants with normal tHcy concentrations. At follow-up, stroop scores decreased by 22% in participants whose tHcy concentrations increased by 40% from baseline (Garcia et al., 2004a). Clarke and colleagues examined 164 patients with histological confirmed AD and found that those with baseline tHcy ≥14 μM had significantly more temporal lobe atrophy after 3 years than those with tHcy ≤11 μM, suggesting that elevated tHcy concentrations may causally related to the progression of the disease (Clarke et al., 1998). In addition, in the Framingham Study, a follow-up study of eight years, results showed that the risk of dementia increased by 40% for each 5 μM increase in plasma tHcy (Seshadri et al., 2002).

On the other hand, Miller and collegues suggested that elevated plasma tHcy concentrations are not a causative factor in dementia and AD but are only a marker for concomitant vascular disease, independently of cognitive status (Miller et al., 2002). Moreover, other studies showed no correlation between Hcy and cognitive functioning. For example, in the Rotterdam study, a follow-up study of 2.7 years, no association between baseline tHcy and decreases in the Mini-Mental Status Examination test (MMSE) score was found. However, the short follow-up time is a major limitation of this study (Kalmijn et al., 1999).

1-2. Pathophysiology of dementia and Alzheimer disease:

One of the most intriguing aspects of neurodegenerative diseases is protein misfolding and aggregation. One century ago, Alois Alzheimer described the typical neuropathological hallmarks of the disease takes his name; neuritic amyloid plaques and neurofibrillary tangles (NFTs). Amyloid in senile plaques is the product of cleavage of a much larger protein, the amyloid precursor protein (APP), by β- and γ-secretases (Hutton et al., 1998). The γ-secretases, in particular, appear to be responsible for generating amyloid peptides Aβ (1-42) and Aβ (1-40). Aβ (1-42) is 42 amino acids in length and has pathogenic importance, as it forms insoluble toxic fibrils and accumulates in senile plaques (Esler and Wolfe, 2001).

NFTs are mainly paired helical filaments (PHF) found in cell bodies and dendrites. The core protein of these filaments is tau, a microtubule-associated protein. Under physiological conditions, tau is a phosphoprotein, but it is phosphorylated to a higher degree under pathological conditions such as AD. Hyperphosphorylated tau tends to dissociate from microtubules, self-aggregates, and participates in NFTs formation (Alonso et al., 1996).

Selective neuronal loss, synaptic alterations, and neuroinflammation are typical features of neurodegenerative diseases. Neuronal loss occurs by programmed cell death or apoptosis

(Mattson, 2000). Many studies have suggested that protein misfolding and aggregation might be involved in neuronal apoptosis by different mechanisms. The most widely accepted mechanism is that misfolded aggregates have a neurotoxic activity that operates in different pathways. Extracellular aggregates, such as Aβ plaques, cause membrane disruption and depolarization mediated by ion-channel formation, resulting in alteration of ion homeostasis and dysregulation of cellular signal transduction, leading to cell death (Arispe et al., 1993). In addition, aggregates might induce oxidative stress by producing reactive oxygen species (ROS), resulting in protein and lipid peroxidation, elevation of intracellular calcium, and mitochondrial dysfunction (Behl et al., 1994).

Another proposed mechanism is that protein misfolding causes lack of its biological activity. Tau hyperphosphorylation and NFTs formation result in loss of tau main function in stabilizing axonal microtubules. This process causes impairment in microtubule-dependent axonal transport and cognitive decline (Vandebroek et al., 2006).

The brain inflammation hypothesis is another mechanism by which misfolded-aggregated proteins are involved in neuronal death. In this hypothesis, abnormal protein aggregates act as irritants and cause a chronic inflammatory reaction in the brain that leads to neuronal death and synaptic changes (Wyss-Coray and Mucke, 2002). The presence of early components of the complement cascade in association with senile plaques and NFTs of AD has been reported (Bergamaschini et al., 1999). Consequently, membrane attack complex (MAC) are formed and inserted into cell membranes, causing lysis and death of the neuronal cells. A recent study, including 691 cognitively intact community-dwelling participants, showed that higher production of interleukin 1 or tumor necrosis factor alpha (TNFα) by peripheral blood mononuclear cells may be a marker of future risk of AD in older individuals (Tan et al., 2007).

In addition to neuritic plaques and NFTs, many studies suggest that different microvascular disorders contribute to AD pathogenesis. Cerebral amyloid angiopathy (CAA) is now well understood in terms of deposition of Aβ in the vessel wall and degeneration of smooth muscle cells causing blood vessel rupture and hemodynamic change (Alonzo et al., 1998). It has been shown that macrophages in AD patients appear to shuttle Aβ from neurons to vessels causing fibrillar Aβ production which contributes to CAA (Zaghi et al., 2009).

Aβ is cleared physiologically across the blood brain barrier (BBB) by low-density lipoprotein receptor-related protein-1. The vascular deposition of Aβ is likely to be related to the lack of clearance (Weller et al., 1998). Irrespective of the mechanism of CAA, it is likely that the characteristic vascular deposition in AD compromises BBB function and promotes chronic

hypoperfusion (De Jong et al., 1997). In addition, the phenomenon characterized by selective degeneration of the endothelium in capillary profiles was observed in virtually all Aβ-laden cortical lobes of almost all AD patients (Kalaria and Hedera, 1996).

White matter lesions, microvascular brain injury marker, are found to be present in more than 60% of AD patients (Barber et al., 1999). Plasma Aβ concentration is independently associated with extent of white matter hyperintensity in subjects with AD (Gurol et al., 2006). These findings support the role of Aβ deposition in the vascular phenomenon along with microangiopathy and its implication in the potency of the brain microvasculature in AD.

Pathogenic mechanisms of dementia are summerized in Figure 1.

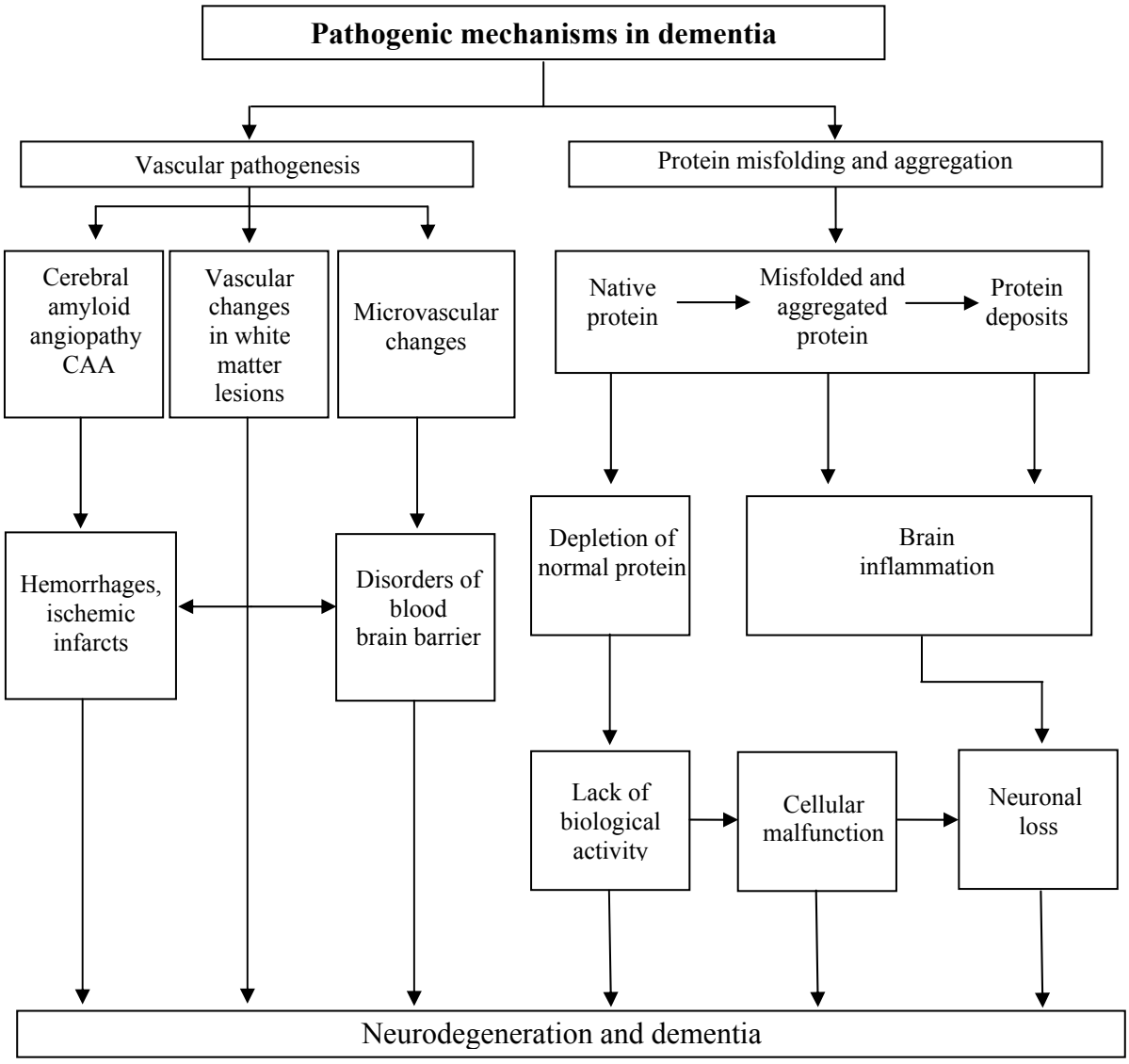


Figure 1: Pathophysiology of dementia

1-3. Risk factors for dementia:

1-3-1. Genetic risk factors:

Early-onset AD, before the age of 60 to 65 years, is uncommon and make up about 6% to 7% of all AD cases. About 7% of early-onset cases are familial (Nussbaum and Ellis, 2003). Familial Alzheimer's disease (FAD) is inherited in an autosomal dominant manner. Three genes have been identified to be involved in FAD: APP gene on the chromosome 21, presenilin-1 (PS-1) gene on chromosome 14, and presenilin-2 (PS-2) gene on chromosome 1.

Mutation in one of these genes can shift the cleavage of APP to favour the γ-secretase site and increased production of the toxic Aβ (1-42) peptide over the shorter, less toxic Aβ (1-40) peptide (Borchelt et al., 1996). The DNA from 34 families with FAD (obtained from all over France) was analyzed for causative mutations (Campion et al., 1999). Half of the patients had mutation in PS-1, whereas approximately 16% of the families had mutation in APP gene. PS-2 mutation was not found, and the genes responsible for the remaining cases were unknown.

The human ApoE gene is located on chromosom 19 and has three different alleles: ε4 allele, ε2 allele, and ε3 allele. ApoE is a protein with roles in lipid metabolism and tissue repair. Its primary site of biosynthesis is the liver, but the second major site of synthesis is the brain. For late-onset AD, apolipoprotein E-ε4 (ApoE-ε4) has been confirmed as a genetic risk factor in large population-based studies (Hsiung and Sadovnick, 2007). The frequency of ApoE-ε4 in patients with AD has been found to be greater than that in age-matched controls (Strittmatter et al., 1993). It has been reported that ApoE-ε4 facilitates the deposition of Aβ in the brain (Bogdanovic et al., 2002) and increases the rate of atrophy in the brain (Wahlund et al., 1999). In addition, there is evidence that cognitive function is lower in individuals possessing the ApoE-ε4 allele in combination with a second factor that may be related to brain pathology. For example, greater cognitive deficits have been observed in ApoE-ε4 carrying older adults who also suffered from peripheral vascular disease or atherosclerosis (Haan et al., 1999).

1-3-2. Non-genetic risk factors:

Demographic factors:

Age is considered the most important risk factor for the development of AD. The prevalence as well as the incidence of AD increases with advancing age and the occurrence doubles every five years after 65 up to 90 years of age, and remains stable after the age of 90 years (Fratiglioni et al., 2000).

Furthermore, female gender has been found to be significantly at higher risk of AD than male gender (Andersen et al., 1999).

In addition, different longitudinal cohort studies showed that the risk of AD is increased among people with low education level. For example, participants who had more than 15 years of education, had a reduced risk of AD when compared with those who had less than 12 years of education (Kukull et al., 2002).

Vascular risk factors:

Many studies have suggested a strong association between different vascular risk factors and AD. For example hypertension may cause AD through causing cerebrovascular lesions. The results of a follow-up study of 2 years showed that in elderly people with isolated systolic hypertension, antihypertensive treatment was associated with a lower incidence of dementia (Forette et al., 1998).

Heart disease is linked with the ApoE-ε4 allele and is known to be a risk factor for AD. In line with this, the Rotterdam Study observed a 1.8 fold increased risk for AD in patients with arterial fibrillation (Breteler, 2000).

Smoking is an important cardiovascular and cerebrovascular risk factor and could therefore increase the risk of AD. For example, current smoking was found to be strongly related to a higher risk of AD (Luchsinger et al., 2005).

Casserly and Topol have summarized the common risk factors for AD and atherosclerosis including hypercholesterolemia, hypertension, diabetes mellitus, systemic inflammation, increased fat intake, obesity, and hyperhomocysteinemia (Casserly and Topol, 2004).

The role of HHcy as an independent risk factor for neurodegeneration has been established in many studies and this will be discussed separately.

Figure 2 summarizes the risk factors for dementia.

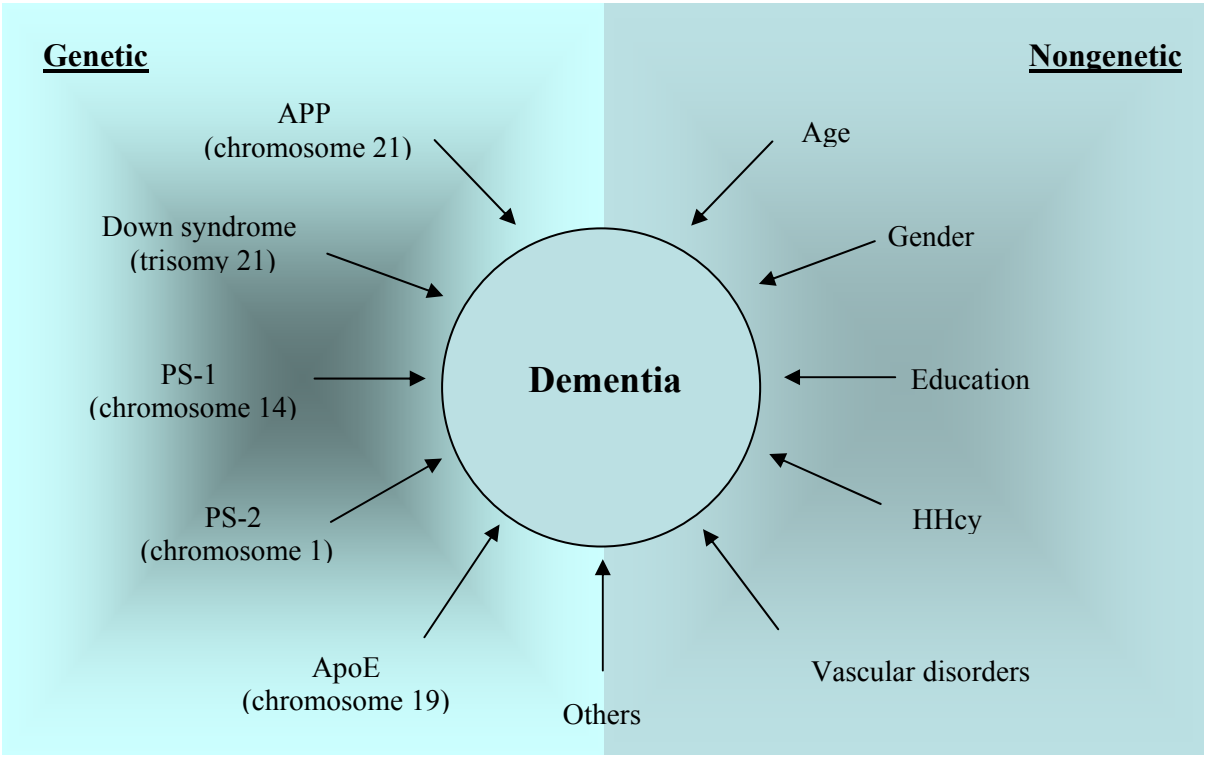


Figure 2: Risk factors for dementia

1-4. Hyperhomocysteinemia as a risk factor for dementia:

HHcy and homocystinuria due to abnormal Hcy metabolism were first described by Carson and Neil in 1962 (CARSON and Neill, 1962). In 1969, McCully proposed that HHcy might be a vascular risk factor. This proposition was based on autopsy evidence in two children with HHcy and homocystinuria who had widespread atherosclerotic changes (McCully, 1969). McCully's observation has been confirmed later by a numerous epidemiological studies showing that HHcy is associated with an increased risk of vascular diseases, including cardiovascular, peripheral vascular, and cerebral vascular diseases. Elevated tHcy is also a recognized risk factor for vascular dementia. Moreover, a series of studies proved that elevated tHcy is strongly associated with an increased risk of AD and cognitive dysfunction. A number of these studies are summarized in Table 1.

In a population sample of the Framingham cohort, individuals with tHcy concentrations ≥14 μM were found to have a two fold increased risk of AD after adjustment for age (Seshadri et al., 2002). Other studies have reported higher tHcy concentrations in elderly demented patients with a clinical diagnosis of AD or VaD compared to concentrations found in control subjects (Quadri et al., 2004). In addition, a relation between elevated plasma tHcy and decreased cognitive performance has been observed in the normal aging population (Haan et

al., 2007). According to the Hordaland Homocysteine Study, a prospective study of 7031 subjects who constituted 2 age groups at baseline (41–42 and 65–67 years), the risk of memory deficit increased according to quartiles of tHcy both at baseline and at follow-up period of 6 years. A decline in tHcy or an increase in folate was associated with a higher memory test scores and vice versa (Nurk et al., 2005).

Some studies have suggested that HHcy is not a causative factor in dementia and AD but it is only a marker for concomitant vascular disease, independently of cognitive status (Mooijaart et al., 2005). However, results from other investigations including the Framingham study argue against this proposition suggesting different mechanisms by which HHcy causing cognitive dysfunction (Ravaglia et al., 2005).

On the other hand, in a prospective analysis of elderly participants in the Rotterdam Study, no significant association between elevated tHcy concentrations and cognitive decline was found (Kalmijn et al., 1999). In this study, however, a plasma concentration of tHcy was assayed in non-fasting samples and the average study follow-up of 2.7 years was probably insufficient to detect significant changes in the cognitive score. Similar observation by the Washington Heights-Inwood Columbia Aging Study showed nonsignificant association between the highest tHcy quartile and risk for AD after adjustment for age and sex (Luchsinger et al., 2004). Insufficient statistical power and methodological issues related to prolonged time between sample collection and processing are major limitations of this study.

It has been hypothesized that the magnitude of association between tHcy concentrations and cognitive performance increases with advancing age. An association between concentrations of tHcy and cognitive performance for subjects in the seventh, but not the sixth, decade of age has been reported (Duthie et al., 2002). Wright and collegues found an inverse association between tHcy concentrations and MMSE scores among individuals aged ≥ 65 years, but not among individuals aged 40 to 64 years (Wright et al., 2004).

Data on the association between elevated tHcy concentrations and MCI are inconsistent. Mean plasma Hcy concentrations were higher in elderly subjects with MCI than in normal non-demented elderly subjects (17.6 ± 7.4 vs. 15.7 ± 4.8 μM) (Kim et al., 2007). In addition, the lowest folate tertile and HHcy (≥14.6 μM) showed a significant association with MCI (adjusted OR=3.1 and 2.6 respectively) (Quadri et al., 2005). In contrast, plasma Hcy concentrations did not differ between normal and MCI elderly subjects, as assessed by the Mayo Clinic criteria in a group of Polish subjects (Religa et al., 2003).

Table 1: Selected studies investigating the relation between Hcy and dementia

Study	Study population	Study design	Tests	Results
(McCaddon et al., 1998)	60 patients with DMS-III-R criteria for AD	Case-control	CAMDEX	tHcy levels inversely related to scores of cognitive testing
(Clarke et al., 1998)	164 demented patients aged > 55 y	Case-control	CERAD CAMDEX MMSE	tHcy >14 μM associated with AD (OR: 4:5, 95% CI: 2.2-9.2). In a 3 years follow-up, radiological evidence of disease progression was greater among patients with higher tHcy concentrations at entry.
(Leblhuber et al., 2000)	31 patients aged 74.8±8.8 y (19 with AD, 12 with VaD) +19 age matched normal controls	Case-control	MMSE	Inverse correlation between the degree of cognitive impairment and Hcy concentrations (r=-0.43) and a correlation between MMSE and folic acid (r=0.37) were found.
(Seshadri et al., 2002) Framingham Study	1092 dementia free patients in Framingham cohort aged 68-97 y	Prospective cohort (median follow-up 8 years)	DSM IV criteria, MMSE Clinical Dementia Rating Scale NINCDS-ADRDA	AD risk for subjects with tHcy >14 μM was nearly doubled.
(Nurk et al., 2005) The Hordaland Homocysteine Study	2189 subjects aged 65-67 y	Prospective cohort (median follow-up 6 years)	Kendrick Object Learning Test (Memory performance)	At base line, subjects with memory deficit had higher concentrations of tHcy and lower concentrations of folate comparing with those without memory deficit. The risk of memory deficit increased according to quintiles of tHcy both at baseline and at follow-up. A decline in tHcy or an increase in folate over a 6-year period was associated with a higher memory test score and vice versa.
(Ravaglia et al., 2005)	816 subjects (mean age= 74 y)	Prospective cohort (median follow-up 4 years)	MMSE	Subjects with tHcy >15 μM had hazard ratio (HR) 2.08 for dementia and 2.11 for AD. Subject with folate < 11.8 nM had HR 1.87 for dementia and 1.98 for AD.
(Garcia et al., 2004a)	180 normal community-dwelling people aged ≥65 y	Prospective cohort (median follow-up 2.3 years)	Stroop Mattis DRS CVLT	tHcy levels were significantly correlated with the stroop scores both at base-line and follow-up. Increasements greater than 40% in tHcy levels from base-line to follow-up were associated with a 22% reduction in the stroop scores.

Table 1: Continued

Study	Study population	Study design	Tests	Results
(Haan et al., 2007)	1779 subjects aged 60-101 y	Prospective cohort (median follow-up 4.5 years)	Neuropsychological test battery MRI DSM NINCDS-ADRDA	High tHcy concentrations were associated with a greater risk of dementia or CIND (HR: 2.39). Plasma vitamin B12 modified the association between tHcy and the outcome. The rates of dementia or CIND associated with tHcy were significantly higher (HR: 1.61, P=0.04) for those in the lowest tertile of vitamin B12 and significantly lower (HR: 0.94, P=0.015) for those in the highest tertile of vitamin B12 comparing to those in the middle terttile.
(Wright et al., 2004) The Northen Manhaten Study	2871 strock free subjects older than 40 y Three ethnic populations	Retrospective cross-sectional study	MMSE	tHcy was associated with lower mean MMSE scores with a drop of 3.5 points among those over 65 y and almost 2.0 points for those under 65 y. Subjects over 65 y, with tHcy >15 μM had 1.5 MMSE points lower than those with tHcy < 10 μM. This correlation was not found in subjects less than 65 y old.
(Stewart et al., 2002)	238 African-Caribbean adults aged 55-75 y	Retrospective cross-sectional study	CERAD MMSE	Raised tHcy (highest quartile >13.85 μM) was significantly associated with cognitive impairment (OR= 2.86)
(Quadri et al., 2004)	228 patients (81 with MCI, 74 with AD, 18 with VaD and 55 non demented)	Retrospective cross-sectional study	MMSE	Subjects with low folate status had significantly higher adjusted OR for mild cognitive impairment (OR: 3.1; 95%CI: 1.2, 8.1) and dementia (3.8; 1.3, 11.2). HHcy was significantly associated with dementia (adjusted OR: 4.3; 1.3, 14.7) and AD (adjusted OR: 3.7; 1.1, 13.1).
(Ravaglia et al., 2003)	650 community-dwelling people (mean age 73 y) with normal cognitive function in Conselice Study	Retrospective cross-sectional study	MMSE	Inverse relation between OR of tHcy >15 μM and MMSE scores

AD: Alzheimer's disease, CERAD: Consortium to Establish a Registry for Alzheimer's disease, DSM: Diagnostic and Statistical Manual of Mental Disorders, MMSE: Mini-Mental Status Examination, NINCDS-ADRDA. National Institute of Neurological and Communication Disorders and Stroke/Alzheimer's Disease and Related Disorders Association, MRI: Magnetic Resonance Imaging, Mattis DRS: Mattis Dementia Rating Scale, CVLT: California Verbal Learning Test, CAMDEX: Cambridge Examination for Mental Disorders of the Elderly, CIND: Cognitive impairment non Demented, CAMCOG: Cognitive and self-contained part of the Cambridge Examination for Mental Disorders of the Elderly, CI: Confidence Intervals, OR: odds ratio, HR: hazard ratio.

Substantial evidence is accumulating suggesting that HHcy is a risk factor for stroke. In a prospective nested case-control study, tHcy concentrations were significantly higher in stroke cases than controls and a graded increase in the relative risk of stroke in the second (10.3-12.49 μM) , third (12.5-15.39 μM), and fourth (≥15.4 μM) quartiles of tHcy concentrations comparing to the first (<10.3 μM) was reported (Perry et al., 1995). In a follow-up study of 5 years, lowering of Hcy with folic acid and vitamins B6 and B12 did reduce the risk of stroke, but not stroke severity or disability (Saposnik et al., 2009).

In addition to dementia, Parkinson's disease (PD) is primarily a degenerative disorder of the central nervous system (CNS) with average age of onset of 55 to 66 years. It is considered the second most common neurodegenerative disease next to AD and characterized by bradykinesia, rigidity, tremor, and postural instability. Long-term treatment of levodopa in patients with PD is known to cause elevation in plasma concentrations of tHcy (Muller et al., 1999). A positive correlation between HHcy and unified Parkinson's disease rating scale (UPDRS) motor section was reported (Ozer et al., 2006). In this study, the subgroup with concentrations of Hcy >14 μM had a significantly poorer performance in frontal and memory tests comparing to individuals with concentrations of Hcy <14 μM . In addition, markers of neurodegeneration, APP and alpha-synuclein, were found to be related to markers of methylation, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), in patients with PD. Better cognitive function was related to higher SAM/SAH ratio (Obeid et al., 2009).

1-5. Homocysteine metabolism and regulation:

Hcy is a thiol-containing amino acid derived primarily from proteins of animal origin (methionine). Hcy is produced entirely from the methylation cycle as it is totally absent from any dietary source (Finkelstein, 1998). Hcy occurs in blood in three forms, 80-90 % of it is bound to albumin through disulfide bonds, 1-2% occurs as a free thiol, whereas the remain occur in non-protein bound forms, predominantly with cysteine or as the Hcy dimer, homocystine (Refsum et al., 2004).

Methionine is converted to SAM in Mg^{+2}, K^{+2}, and adenosine triphosphate (ATP) requiring reaction catalyzed by methionine-S-adenosyltransferase, which occurs in most tissues. SAM donates its methyl group to a large variety of substrates resulting in different compounds, which are vitally essential in many important physiological functions, for example nucleic acid (DNA and RNA), phospholipids, myelin, catecholamins, neurotransmitters, and proteins. When methyl groups are transferred from SAM, SAH is formed, which is then hydrolyzed in

a reversible reaction by the widely distributed SAH hydrolase to release the adenosine and results in the formation of Hcy. The reverse reaction forming SAH is favored over that forming Hcy (Finkelstein, 1998). Hcy is metabolized further in two pathways: remethylation and transulfuration (Figure 3).

The re-methylation pathway is comprised of two biochemical pathways; the first reaction occurs in all tissues and is directly dependent on the enzyme methionine synthase (MS) that requires B12 as a co-factor and 5-methyltetrahydrofolate (5-MTHF) as a substrate. This reaction is indirectly regulated by the activity of 5-MTHFR, which in turn uses flavin adenine dinucleotide (FAD; a biologically active form of vitamin B2) as a co-factor (Guenther et al., 1999). In the alternative re-methylation route, which is mainly expressed in the liver and kidney, betaine is used as a methyl donor by the enzyme betaine-homocysteine methyltransferase (BHMT).

Under conditions in which excess of methionine is present or if cysteine synthesis is required, Hcy enters the transsulfuration pathway by which it condenses with serine to form cystathionine (Cys). This reaction is catalysed by cystathionine β-synthase (CβS), an enzyme that depends on vitamin B6 as a cofactor. Cys is broken down by another vitamin B6 requiring enzyme, γ-cystathionase, forming α-oxobutyrate and cysteine, which is a precursor of glutathione, the major cellular redox buffer (Finkelstein, 1990).

Hcy metabolism in the brain undergoes the same steps as described before with two major exceptions. Firstly, BHMT is not expressed in neural tissue; therefore MS represents the only enzyme in the brain capable of Hcy remethylation to methionine (Chadwick et al., 2000). Secondly, Hcy catabolism by the transsulfuration pathway is probably incomplete and substantially blocked beyond the formation of Cys (Finkelstein, 1998).

Hcy in the brain can be either produced in the brain itself, or it can be imported from the plasma to the brain and vice versa probably via specific, bi-directional cellular transporters (Grieve et al., 1992).

Hcy metabolism is tightly controlled via several mechanisms. SAM is an inhibitor of BHMT (Finkelstein and Martin, 1984) and an activator of CβS (Finkelstein et al., 1975). When SAM concentration is low, the synthesis of 5-MTHFR will be activated whereas Cys synthesis will be suppressed thus stimulating the methionine synthesis from Hcy to deliver more SAM. Conversely, when SAM concentration is high, Hcy is diverted through the transsulfuration pathway because of the inhibition of 5-MTHFR synthesis. Thus, the ability of SAM to act as an enzymatic effector of Hcy metabolism provides a mechanism by which remethylation and transsulfuration pathways can be coordinated (Selhub, 1999).

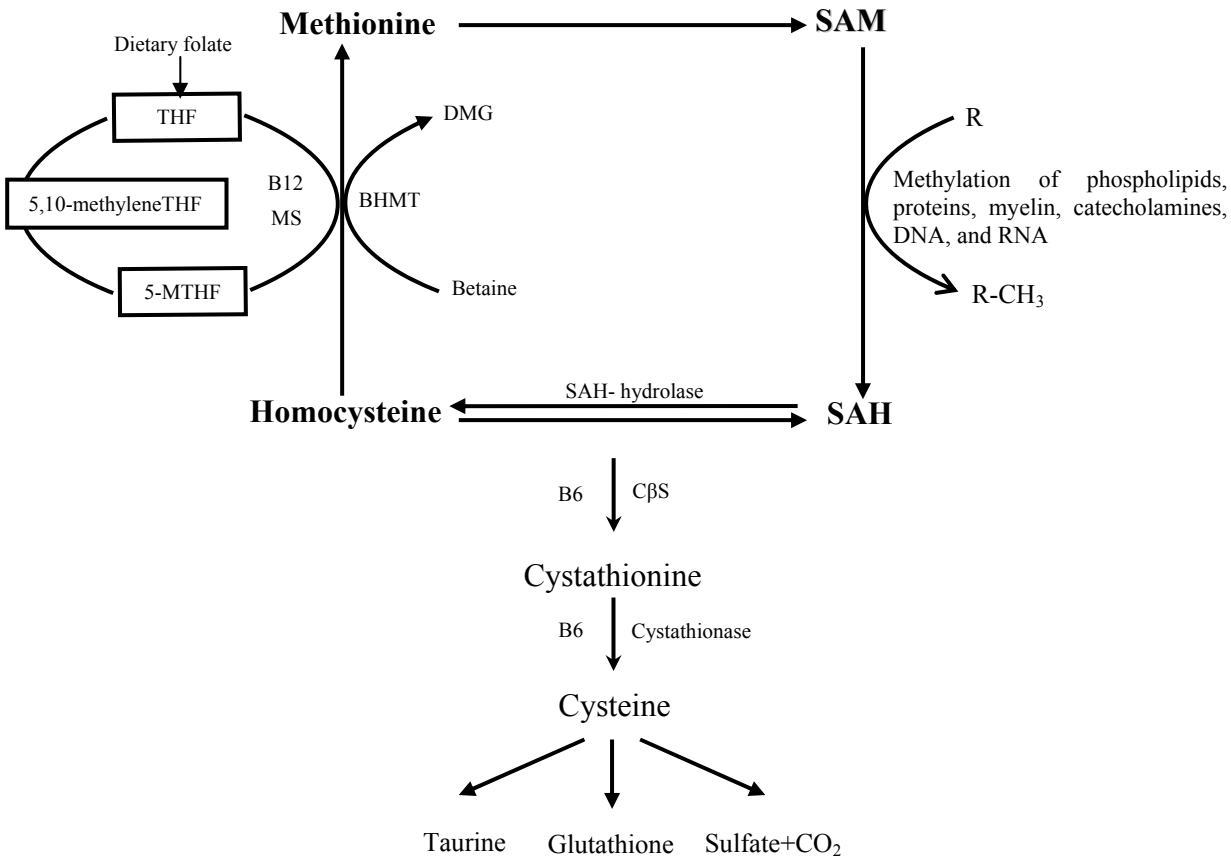


Figure 3: Homocysteine metabolism

CβS: cystathionine β synthase; MS: methionine synthase; 5;10-MTHFR: 5,10-methylene tetrahydrofolate reductase; SAM: *S*-adenosylmethionine; SAH: *S*-adenosylhomocysteine; THF: tetrahydrofolate; BHMT: betaine- homocysteine methyltransferases; DMG: dimethyl glycine.

1-6. Definition of fasting and post methionin loading hyper-homocysteinemia:

Fasting concentrations of tHcy:

Plasma tHcy values less than 12 μM are considered optimal. The range (12.0-15.0 μM) is termed as borderline. Hcy elevation is divided into moderate (15.0-30.0 μM), intermediate increase (30.0-100.0 μM), and severe HHcy when tHcy concentrations are above 100 μM (Welch and Loscalzo, 1998).

Hcy post-methionine loading (PML) concentrations:

The methionine loading test was originally introduced to detect the heterozygous for CβS deficiency, especially in affected families (Brenton et al., 1966). Currently, it is also used to detect mild abnormalities of methionine metabolism. Furthermore, several studies showed that post methionine loading test (PMLT) may identify HHcy in more than 50% of subjects who have not been diagnosed by screening with fasting plasma tHcy (van der et al., 2002). This test is performed by giving oral methionine as L-isomer (0.1 g/Kg body weight) after an over night fasting. tHcy is usually measured after a time interval of 4 or 6 hours. Subjects with PML values more than 38 μM are considered to have elevated PML tHcy concentrations (Graham et al., 1997).

1-7. Causes of hyperhomocysteinemia:

1.7.1. Acquired conditions:

B vitamins deficiency:

B vitamins: folic acid, vitamin B12 (cobalamin)(Cbl), and vitamin B6 affect Hcy concentrations through their roles as cofactors for the enzymes involved in methionine metabolism (Figure 3). Several studies have provided evidence for a significant inverse correlation between plasma concentrations of tHcy and plasma concentrations of folate and Cbl even within the normal range (Jacques et al., 2001;Stabler et al., 1988).

In a randomized, placebo-controlled pilot study of treatment with monthly injections of Cbl for 6 months, tHcy concentrations were significantly lower in the treatment group than in the placebo group (Garcia et al., 2004b). In the general population, oral folic acid supplementation have stronger effect on tHcy concentrations than does Cbl supplementation, therefore, the concentration of serum folate is a stronger predictor of HHcy than that of Cbl. However, co-administration of folic acid and Cbl results in a greater reduction in plasma tHcy concentrations than does folic acid alone (Ubbink et al., 1994).

Vitamin B6 is a cofactor in the metabolism of Hcy through the transsulfuration pathway. The impact of vitamin B6 treatment on tHcy concentrations is controversial. In one study, high-dose vitamin B6 therapy (100-200 mg/day) was reported to be effective in reducing cardiovascular events in patients with elevated tHcy concentrations (Wilcken and Wilcken, 1998); however, such treatment was not effective in reducing tHcy concentrations in adults with mild HHcy (van der et al., 2000; van der et al., 2000).

Renal function:

A normal kidney plays a major role in the clearance and metabolism of most of amino acids. The amount of urinary Hcy excretion in healthy subjects is 3.5–10 μM daily, that is about 0.1% of the total production of Hcy (Ueland and Refsum, 1989). The rest of Hcy is reabsorbed in the tubules and then oxidatively catabolized to carbon dioxide and sulfate in the kidney cells (House et al., 1998). Plasma Hcy concentrations are strongly correlated with glomerular filtration rate (GFR) and this association seems to be linear (Veldman et al., 2005). The precise mechanism by which GFR is related to plasma Hcy concentrations is not definitively established. One possible mechanism is the imbalance between the remethylation and transsulfuration of Hcy (Henning et al., 1999). Improved folate status has been shown to induce considered decrease in Hcy concentrations in dialysis patients (Sunder-Plassmann et al., 2000). In addition, tHcy concentrations decreased significantly by 51% in dialysis patients supplemented with a combination of folic acid, vitamin B12, and vitamin B6 for 4 weeks (Obeid et al., 2005). In the same study, twenty weeks after vitamin withdrawal, tHcy concentrations returned to values comparable to baseline.

Further factors that affect tHcy concentrations are summarized in Table 2.

Table 2: Physiologic and lifestyle determinants of plasma tHcy level

Factor	Effect	Reference
Physiologic factors		
Male sex	↑	(Silberberg et al., 1997)
Body weight	↑	(Nurk et al., 2004)
Postmenopausal women	↑	(Hak et al., 2000)
Old age	↑	(Herrmann et al., 1999)
Pregnancy	↓	(Walker et al., 1999b)
Lifestyle factors		
Vegetarian diet	↑	(Herrmann et al., 2001)
Alcohol consumption	↑	(Jacques et al., 2001)
Smoking	↑	(de et al., 2001)
Coffee consumption	↑	(Nygard et al., 1997)
Acute exercise	↑	(Herrmann et al., 2003)
Drugs		
Lipid lowering drugs	↑	(Desouza et al., 2002)
Nitrous oxide	↑	(Ermens et al., 1991)

1.7.2. Genetic polymorphisms:

Remethylation of Hcy to methionine requires the enzyme 5,10-methylene tetrahydrofolate reductase (5,10-MTHFR). A common MTHFR gene mutation (C-to-T substitution at codon 677 (C677T) results in increased thermolability and decreased activity of the MTHFR. People

homozygous for the C677T variant exhibit significantly reduced MTHFR activity and higher tHcy concentrations than heterozygous and normal subjects do (Frosst et al., 1995). There is evidence to suggest that the effects of this mutation on tHcy concentrations may be most evident when they are combined with low concenrations of folate (Jacques et al., 1996), and possibly also with low riboflavin concentrations, in the younger subject (Skoupy et al., 2002). The MTHFR C677T mutation has been described in patients with cardiovascular disease (Ma et al., 1996). In addition, this mutation has been found to increase the risk of stroke and vascular dementia (McIlroy et al., 2002).

CβS is the enzyme that catalyzes the conversion of Hcy to Cys, using vitamin B6 as a cofactor. Homozygosity for CβS is one of the rare autosomal recessive genetic disorders of Hcy metabolism that causes homocystinuria and severe HHcy (>100 μM)) (Kozich et al., 1995). Heterozygous CβS mutations occur in 0.5-1.5 % of the general population and can be associated with normal plasma concentrations of tHcy, but the PML tHcy may be elevated (Tsai et al., 1996).

CβS deficiency is characterized biochemically by severe HHcy, hypermethioninemia, and hypocysteinemia. Among the pathological manifestations of human CβS deficiency, which include mental retardation, ectopia lentis, and osteoporosis, vascular complications remain the major cause of morbidity and mortality in untreated CβS-deficient patients. The first therapy choice in CβS deficient patients is administration of supraphysiological doses of vitamin B6. About 44% of subjects with CβS deficiency respond to vitamin B6 therapy (Mudd et al., 1985). Certain mutations of CβS are vitamin B6 responsive, whereas others are non-responsive. Those patients can be treated with a combination of folic acid, hydroxycobalamin, and betaine to stimulate the remethylation of Hcy to methionine (Kraus et al., 1999).

1-8. Homocysteine as a risk factor for diseases:

By the early 1990s, elevated concentration of plasma tHcy was considered an independent risk factor for cardiovascular disease. In a prospective-cohort study following 5066 participants for over 4 years, HHcy was directly related to higher mortality (Vollset et al., 2001). In total, nearly 100 retrospective and prospective clinical studies link HHcy with increased risk of cardiovascular outcomes (Herrmann, 2001;Ford et al., 2002). According to a recent meta-analysis, a causal relationship between Hcy and cardiovascular disease is highly likely (Wald et al., 2002). This study estimated that lowering plasma tHcy by 3 μM would

reduce the risk of stroke by 24%, deep vein thrombosis by 25%, and ischemic heart disease by 16%.

Chronic heart failure (CHF) is a major public health problem causing considerable morbidity and mortality (Kannel and Belanger, 1991). A major clinical impact of HHcy in CHF was first reported in the Hordaland Homocysteine Study (Ueland et al., 2001). Although several studies have later demonstrated that HHcy is significantly associated with the incidence and the severity of CHF (Vasan et al., 2003;Herrmann et al., 2005a), the mechanisms by which HHcy involves in CHF pathology remain unclear.

Early abortion, pregnancy complications, and poor pregnancy outcomes have been linked to HHcy and low folate or vitamin B12 status. Preeclampsia is a pregnancy condition in which high blood pressure and protein in the urine develop after the 20th week. Findings in pregnant women have demonstrated that higher concentrations of tHcy and Cys and lower concentrations of folate were observed in the preeclamptic group than in the asymptomatic group (Herrmann et al., 2005c).

Osteoporosis is a disease of bone that leads to an increased risk of fracture at higher age. The underlying mechanism in all cases of osteoporosis is an imbalance between bone resorption and bone formation. Bone is resorbed by osteoclast cells; after which new bone is deposited by osteoblast cells (Raisz, 2005). Osteoporosis was found to be associated with homocysteinuria in children with cystathionine synthase deficiency (Tamburrini et al., 1984). The effect of Hcy on osteoclast activity has been tested in vitro. Higher tHcy concentrations induced increased activity of enzymes involved in bone remodeling and resorption (tartrate-resistant acid phosphatase and cathepsin K) suggesting enhanced bone resorption (Herrmann et al., 2005b). In an intervention study, combined treatment with folate and vitamin B12 resulted in a reduced risk of a hip fracture in elderly stroke patients (Sato et al., 2005).

1-9. Folates and vitamin B12:

1.9.1. Folates:

Folates are essential cofactors for one-carbon transfer reactions in most living organisms. Unlike plants and microorganisms, humans cannot synthesize folates de novo and must acquire them from the diet, primarily from plant foods. Folates occur almost exclusively in food as polyglutamyl derivatives of tetrahydrofolic acid and they can be easily oxidized and lose their biological effect by cooking or by preservation processes. Therefore, folates intake and bioavailability depend not only on the food folates content, but also on the preparation of

the food. Folic acid is a stable synthetic product that is frequently used in fortified foods or multivitamin tablets (Lucock, 2000). Most folates in food are polyglutamate derivatives and must be deconjugated to monoglutamate forms in the gut by enzymatic cleavage prior to absorption, which occurs in the jejunum. After extensive jejunal resection, the transport system can be induced in the ileum. The liver is the principal storage site of folates. Distribution of folates to other tissues occurs in a methylated form, which is reabsorbed from the bile into the serum (Mason et al., 1990). Most of folates are bound unspecifically to low-affinity proteins (albumin, transferrin, α_2-macroglobulin) or specifically to high-affinity folate binding proteins (Holm et al., 1992). The bioavailability of folates is approximately 50% from naturally occurring folates in food, whereas, the bioavailability of synthetic folic acid is about 85%. This difference should be considered for the current dietary reference intakes (DRIs) (Gregory, III, 1997)

Folate coenzymes participate in single carbon group transfers, including two reactions of particular importance. The first reaction is the synthesis of purines and pyrimidines that are incorporated into DNA and RNA. The failure of this reaction in case of folates deficiency ultimately leads to the characteristic megaloblastic anemia. The second reaction is the remethylation of Hcy to methionine (Lucock, 2000).

1.9.2. Vitamin B12:

Humans are unable to synthesize vitamin B12 (Cbl), therefore, food sources in human nutrition are limited to animal source products (Herbert, 1988). Cbl is sensitive to light and heat. Cooking meat or boiling milk for long time may considerably reduce their contents of Cbl. Cyanocobalamin is the form of the vitamin commonly used for fortification of foods and in nutritional supplements.

Cbl presented in human food binds usually with food proteins. Cbl high affinity protein (R-binder) and some enzymes in saliva stimulate releasing of Cbl (Seetharam, 1999). Arriving the stomach, the presence of food stimulates the secretion of intrinsic factor (IF), a glycoprotein produced by gastric parietal cells. IF, in the neutral pH of the duodenum, displaces the R-binder with the aid of pancreatic enzymes. The IF-Cbl complex is taken up by a specific IF receptor in the terminal ileum. In the enterocytes, Cbl is transferred to another binding protein transcobalamin (TC); a non-glycoprotein produced by many cell types and is known for its role in delivering Cbl into all DNA-synthesing cells. Holotranscobalamin (holoTC), a TC-Cbl complex, enters the cell through a specific receptor, the holoTC receptor (Seetharam and Li, 2000). In the cell, after dissociation of the TC-Cbl complex in the

lysosomes, Cbl is transformed to the coenzymes methyl- and adenosyl-Cbl (Ado-Cbl) in the cytoplasm and in the mitochondria, respectively. Ado-Cbl is required for the conversion of methylmalonic acid (MMA) to succinyl-Co A and methylcobalamin is the cofactor for methionine synthase that mediates the conversion of Hcy to methionine (Carmel, 2000).
Cbl in blood is either bound to the TC which account for only 6-20% of the total serum Cbl concentrations, or to haptocorrin that binds approximately 80% of the total serum Cbl and thought to be a circulating storage protein that reflects the liver stores (Seetharam, 1999).

1.9.3. B vitamins and cognitive function:

Folate, cobalamin, and vitamin B6 play important roles as co-factors for specific enzymes in one-carbon metabolism. Epidemiologic evidence linking low B vitamins status or intake with decline in cognitive function in elderly people was first described by Goodwin and collegues (Goodwin et al., 1983). This linking has been later confirmed by other studies (Table 3).
Since cell replication is of low order in the adult brain (Eriksson et al., 1998), purine and pyrimidine synthesis are minimal. Therefore, the principal mechanism whereby B vitamins influence brain function is probably through the methylation cycle.
The hypomethylation hypothesis assumes that, the disruption of the one-carbon metabolism interferes with the synthesis of SAM. Methylation reactions in the brain include synthesis of neurotransmitters and methylation of phospholipids and myelin. Hence, it is obvious that a disturbance in SAM availability in the CNS may impact cognitive functioning as well as on other psychological statuses (Brosnan et al., 2004;Calvaresi and Bryan, 2001).
The Hcy hypothesis suggests that cognitive deterioration, associated with low status of B vitamins, is caused by increased concentrations of tHcy. The mechanisms by which HHcy causes neurodegenerative events are different and this will be disscused in the next paragraph.
Ellinson and colleagues have reviewed a total of six studies and found that the relationship between serum folate and vitamin B12 status with cognitive impairment in older adults was heterogeneous. Only one case control study reported decreasing cognitive scores with increasing serum vitamin B12 (Ellinson et al., 2004). Further research to clarify mechanisms linking vitamin B12 and folate deficiency to cognitive impairment before supplementation are recommended.

Table 3: Selected studies investigating the relation between B vitamins and cognitive function in elderly people

Study	Study population	Study design	Tests	Results
(Li et al., 2008)	191 subjects mean age= 72.2y	Cross-sectional	MMSE	Inverse relationship between MMSE scores and plasma tHcy concentrations (p=0.024). This relation became non-significant after adjustment for plasma concentrations of vitamin B12 and folate (p=0.136).
(Kim et al., 2008)	518 elderly (≥ 65 y)	Prospective cohort (follow-up 2.4 years)	MMSE Clinical dementia rating scale Instrumental activities of daily living scale	Only baseline lower folate concentrations predicted incident dementia. The onset of dementia was significantly associated with low folate status, higher tHcy concentrations, and weaker increase in vitamin B12 concentrations over the follow-up period.
(Morris et al., 2007)	1302 subjects (≥ 60 y)	Retrospective	Digit symbol coding subtest of the Wechsler adult intelligence scale II	Low versus normal vitamin B12 status was associated with cognitive impairment (OR: 2.5). In the group with low B12, serum folate>59 nM; as opposed to ≤ 59 nM, OR for cognitive impairment was 2.6.
(Kado et al., 2005)	499 community-dwelling, aged (70-79 y)	Cross-sectional longitudinal cohort (follow-up 7 years)	Test of multiple cognitive domains	Subjects with lowest folate status had worst cognitive decline.
(Duthie et al., 2002)	2 cohorts, the first one (ABC 36) contained 150 subjects aged ≥ 63 y and the second one (ABC 21) contained 150 subjects aged ≥ 78 y	Cross-sectional	MMSE RPM AVLT DS BD	MMSE, RPM, AVLT, DS, and BD scores were higher in ABC36 comparing to ABC21. In the ABC21, folate, vitamin B12, and MMSE score were positively correlated and homocysteine was negatively correlated with RPM, DS, and BD scores. In the ABC36, folate was positively correlated with BD score.
(Clarke et al., 1998)	164 subjects with confirmed AD age≥ 55 y	Case-control	MMSE CAMDEX CAMCOG	Concentrations of folate and vitamin B12 were lower in patients than in controls. Corresponding OR for the lower third with the upper third was 3.3 for folate and 4.3 of vitamin B12.

AD: Alzheimer's disease, MMSE: Mini-Mental Status Examination, CAMDEX: Cambridge Examination for Mental Disorders of the Elderly, OR: Odds Ratio, CAMCOG: Cambridge Examination for Mental Disorders. RPM: Raven's Progressive Matrices, AVLT: Auditory Verbal Learning Test, DS: digit symbol subtest, BD: block design subtest.

1-10. Pathomechanisms of homocysteine neurotoxicity:

1-10-1. Direct effects of homocysteine on the nervous system:

Protein N-homocysteinylation:

Hcy is metabolized to the cyclic thioester Hcy-thiolactone when remethylation or transsulfuration reactions are impaired by genetic alterations of enzymes involved in Hcy metabolism (Jakubowski, 2004). Hcy-thiolactone is a reactive intermediate that causes N-homocysteinylation through the formation of amide bonds with ε-amino groups of protein lysine residues. Recently, it has been shown that Hcy-thiolactone is detectable in human vascular endothelial cells, where Hcy is incorporated into proteins and the extent of thiolactone formation and protein-homocysteinylation depends on the extracellular concentrations of Hcy and folate (Jakubowski et al., 2000). Hcy-thiolactone is also known to be acutely toxic to the CNS in experimental animals. Hcy-thiolactone, injected intravenously in 1 dose into mice and rats as a possible radioprotectant in studies of tumor therapy, is extremely neurotoxic (Spence et al., 1995). For example, at 200 mg/kg Hcy-thiolactone, many mice developed immediate seizures followed by death within minutes. At 350 mg/kg, all animals developed seizures and died. At doses of 100 mg/kg or below, mice developed only mild somnolescence, and no long-term effects were observed within 30 day.

N-methyl-D-aspartate (NMDA) receptors and excitotoxcity:

The N-methyl-D-aspartate (NMDA) receptor, a glutamate receptor, has critical roles in synaptic transmission, plasticity, and excitotoxicity in the CNS. Hcy acts as a partial antagonist of the glycine site of the NMDA and therefore inhibits NMDA receptor-mediated activity. In addition, Hcy is also an agonist at the glutamate site of the NMDA receptor and is therefore a potential excitotoxin. Under conditions of normal glycine concentrations, the agonist action of Hcy would only occur if its level approached millimolar concentrations. In case of elevated glycine levels, such as ischemia or head injury, a relatively low Hcy concentrations can stimulate NMDA receptors (Lipton et al., 1997).

NMDA receptor stimulation leads to transient rise in intracellular calcium concentrations, which in turn activate calcium-activated proteases and cause potentially damaging effects on neuronal cells. At their worst, these effects can include cell death by apoptosis, and it is possible that they can have localized function limited to dendrites (Gilman and Mattson, 2002). In cultured neurons, Hcy treatment increases cytosolic calcium and treatment with

calcium-channel blockers attenuates this increase (Ho et al., 2002). Hcy potentiates glutamate neurotoxicity, and the toxicity of Hcy itself is attenuated by antagonists of metabotropic glutamate receptors (Kruman et al., 2000).

1-10-2. Indirect effects of homocysteine on the nervous system:

Homocysteine and hypomethylation:

Methylation reactions in the brain, including synthesis of phospholipids, nucleic acids, neurotransmitters, regulation of gene expression, and modification of protein function can not occur without the proper function of methyltransferases. Under conditions of Hcy excess, reversibility of the hydrolytic reaction causes accumulation of SAH, a potent inhibitor of methyltransferases (Mudd et al., 1995). Thus, it has been suggested that one of the basic biochemical mechanisms of HHcy toxicity is a hypomethylation through SAH accumulation (Hultberg et al., 2000).

Disturbed SAH, SAM, or their ratio have been reported in patients with dementia or AD (Bottiglieri et al., 1990;Kennedy et al., 2004). In addition, depressed patients with raised tHcy concentrations were found to have significantly lower concentrations of CSF-folate, CSF-SAM and all CSF monoamine metabolites (Bottiglieri et al., 2000a).

Myelin forms the myelin sheath around the axon of the neuron. It is essential for the proper function of the nervous system. Methylation is essential for myelin formation and function since 19% of myelin basic proteins are methylated (Baldwin and Carnegie, 1971). Subacute combined degeneration of the cord and brain, occured as a result of demyelination, was reported in children with inborn errors of the one-carbon transfer pathway (Surtees et al., 1997).

The alternative pathway for the methylation of Hcy to form methionine is catalyzed by BHMT. Betaine, which is derived from dietary choline by the action of choline dehydrogenase, is the methyl group donor in this reaction and supplemental oral betaine can lower plasma tHcy concentrations (Steenge et al., 2003). Twelve patients (median age 6 years), with neurologic disease due to remethylation defects, showed to have an isolated brain choline deficiency probably secondary to depletion of labile methyl groups produced by the transmethylation pathway (Debray et al., 2008).

Hypomethylation of DNA and altered gene expression play critical roles in neuronal damage. DNA damage, caused by elevated Hcy, triggers a cell death pathway involving poly ADP-

ribose polymerase (PARP) and the tumor suppressor protein p53, leading to mitochondrial dysfunction and activation of all death proteases called caspases (Kruman et al., 2000).

Homocysteine and oxidative stress:

Oxidative stress is an important event that has been implicated in the pathogenesis of neurodegenerative diseases. Many studies have elucidated Hcy neurotoxicity through generating a status of oxidative stress. It has been reported that HHcy leads directly to a rise in the formation of superoxide and hydrogen peroxide by Hcy autoxidation or by cysteine autoxidation (Hogg, 1999) and that excitotoxicity indirectly provokes intracellulary increase of free radical production. Similar consequences such as apoptosis and increased cytosolic calcium and ROS were observed following direct addition of Hcy to cultured neuronal cells (Kruman et al., 2000).

Cytoplasmic calcium influx, a consequence of both excitotoxicity and oxidative stress, is associated with Hcy exposure (Zieminska et al., 2003). Calcium can induce cell death by causing metabolic aberrations in the mitochondria and modulating gene transcription in the nucleus (Ermak and Davies, 2002).

The generation of ROS in normal cells, including neurons, is under tight homeostatic control. Excessive ROS can lead to the destruction of cellular components including lipids, protein, and DNA, and ultimately cell death via apoptosis or necrosis (Kannan and Jain, 2000). Folate deprivation and consequently elevated Hcy concentrations have been found to increase ROS and induce mitochondrial degeneration in cultured cortical neurons (Ho et al., 2003).

An interesting recent development is a proposed relation ship between HHcy and asymmetric dimethylarginine (ADMA), an inhibitor of endothelial nitric oxide (NO) synthase, and thereby reduces the synthesis of NO. A significant negative correlation was detected between the plasma concentrations of NO and both plasma concentrations of Hcy and ADMA in subjects with AD (Selley et al., 2002).

Homocysteine and tau protein:

Tau protein occurs predominantly in neuronal axons, where it binds to microtubules and regulates their length and activity. The biological activity of tau in stabilizing microtubules correlates inversely with its degree of phosphorylation. Tau hyperphosphorylation disrupts the normal colocalization of it with microtubules, leading to the probability of tau-tau interaction and the formation of PHF, and their subsequent aggregation into NFTs, a major hallmark of AD (Stoothoff and Johnson, 2005) (Figure 4).

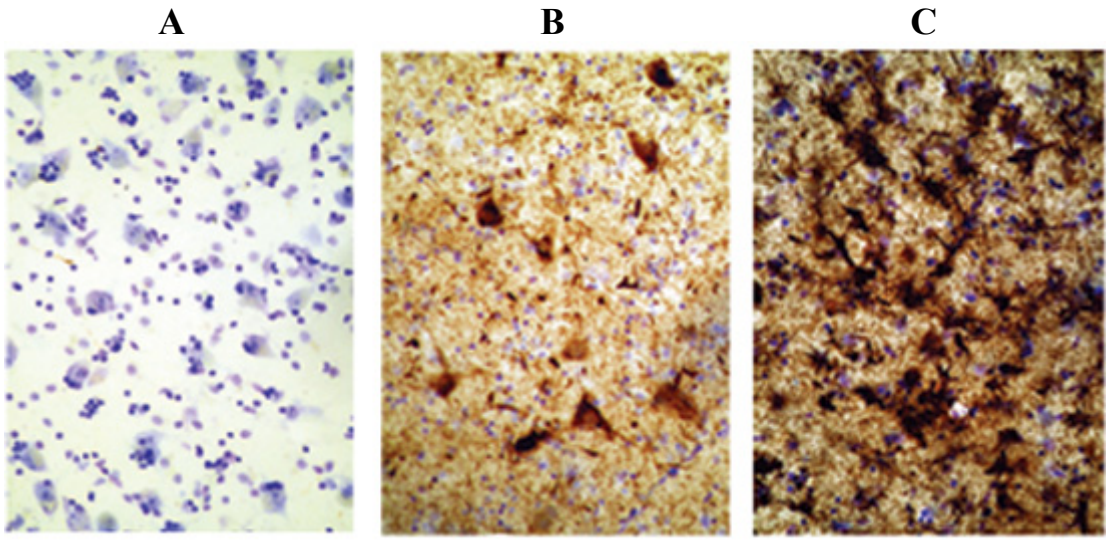


Figure 4: Tau immunostained microscopic sections

A: Normal brain, B: Brain with mild dementia, C: Brain with severe dementia

(Image is adapted from: www.bu.edu/alzresearch/cste)

Phosphorylation of tau is regulated by a host of kinases and phosphatases. Glycogen synthase kinase 3β (GSK 3β), phosphatidyl nositol 3-kinase (PI3K), and mitogen-activated protein (MAP) kinases are among several kinases found to be activated by elevated Aβ status (Ferreira et al., 1997).

Protein phosphatase-2A (PP2A), which is found in association with microtubules in the brain, plays an important role in tau dephosphorylation (Wang et al., 2007). Studies suggest that a decrease in PP2A activity, rather than an increase in kinase activity, is crucial for the elevated levels of tau phosphorylation associated with NFTs formation (Planel et al., 2001). Methylation is considered an important process in regulating PP2A activity. PP2A methylation is controlled by a specific SAM-dependent methyltransferase (Lee and Stock, 1993). PP2A is a multimeric protein complex consisting of 3 subunits. A subunit acts as a scaffold for the association of catalytic C subunit and one of a variety of regulatory B subunits. B subunits control the substrate specificity and subcellular localization of PP2A (Sontag et al., 1999). The assembly of ABC heterotrimers proceeds as a multistep process with AC dimer methylation followed by binding of regulatory B subunit.

The importance of SAM in regulating PP2A activity provided the link between HHcy and tau hyperphosphorylation, NFTs formation, and neurodegeneration (Vafai and Stock, 2002). Sontag and colleagues showed that incubation of neuroblastoma cells with SAH results in reduced methylation of PP2A, thereby affecting PP2A substrate specificity and accumulation of both phosphorylated tau and APP isoforms. Conversely, incubation of N2a cells with SAM

enhances PP2A methylation and accumulation of dephosphorylated tau (Sontag et al., 2007). After treatment of rat primary neuron cultures with a folate antagonist, methotrexate, levels of phosphorylated tau and APP were increased and the neuronal viability was reduced. Interestingly, levels of methylated PP2A were reduced. These novel findings indicate that folate deficiency increases the characteristic AD pathology including tau phosphorylation presumably by PP2A inactivation (Yoon et al., 2007).

In addition, high plasma Hcy induced by vena caudalis injection for 2 weeks could induce AD-like tau hyperphosphorylation at multiple sites in rat brain hippocampus (Zhang et al., 2008). A simultaneous supplement of folate and vitamin B12 restored partially the plasma Hcy concentrations and thus significantly antagonized the Hcy-induced tau hyperphosphorylation and as well as PP2A inactivation. These results suggest that Hcy may be an upstream effector to induce AD-like tau hyperphosphorylation through inactivating PP2A.

1-11. Homocysteine and amyloid beta protein:

1-11-1. Amyloid precursor protein:

The human APP gene is located on chromosome 21 and spans approximately 240 Kb. APP is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. Its normal functions are not fully understood, but increasing evidence suggests that it plays important roles in regulating neuronal survival, neuritis outgrowth, and synaptic plasticity (Mattson, 1997).

APP consists of a membrane-spanning segment, a large extracellular N-terminal region, and a shorter intracellular carboxy terminus. The β-peptide consists of 42 amino acid stretch of APP that lies partially extracellular and partially within the plasma membrane. Proteolytic processing of APP occurs by three proteases (α-, β-, and γ-secretases) via two major pathways, amyloidogenic and non-amyloidogenic (Figure 5). The non-amyloidogenic cleavage occurs when α-secretase splits APP in the middle of the Aβ domain, precludes the release of the plaque-forming Aβ fragment and liberates sAPPα and C83, the latter being cleaved by γ-secretase to generate p3. β- and γ-secretases operate in the amyloidogenic cleavage thus liberating sAPPβ and C99 moieties. C99 is further cleaved within the transmembrane domain and two major forms of 40 and 42 amino acids with different C-termini [Aβ (1-40) and Aβ (1-42)] are generated (Suh and Checler, 2002).

Mutations in three genes, each inherited in an autosomal dominant manner, can cause early onset forms of AD. One gene encodes APP, and the other two genes encode PS-1 and PS-2. PS mutations promote neuronal degeneration by enhancing γ-secretase cleavage of APP, thereby increasing production and accumulation of neurotoxic Aβ (Haass and De, 1999).

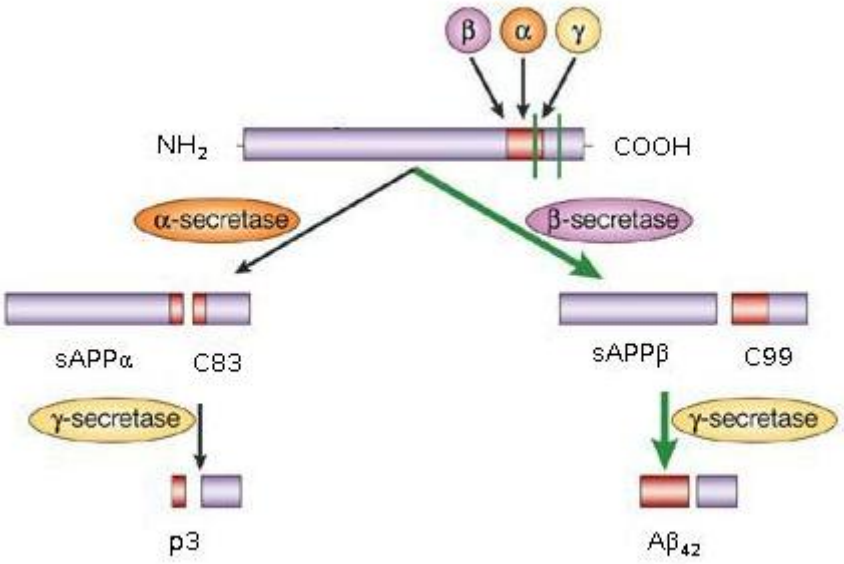


Figure 5: Proteolytic processing of APP

sAPPα: soluble APP after α-secretase cleavage; sAPPβ: soluble APP after β-secretase cleavage.

1-11-2. The role of amyloid cascade in Alzheimer's disease:

Studies of neuronal culture and mouse models identified that increased production, aggregation, and accumulation of Aβ initiate a cascade of events leading to neurotoxicity (Hardy and Selkoe, 2002). Exposure of cultured neurons to Aβ can induce apoptosis (Loo et al., 1993), and increase their vulnerability to death by oxidative stress and reduced energy availability that are known to occur in the brain during aging (Mattson and Pedersen, 1998).

Aβ can sensitize neurons to death by different mechanisms (Figure 6). One major mechanism is disrupting calcium homeostasis. Calcium is a key second messenger capable of mediating fundamental processes in neuronal function, synaptic transmission, plasticity, and the regulation of various metabolic pathways (Kater et al., 1988). Disturbed processing of APP may destabilize calcium homeostasis in neurons by increased production of Aβ (1-42) and by decreased sAPPα levels. Aβ induced-oxidative stress impairs membrane calcium pumps and enhances calcium influx through voltage-dependent channels and ionotropic glutamate receptor (Mattson and Chan, 2003). Additionally, Aβ was shown to promote calcium influx

by forming channels in cell membranes or by activating cell surface receptors coupled to calcium influx (Hartmann et al., 1993).

Another mechanism of Aβ neurotoxicity is via enhancing oxidative stress. Aβ enhances free radicals formation by binding metals such as zinc, copper, and iron (Huang et al., 1999). Aβ is believed to contact or insert into the neuronal and glial membrane bilayer and generate oxygen-dependent free radicals that can cause lipid peroxidation and protein oxidation (Varadarajan et al., 2000). Lipids are structural components of cell membranes and serve as intra- and intercellular signaling molecules. Aβ-induced lipid peroxidation impairs the function of ion-motive ATPases and glucose and glutamate transporters (Mattson, 1997).

Aβ can be neurotoxic by stimulating neuroinflammation including glial activation which plays an important role in the pathogenesis of AD (Calingasan et al., 2002). A significant, dose-dependent, increase in the production of different inflammatory mediators was obtained in cultures of microglia from rapid (mean of 2 h 55 min) autopsies of patients with AD and non-demented elderly controls after exposure to Aβ (Lue et al., 2001). This increase was significantly higher in AD compared with microglia from controls. Moreover, the production of neurotrophic factors such as basic fibroblast growth factor increased in astrocytes associated with Aβ deposits (Cummings et al., 1993).

Aβ might activate some intracellular signalling pathways thus enhancing the neurotoxicity. For example, Aβ was shown to induce sustained activation of the mitogen-activated protein kinases (MAPK) followed by hyperphosphorylation of tau protein in aging hippocampal neurons (Rapoport and Ferreira, 2000). Furthermore, in the same study, the blockage of MAPK activation using specific inhibitors prevented neurite degeneration in these cells. These results suggested that the MAPK signal transduction pathway could play a key role in Aβ-induced neuritis degeneration.

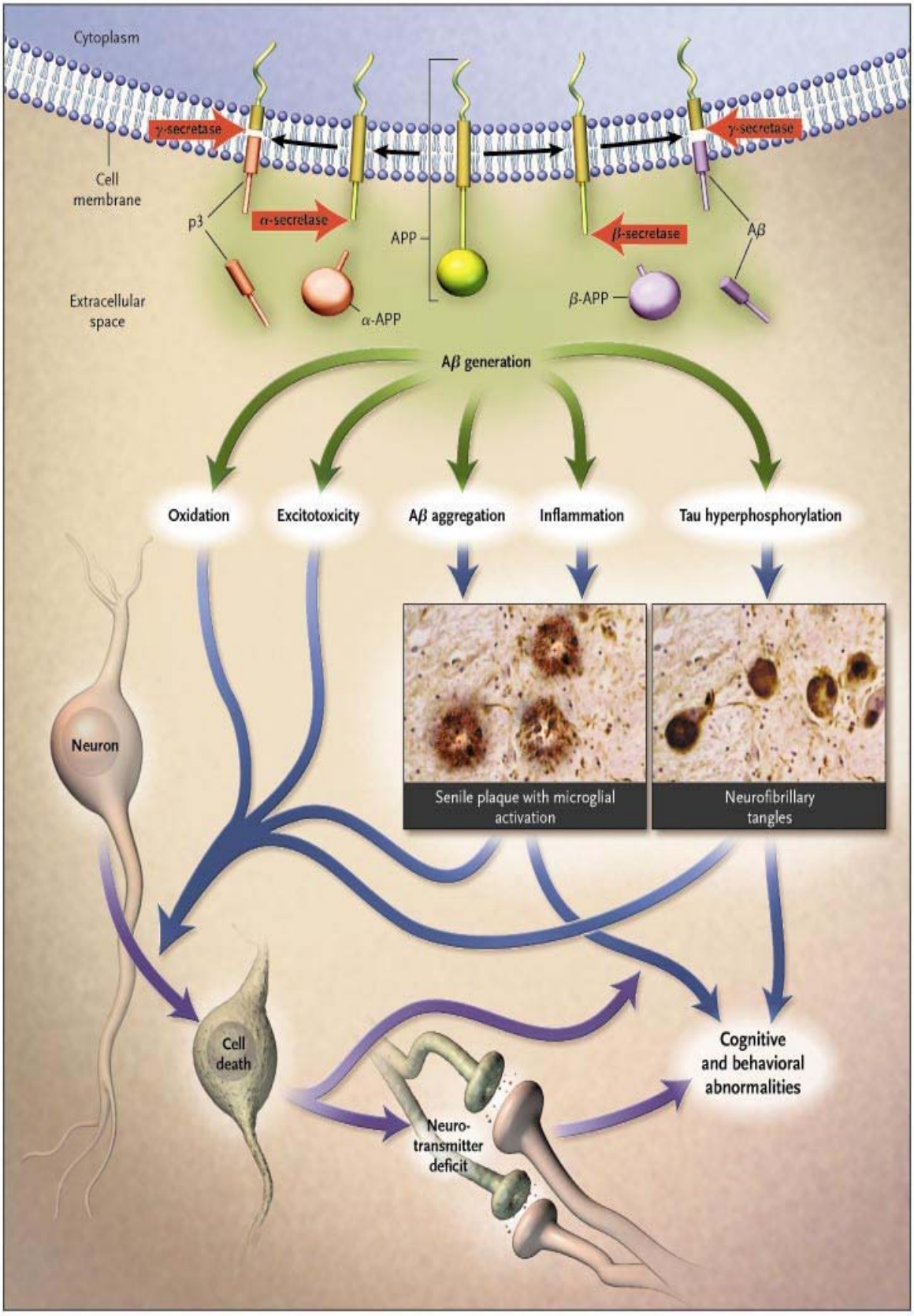


Figure 6: Amyloid cascade (Cummings, 2004)

1-11-3. Homocysteine and Aβ protein:

Different studies have reported the synergistic influence of Hcy and Aβ (Table 4).

Table 4: A: Selected studies on the association of Hcy and Aβ

Study	Subjects/cells	Results
(Irizarry et al., 2005)	465 patients (AD, MCI, PD, CAA, hICH)	Plasma concentrations of tHcy were positively correlated to that of Aβ (1-42) and Aβ (1-40) after adjusting for age.
(Flicker et al., 2004)	299 elderly mean age:78.9 y	Plasma concentrations of tHcy and Aβ (1-40) were positively correlated after adjusting for GFR. Doubled tHcy levels were associated with 24% increase in Aβ (1-40) levels.
(Sai et al., 2002)	PS deficient fibroblasts HEK 293 cells	Hcy increased Aβ levels by upregulating a presenilin-interacting endoplasmic reticulum stress protein (HERP) (Hcy-induced protein).
(Scarpa et al., 2003)	Human neuroblastoma	Deficient methylation upregulated PS gene function and Aβ generation.
(Hasegawa et al., 2005)	Cortical neurons	Neuron exposure to HA enhanced Aβ (1-42) accumulation inside the cells. This was prevented by γ-secreatase inhibitor.

B: Selected studies on the synergistic influence of Hcy and Aβ

Study	Cells	Results
(Ho et al., 2001)	SH-SY neuroblastoma	Hcy potentiated the effects of Aβ on cytosolic calcium and neuronal apoptosis.
(White et al., 2001)	Primary mouse neuronal cultures	Hcy generated high level of hydrogen peroxide in the presence of Cu and promoted Aβ/Cu mediated hydrogen peroxide production and neurotoxicity.
(Kruman et al., 2002)	Hippocampal and cortical cell cultures	Cell exposure to Aβ (1-42) in control medium for 48 hours caused 30% cell death versus 70% in methyl donor deficient medium via the impairement of DNA repair.
(Ho et al., 2003)	SH-SY neuroblastoma	Cells cultured in folate deprivation conditions showed (mean±SD: 64±5%) increase in ROS versus cells cultured in the presence of folate, while Aβ treatment in the presence of folate induced 34±4% increase. However, Aβ treatment of folate-deprived cells induced a 144±10% increase.

AD: Alzheimer's disease, MCI: mild cognitive impairment, PD: Parkinson disease, CAA: cerebral amyloid angiopathy, hICH: hypertensive intracerebral hemorrhage, PS: presenilin, HA: homocysteic acid, GFR: glomerular filtration rate.

1-12. Prevention of dementia:

Several studies confirmed the association between HHcy and cognitive function. The effect of lowering homocysteine concentrations on cognitive performance has been widely tested and inconclusive outcomes were obtained (Ellinson et al., 2004).

In a systematic review of randomized trials in a variety of doses, route of administration, and population, one of three trials of folic acid found a benefit in cognitive function in people with cognitive impairment and low baseline serum folate levels. Six trials with combinations of B vitamins concluded that the supplementation had no effect on cognitive function (Balk et al., 2007). However, major limitations of these studies and others preclude a firm conclusion that B vitamins supplementation has no effect on cognitive performance. Long-term duration, large participant's number, standardized cognitive tests that distinguish different cognitive domains, adjusting for demographic factors such as age and gender, doses and forms of the vitamins, and start of the treatments are all critical factors that have to be considered for judging available results or for future studies. Table 5 summarizes some of B vitamins intervention studies related to cognitive function.

3-deazaadenosine (DZA) is an adenosine analogue that reduces Hcy accumulation via the inhibition of SAH hydrolysis (Chiang et al., 1977). DZA exerts a number of biological properties, such as anti-human immunodeficiency virus (HIV) activity (Gordon et al., 2003) and immunosuppressive and anti-inflammatory effects (Fingerhuth et al., 2004). In addition, it has been shown that treatment with DZA provides neuroprotection in normal and ApoE deficient mice and in cultured neuronal cells deprived of folate and vitamin E and subjected to oxidative challenge (Tchantchou et al., 2004).

The mechanisms underlying DZA actions are generally thought to be mediated through the inhibition of cellular methylation reactions (Walker et al., 1999a). Dietary supplementation with DZA prevented both the increase in oxidative damage and impaired cognition of ApoE deficient mice following folate deprivation (Shea et al., 2004). Folate deprivation induced Hcy accumulation, while addition of DZA prevented both this increase and the increased generation of ROS that normally accompanies folate deprivation (Ho et al., 2003). These findings demonstrate that DZA can provide neuroprotection effect via mechanism related to its antioxidant properties.

Table 5: Studies investigating the possible relation between B vitamin supplementation and improvement of cognitive function

Study	population	Supplements and duration	Tests	Results
(Durga et al., 2007)	818 subjects	800 µg folic acid/placebo 3 years	Performance for memory Sensomotor speed Complex speed Word fluency Information processing speed	Improvement in domains of cognitive function that tend to decline with age (change in memory, Information processing speed, and sensomotor speed).
(Bryan et al., 2002)	211 women (aged 20-92y)	750 µg folic acid 15 µg vitamin B12 75 mg vitamin B6/placebo 5 weeks	Information processing speed Working memory Executive function Verbal ability Mood measures	Positive effects were seen mainly for memory performance but not for mood. Short time of supplementation, lack measures of B vitamins at baseline and testing effects of single nutrient are major limitations of this study.
(La et al., 1997)	137 healthy, dementia free elderly (aged 66-90 y)	vitamins A, E, B6, B12, C, and folate (Longitudinal study carried out over 6 years).	Wechsler memory scale, Rey-Osterrieth complex figure Shipley-Hartford abstraction test	Correlation between improved abstraction performance and high thiamine, riboflavin, niacin, and folate intakes was found.
(McMahon et al., 2006)	276 healthy, dementia free elderly (aged>65y)	1000 µg folic acid 500 µg vitamin B12 10 mg vitamin B6/placebo 2 years	MMSE Rey-verbal learning test Paragraph-recall test	Scores of cognition tests showed no significant differences after the supplementation. Relatively short duration, few numbers of participants, and the intact cognitive scores in placebo group are limitations of this study.
(Aisen et al., 2008)	340 subjects with mild to moderate AD	5 mg folate 1 mg vitamin B12 25 mg vitamin B6 Placebo / 18 months	Cognitive subscale of Alzheimer disease assessment scale (ADAS-cog)	No beneficial effects on cognitive performance was found.
(Eussen et al., 2006)	195 elderly subjects (aged>75y) with vitamin B12 deficiency	1 mg vitamin B12 0.4 mg folic acid 1 mg vitamin B12 / placebo 24 weeks	Neuropsychological test battery that included the domains of attention, construction, sensomotor speed, and memory	No improvement in the cognitive status was obtained.
(Lewerin et al., 2005)	195 elderly subjects (mean age=76y)	3 mg vitamin B6 0.8 mg folic acid 0.5 mg vitamin B12 Placebo / 4 months	Movement and postural-locomotor-manual test Battery of cognitive test	Vitamins supplementation caused no improvement in tests scores. Short duration, relatively small sample size, and insufficient dosage are the limitations of this study.

Another important therapeutic strategy in dementia is cholesterol-altering drugs. Individuals that consume diets high in cholesterol and those with increased cholesterol levels may be at increased risk of AD, whereas those who take cholesterol–lowering drugs (statins) may be at reduced risk (Puglielli et al., 2003;Wolozin et al., 2000). Accumulating data suggest that cholesterol may contribute directly into the amyloid cascade by promoting amyloidogenic processing of APP. In culture and animal model systems, statins and other cholesterol-lowering agents decrease Aβ levels and Aβ deposition (Fassbender et al., 2001), whereas high-cholesterol diets in APP transgenic mice increase Aβ deposition (Refolo et al., 2000). Lipid bilayer of plasma membranes, which are enriched in cholesterol and sphingomyelin, contain several molecular events implicated in AD pathogenesis, including signal transduction, initiation of apoptosis, and APP processing (Ehehalt et al., 2003). Cholesterol-modulating drugs could directly influence Aβ deposition and production through alteration in secretase activity or indirectly influence Aβ deposition by altering levels of factors such as ApoE (Wolozin et al., 2000).

2. STUDY AIMS

Study (1):

Markers of methylation are related to that of neurodegeneration:

HHcy is a risk factor for neurodegenerative and psychiatric disorders, but causal relationship remains elusive. Elevated concentrations of tHcy in plasma are associated with increased concentrations of tHcy and SAH in the brain. Vitamin B12 and folate play an important role for Hcy recycling to methionine, therefore participating in methyl group synthesis or metabolism. Methylation reactions in the brain include methylation of myelin, synthesis and catabolism of neurotransmitters, methylation of phospholipids, and methylation of proteins, RNA, and DNA.

Neurodegenerative diseases share a common feature, accumulation of misfolded proteins. Tau and Aβ are two examples of proteins that accumulate in brains of patients with dementia. Our study aimed at investigating the relationship between markers of neurodegeneration, and that of methylation in CSF samples from patients with neurological disorders.

Study (2):

B vitamins may improve cognitive function in elderly:

Age-related changes in absorption, metabolic pathways, and physiologic systems may result in older persons obtaining insufficient dietary intake of B vitamins. Poor folate status has been associated with depression and dementia in the elderly, and folate metabolism is linked to a variety of neurochemical processes. Vitamin B12 deficiency may induce neuropsychiatric complications in the absence of anaemia. Lower plasma concentration of vitamin B12 has been related to brain shrinkage and worse cognitive performance. Vitamin B6 status declines with age, and low blood concentrations of vitamin B6 have been associated with impaired cognitive function and AD. Results from intervention studies with B vitamins are not consistent. Several studies have suggested that early intervention is necessary to prevent cognitive decline. The aim of this part of our study was to investigate the effect of the therapeutic doses of B vitamins on cognitive performance in elderly subjects.

Study (3):

One-carbon cycle metabolites might affect APP level and/or its processing:

A prominent feature of AD brain is the widespread cerebral deposition of Aβ within senile plaques and in cerebral and meningeal blood vessel. Deposits of amyloid fibrils cause neuronal death and memory decline in patients with AD. Aβ is formed from APP by a consequent splitting by means of β- and γ-secretases. Results from different studies suggest that alterations in Hcy metabolism are related to increased accumulation of Aβ and may contribute to the amyloid pathology in normal aging and in AD. In the current study, we aimed at testing the hypothesis that the methyl group metabolism affects APP level and/or its processing through the amyloidgenic pathway. We utilized Down syndrome fibroblasts as a culture model with enhanced risk for neurodegeneration.

3. SUBJECTS AND METHODS:

3-1. Subjects and samples:

The first part of this study included 182 patients who were administered to the department of neurology/university hospital of the Saarland during April 2002 and April 2004. The study included 31 patients with multiple sclerosis, 19 patients with stroke, 31 patients with dementia (8 had Alzheimer disease), and 36 patients with peripheral neuropathy. Additionally, a control group consisted of 65 patients with various neurological diseases other than dementia, PD, polyneuropathy, multiple sclerosis, AD, and depression. Exclusion criteria included renal or liver dysfunction and alcoholism. Patients with depression, brain tumor, and PD were not included in this study.

CSF samples contaminated with peripheral blood or hemoglobin were excluded from the study. Non-fasting blood samples were collected from all patients. Serum and EDTA plasma were available. CSF samples were obtained during clinically indicated lumbar puncture. Blood and CSF samples were collected within 24 hours. Blood and CSF samples were directly centrifuged and several aliquots were prepared and stored at -70°C until analysis. Aliquots of the EDTA plasma and CSF were immediately deproteinized using perchloric acid (10%). The samples were stored at -70°C and were used for SAM and SAH assays.

The second part of the study was triple-blind placebo controlled. Sixty-nine patients (mean age 78 years, 62 females) agreed to participate and were randomly allocated to receive vitamins (1000 µg B12) or placebo. The placebo or the vitamins were injected subcutaneous three times a week for 3 weeks. Additionally, participants received oral placebo or vitamins (20 mg B6, 500 µg B12, and 2.5 mg folic acid) from day 0 until the end of the intervention that lasted 45 days. Placebo and vitamins capsules were similar in shape and color. The injections had different colors but they were injected into the patients in a way that he/she could not see the color.

During the first three weeks the patients were hospitalized (Geriatrische Rehabilitationsklinik St. Ingbert). At the end of the intervention, patients were interviewed at home and blood samples were collected. Sixty participants completed the whole treatment phase. Detailed study design is illustrated in Figure 7.

Blood samples were stored at 4°C for no longer than one hour then centrifuged at 2000 g, for 10 min, at room temperature. The plasma and serum were separated and stored at -70°C until analysis.

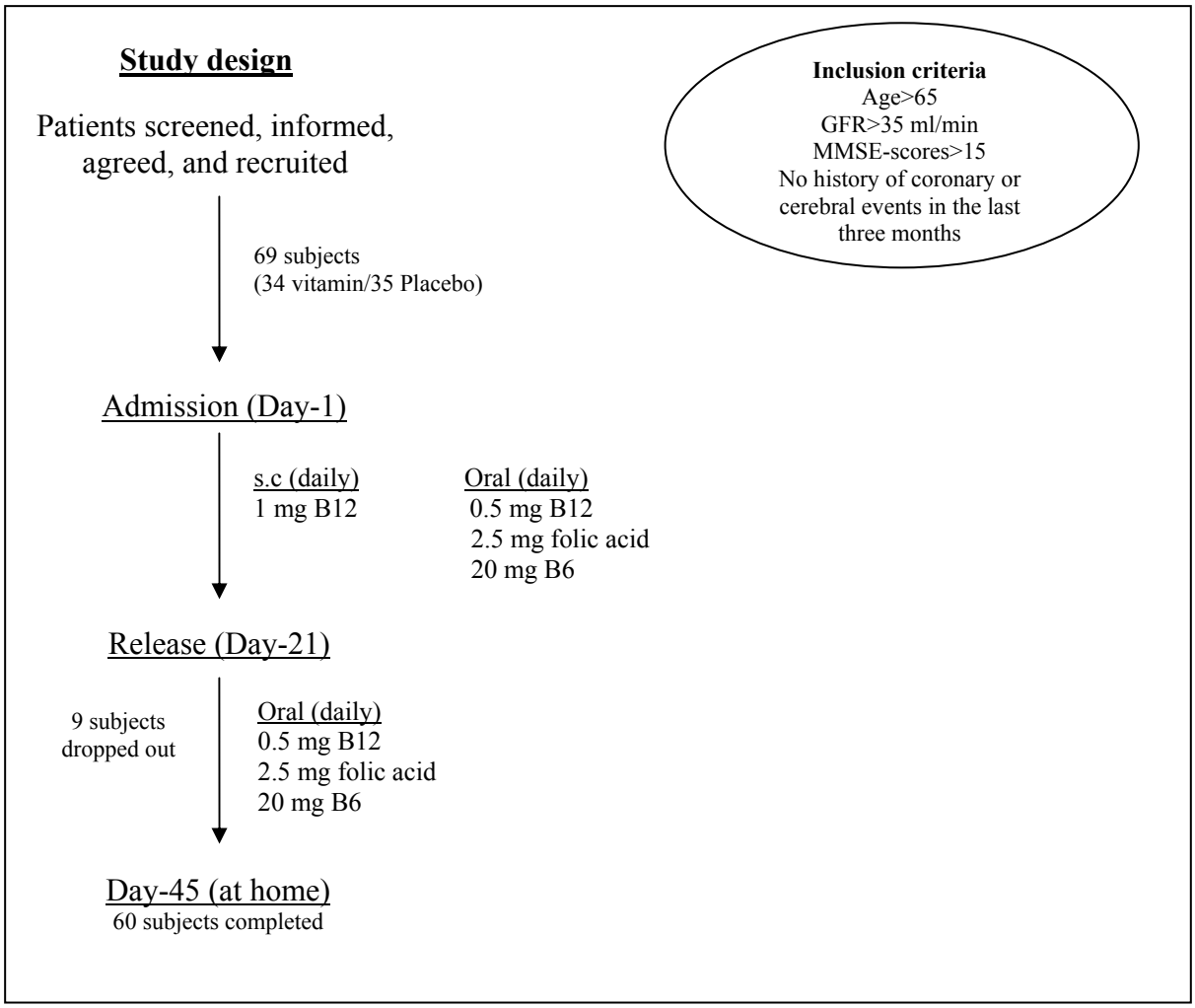


Figure 7: Study design

Cognitive function was investigated by using MMSE and the Structured Interview for Diagnosis of Dementia of Alzheimer Type, Multi-infarct Dementia and Dementia of other ethiology according to ICD-10 (SIDAM).

MMSE is a brief 30-point questionnaire test that is used to estimate the severity of cognitive impairment at a given point in time and to follow the course of cognitive changes in an individual over time, thus making it an effective way to document an individual's response to treatment. Any score over 27 (out of 30) is considered normal. A score between 20 and 26 indicates mild cognitive impairment, 10 and 19 moderate to severe cognitive impairment, and below 10 dementia.

SIDAM comprises a brief structured clinical interview, a range of cognitive tests (e.g. including the MMSE) which constitute a short neuropsychological battery and a section for clinical judgement. It is a brief (average of 28 min), practical and easily scored diagnostic instrument, which reliably separates subjects with Diagnostic and Statistical Manual of

Mental Disorders (DSM-III-R) and International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) dementia from those without such a disorder. Furthermore, the SIDAM score allows a detailed measurement of even low levels of cognitive impairment and provides quantification of severity grading of cognitive dysfunction. In our study, we utilized one part of the SIDAM, which tested the cognitive performance in a standardized form with 55 questions, including MMSE. Furthermore, this test evaluates orientation, short term and long-term memory, global memory, intellectual performance, verbal/mathematical ability, ability for three dimensional design, aphasia and apraxia as well as higher cortical functions.

The third part of the thesis included cell culture experiments that will be explained later.

3-2. Methods:

3-2-1. Homocysteine, cystathionine, and methylmalonic acid assays:

This assay was performed by Gas Chromatography Mass spectrometry (GCMS), utilizing a slightly modified protocol as previously described (Stabler et al., 1993).

GCMS is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. In order for a compound to be analysed by GCMS it must be sufficiently volatile and thermally stable. In addition, functionalized compounds may require chemical modification (derivatization) prior to analysis, to eliminate undesirable adsorption effects that would otherwise affect the quality of the data obtained. The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas (usually helium). The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions. Electron ionization is the most frequently used method for ion production. A beam of electrons ionize the sample molecules resulting in the loss of one electron. A molecule with one electron missing is called the molecular ion and is represented by M^+ (radical cation). Due to the large amount of energy imparted to the molecular ion it usually fragments producing further smaller ions with characteristic relative abundances that provide a 'fingerprint' for that molecular structure. As the ions continue

through the MS, they travel through an electromagnetic field that filters the ions based on mass. The range of masses that should be allowed through the filter is specified by the analyst. After the ions are separated they enter a detector, and the output is amplified to boost the signal. The detector sends information to a computer that records all of the data produced, converts the electrical impulses into visual and hard copy displays.

Materials:

Mass spectrometry with electron ionizer system.

Gas chromatography-column; HP 5MS (crosslinked 5% PH ME Siloxane). Column length 30 m, film thickness 0.25 µm, phase ratio 250, column ID 0.25 mm. (Cat-N: 19091S-433. Agilent Technologies®).

Poly-prep chromatography columns: Cat-N: 731-1550 (BIO-RAD®).

Anionic resin: AG MP-1M Resin (BIO-RAD®).

Derivatizing agent: N-methyl-butyldimethylsilyl-Tri-fluoroacetamide (MBDSTFA, Machery and Nagel®).

Reducing agent: 1,4-Dithiothreitol ($C_4H_{10}O_2S_2$, MW=154.2; DTT) from ROTH® (Cat-6908.1). Working concentration: 10 mg/mL (in 1 N; NaOH).

Internal standards working concentrations were 392 µM for DL-Hcy (3,3,3`,3`,4,4,4`,4`, D-8) (MW=276.36), 413.36 pmol/50 µL (2-amino-2-carboxythyl) (MW=226.3) for DL-Hcy = DL-Cys, and 1.635 nM for DL-MMA (MW=121.11).

Acetonitril, methanol, acetic acid, and chromatography water were all from MERCK®.

Sample preparation:

Hcy and Cys were simultaneously assayed in serum as following:

- Sample was prepared in 5 mL glass tube by adding 1 mL HPLC grade water + 250 µL serum + 15 µL DL-Hcy + 20 µL DL-Cys + 30 µL reducing agent.
- Mixture was incubated for 35 min at 45˚C for Hcy reduction.
- Sample was then loaded on the anionic resin containing 100 mg (dry weight) of an anion exchange resin, pre-equilibrated with 1 mL methanol, then 3 mL water (The anionic resin should be pre-washed with HCl (1N) and methanol and left to dry by heating 60˚C for 4 hours).
- The column was washed three times with 3 mL water and once with 3 mL methanol.
- Hcy and Cys were eluted with 1.1 mL of 0.4 N acetic acid/methanol solution.

- Eluates were dried at 45°C in an Eppendorf concentrator 5301(Eppendorf, Germany) for 2-3 hours.
- Dried eluates were derivatized by adding 30 µL of derivatizing agent and acetonitril (1:2 V/V), mixed and left in a microwave oven at 440 volt for 5 min.
- Samples were loaded into the GCMS for the final assay.

MMA quantification was separately performed as described for Hcy and Cys assay with the following modifications: we added in a 5 mL glass tube 1mL water + 250 µL serum + 15 µL DL-MMA. After transferring to chromatography column, sample was washed once with water and three times with mixture of acetic acid (0.01N) and methanol. Elution was performed using 1.1 mL of elution solution containing 10 mL Hcl (1N) and 90 mL acetic acid (4N).

Chromatography conditions:

- Column head pressure 53.3 Psi. Intial/Max temperature 80/310˚C with rise rate 15˚C/min.
- Major ion fragments (mass/charge) (m/z) for Hcy, Cys, and MMA were respectively 424/396, 366/625, 292/334 for the labeled molecules and 420/392/318, 362/621/303, 289/331/189/147 for the main molecules.
- The retention times were 13.4 min for Hcy, 17.2 min for Cys, and 9 min for MMA.
- Tested sample concentration was calculated as follows:

Conc. (nmol/L) = (Area under the curve of the sample/area under the curve of the internal standard) × correction factor

(Correction factor is 39.2 for Hcy, 600 for Cys and 4087.5 for MMA).

Assay quality control:

Day-to-day imprecision (CV%)		
	Serum	CSF
Hcy	<5% (at level 8.0 and 16.0 µM)	<10% (at level 0.30 µM)
Cys	<8% (at level 300 nM)	<10% (at level 60 nM)
MMA	<6% (at level 290 nM)	<6% (at level 290 nM)

Reference ranges:

Serum/Plasma: Hcy: 2-12 μM

Cys: 65-301 nM

MMA: 73-271 nM

CSF: Hcy: 0.007-0.020 μM (Blom et al., 1993)

Cys: 18-28 μM (Calvani, Jr. et al., 2001)

MMA: 0.14-0.73 μM (Stabler et al., 1991)

3-2-2. SAH and SAM assay:

Concentrations of SAH and SAM were measured by using modified Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) method according to Gellekink and collegues (Gellekink et al., 2005).

The mass spectrometer is an instrument designed to separate gas phase ions according to their m/z value. This is achieved by ionizing the sample and separating ions of differing masses and recording their relative abundance by measuring intensities of ion flux. MS/MS is the combination of two or more MS experiments. The aim is either to get structure information by fragmenting the ions isolated during the first experiments, or to achieve better selectivity and sensitivity for quantitive analysis.

Materials:

LC-MS/MS system; Waters 2795 Separation Module: E01SM9925N

Liquid chromatography-columns; SymmetryShield^{TM}RP18 Column 3.5 μm 2.1×100 mm and SymmetryShield^{TM}RP18 Guard Column 3.5 μm 2.1×10 mm were from Waters.

PH-Meter /INOLAB/.

Solid-phase extraction (SPE) columns (BondElut$^{®}$PBA).

$^{13}C_5$-SAH was from the laboratory of Gellekink group (Laboratory of Pediatrics and Neurology, Radboud University, Netherlands).

$^{2}H_3$-SAM, SAM, and SAH were from Sigma.

Ammonium acetate was from Fluka (for mass spectroscopy, MW 77.08).

HPLC grade water, Acetic acid, and Ammonia solution were from MERCK$^{®}$.

Mobile phase: Acqueous acetic acid (pH: 2.636).

Sample preparation:

Acidified samples were neutralized with a mix (v/v) 5:1 of 20 mM ammoniumacetat and 1M NH3 (pH= 7.4-7.5). Calibrators were prepared by diluting the stock solutions of SAH and SAM in ammonium acetate (pH 7.4) to concentrations of 0, 5, 15, 25, and 50 nM for SAH and 0, 50, 100, 150, and 300 nM for SAM. Controls were prepared by diluting the stock solutions of SAH and SAM in ammonium acetate (pH 7.4) to concentrations of 32 nM for SAH and 160 nM for SAM for the high control and to concentrations of 8 nM for SAH and 40 nM for SAM for the low control. 25 µL of internal standard (212.86 µM for $^{2}H_{3}$-SAM and 58.16 µM for $^{13}C_{5}$-SAH) were added to 500 µL of neutralized samples, calibrators, and controls. Sample cleanup was performed with solid-phase extraction (SPE) columns preconditioned at 500×g for 1 min by addition of five 1 mL volumes of mobile phase and five 1 mL volumes of 20 mM ammonium acetate (pH 7.4). SAM, SAH, and their internal standards bind to this column. Water-soluble impurities were removed by washing the column twice with 1 mL of ammonium acetate (pH 7.4), and SAM and SAH were eluted with 3×350 µL of the mobile phase (250×g for 2 min at 8°C). Eluates were stored at -20°C until analysis.

Samples measuring:

The samples were injected on an equilibrated (mobile phase) SymmetryShield^{TM}RP18 column [3.5 µm 2.1×100 mm] and SymmetryShield^{TM}RP18 Guard Column [3.5 µm 2.1×10 mm] pre-column. The sample injection volume was 20 µL. Column temperature was 25°C and the flow rate was 0.3 mL/min over a total run time of 3 min. The retention times were ~ 2 min and ~ 1 min for SAH and SAM respectively. Optimal multiple-reaction monitoring conditions were obtained for 4 channels: SAM (m/z 399→250), $^{2}H_{3}$-SAM (m/z 402→250), SAH (m/z 385→136), and $^{13}C_{5}$-SAH (m/z 3909→136). Data were acquired and processed by QuanLynx for Windows NT software. Calibration curves were obtained by plotting ratios of the peak area (calibrator/internal standard) against the concentration of the calibrator.

Reference ranges:

Plasma: SAH: 9.1-16.1 nM; SAM: 82.9-122.5 nM

CSF: SAH: 8.9-14.1 nM; SAM: 137-385 nM (Struys et al., 2000)

Assay quality control:

A plasma pool was collected in our laboratory and one aliquot was used each run with the samples. Estimates of interassay imprecision (CV) for SAH (at level 15.6 nM) and SAM (at

level 103 nM) were 7.9% and 4.9% respectively (n=29) and the intraassay CVs were 6.1% and 2.5% respectively (n=9).

3-2-3. Folate assay (ADVIA Centaur®):

This is a competitive chemiluminescence immunoassay that depends on releasing folate in serum sample from its binding proteins. The released folate compensates with advidin-bound folate on limited amount of folate-binding protein (labeled with biotin). The amount of folate in the tested sample correlates inversely with the resulted signal. The CVs for low and high controls were <5%.

Reference range (serum/plasma): 5-14.6 ng/mL

(CSF): 14-42 nM (Blom et al., 1993)

3-2-4. Vitamin B12 assay (ADVIA Centaur®):

This is a chemiluminescence immunoassay that depends on vitamin B12 releasing from its binding proteins in serum to compensate with acridiniumester labeled-vitamin B12 on a limited amount of a solid-phase bound intrinsic factor. The amount of the labeled B12 bound is proportional to the B12 in the tested sample.

The CVs for low and high controls were <5%.

Reference range (serum/plasma): 211-900 pg/mL

(CSF): 2.1-22.9 pM (Blom et al., 1993)

3-2-5. Holotranscobalamin assay:

This assay is based on Micro particle Enzyme Immunoassay technology and the reagents set was from Axis-Shield® (Norway). The first step is the immobilization of holoTC from the serum sample using mouse anti-human TC monoclonal antibodies bound to magnetic micro spheres. The next step is releasing Cbl content of the sequestered holoTC under reducing and alkaline conditions. Released Cbl is converted to the stable cyano form with potassium cyanide, and quantified in a competitive binding assay with (^{57}Co) Cbl as tracer. The competitive assay occurs on limited numbers of binding sites on intrinsic factor-bound to a solid support. Finally, the solid phase bound-Cbl is separated by centrifugation and the pellet is counted in a gamma counter. The concentration of Cbl in the sample is inversely correlated to the measured radioactivity and determined by interpolation from a calibration curve obtained using holoTC calibrators of known concentrations. Quality control sera are applied

by the manufacturer of the kit. CVs for this assay at 37 and 95 pmol/L were 6% and 8% respectively.

Reference range (serum/plasma): 40 pM as the lower cut point (Hvas A-M & Nexo E, 2005).

3-2-6. Vitamin B6 assay (Immundiagnostik®):

The first step in vitamin B6 determination includes the sample preparation with additional derivatization. This was done by addition of 200 µL tested sample (serum, calibrator or controls) in 1.5 mL Eppendorf tube with 50 µL precipitating reagent. The samples were well mixed and left for 10 min at 2-8°C, and then they were centrifuged at 14.000 rpm for 5 min. After centrifugation, 100 µL of the supernatant were taken apart and 250 µL of the derivatizing agent was added. The mixture was incubated for 20 min in a water bath at 60°C, then 15 min at 2-8°C, and finally samples were centrifuged for 5 min at 14000 rpm. Supernatants were transferred into glass vials and loaded on an auto sampler connected with a reverse phase HPLC with a fluorescence-detector. An external standard was used in this method. The quantification is performed by the calibrators; the concentration is calculated via integration of the peak areas.

Chromatographic conditions:

Column material: Bischoff Prontosil Eurobond, 5 µm (125 mm, 4 mm); flow rate: 1.0-1.5 mL/min; temperature: 30°C; injection volume: 20 µL; run time for each sample: 10 min; excitation/ emission wave lengths: 320/415 nm. The HPLC-system was provided by Agilent, (BIO-RAD, Germany). Mobile phase, calibrators, controls, precipitating reagent, and derivatization solution were provided with the kit. The CVs for low and high controls were <5%.

Reference range (serum/plasma): 4.3-17.5 ng/mL

3-2-7. Aβ assay:

The INNOTEST™ β-AMYLOID (1-42) is a solid-phase enzyme immunoassay in which the amyloid peptide is first captured by a monoclonal antibody (21F12) bound on the solid phase. CSF samples were added in 25 µL volumes and subsequently incubated with a biotinylated antibody (3D6). This antigene-antibody complex is then detected by a peroxidase-labeled streptavidine. After addition of substrate working solution, positive samples developed a blue color. The reaction is stopped by the addition of sulfuric acid which produces a yellow color. The absorbance is then measured within 15 minutes at 450 nm.

Reference range (Sjogren et al., 2001): >500 ng/L

3-3. Cell culture:

3-3-1. Equipments:

Analytical balance	METTLER TOLEDO, Switzerland
Bench Top Centrifuge	SIGMA 3 K12, Germany
CO_2 Tank	Air Liquide, Germany
Culture dishes 10 cm2	NUNC, Denmark
Eppendorf table centrifuge	Heraeus Pico 21, Germany
Eppendorf tubes	SARSTEDT, USA
Hypercassette™ Autoradiography	Amersham Biosciences, UK
Hyperfilm™ ECL	Amersham Biosciences, UK
Image Scanner	Amersham Biosciences, UK
Incubator	Heraeus Instruments, Germany
Inverted Microscope	Leica DMIL, Wetzlar GmbH, Germany
Liquid Nitrogen Tank	Thermolyne, USA
Microplate Reader	POLARstar OPTIMA, BMG Labtech, Germany
96 Microwell Plates	NUNC, Denmark
Mini-PROTEIN® 3 Cell	BIO-RAD, USA
Mini Trans-Blot®	BIO-RAD, USA
Neubauer-counting chamber	Cryos International, Denmark
Pipette	BIOHIT, Germany
Shaker	ROTAMAX 120, Heidolph GmbH, Germany
Sterile bench	Heraeus, Germany
Sterile filter	Sartorius
Sterile syringe	Ecoject
Tissue culture flasks 75 cm^2	SARSTEDT, USA
Tissue culture plate 24-well	SARSTEDT, USA
15 ml/ 50 ml Tubes	SARSTEDT, USA
Ultra centrifuge	OPTIMA LE-80K, Beckman Coulter, Germany
Ultrasonic bath	BRANSONIC 12, Germany

3-3-2. Chemicals and laboratory materials:

Reagent	Company / catalogue number
Albumin, BOVINE (BSA)	Sigma / A-7906
ALPHA MEDIUM (×1) (Normal Medium)	BIOCHROM AG / F0915
ALPHA MEDIUM (×1) mod. (Deficient Medium)	BIOCHROM AG / FZ0915
Amyloid Precursor Protein β-Secretase Inhibitor	Calbiochem / 171601
Ammonium Persulfate (APS)	Sigma / A-3678
Bicinchoninic Acid (BCA)	Sigma / B-9643
Bromophenol Blue	Merck / 11746
Complete^{TM} -Protease inhibitor	Roche / 11 836 153 001
Copper(II) sulfate CuSO4.5H_2O	Merck / 1.02790
3-Deazaadenosine	Sigma / D8296
Developer	Sigma / P7042
DL-Homocysteine	Sigma / H4628
Dulbecco's Phosphate Buffered Saline (×1)	PAA Laboratories / H21-002
ECL Plus Western Blotting Detection Reagents	Amersham / RPN2132 - RPN2133
Fixer	Sigma / P7167
Fetal Bovine Serum (FBS)	GIBCO / 10270-106
Glycine	ROTH / 3908
Glutamine	Sigma / G7513
Human beta amyloid 1-42 (ELISA KIT)	Wako / 296-64401
LactaAlbumin Enzymatic Hydrolysate	Sigma / L9010
Liquid Nitrogen	Air Liquide
2-Mercaptoethanol	Sigma / M-3148
Methanol	Merck / 1.06007
Mevinolin (Lovastatin)	Sigma / M 2147
Okadaic acid	Fluka / 75320
Penicillin/Streptomycine	BIOCHROM AG / A2213
Phenylmethylsulfonyl Fluoride	Sigma / P-7626
Precision Plus Protein	BIO-RAD / 161-0374
Polyvinylidene fluoride (PVDF) membranes	Roche / 03010040001
RIPA Lysis Buffer (×10)	UPSTATE / 20-188

Rotiphorese® Gel 40	ROTH / T802.1
S-adenosyl-L-homocysteine	Sigma / A9384
S-adenosyl –L-methionine chloride	Sigma / A7007
γ-Secretase Inhibitor XXI, Compound E	Calbiochem / 565790
Sodium Bicarbonate	Sigma / S6297
Sodium chloride	Saarland University Pharmacy
Sodium dodecyl sulfate (SDS)	SERVA / 20765
Sodium fluoride	Sigma / 71519
Sodium orthovandate	Sigma / 450243
Sodium pyrophosphate	Sigma / 71515
Tetramethylethelenediamine (TEMED)	Sigma / T-9281
Tris	Merck / 1.08382
Trypan blue stain 4%	Gibco / 15250-061
Trypsin/EDTA Solution (×10)	BIOCHROM AG / L2153
Tween 20	SERVA / 37470

3-3-3. Cell line and antibodies:

Cell line name: Detroit 532

Human Caucasian skin Down syndrome (DS fibroblasts)	ECACC, UK / 87032602

Western blot antibodies:

Mouse anti –Alzheimer precursor protein A4 (first antibody against APP)	Chemicon / MAB348
Polyclonal rabbit anti-mouse Immunoglobulins/HRP (secondary antibody against APP)	DakoCytomation / P0161
Polyclonal antibody to amyloid precursor protein/APP (first antibody against C99)	ACRIS/SP7016P
Polyclonal antibody to rabbit IgG (H&L)-HRP	ACRIS/R1364HRP
Beta actin (first antibody)	Abcam / Ab8227
Goat polyclonal to rabbit IgG-H&L (HRP) (secondary antibody against beta actin)	Abcam / Ab6721

3-3-4. Mediums, buffers and solutions:

Vitamin-rich medium	500 mL ALPHA MEDIUM (×1) 500 µg lactalbumin enzymatic hydrolysate (this amount is solubilized in 20 mL medium and sterilized using sterile filter) 50 mL inactivated FBS (prepared by heating FBS at 65°C for 1 hour) 100 µL glutamin (final concentration 2 mM) 5 mL penicillin/streptomycine
Vitamin-free medium	500 mL ALPHA MEDIUM (×1) mod. 500 µg lactalbumin enzymatic hydrolysate (this amount is solubilized in 20 mL medium and sterilized using sterile filter) 50 mL inactivated FBS 100 µL glutamin (final concentration 2 mM) 5 mL penicillin/streptomycine
APS-solution	10% (w/v) ammonium persulfat
Blocking buffer	1% (w/v) BSA in trisbuffered saline-tween (TBST)
Copper(II) sulfate-solution	4% $CuSO4.5H_2O$ in dH_2O This solution must be kept in dark and at 4°C
Collecting gel	100 µL 0.6 M Tris Hcl (pH 6.8) + SDS 0.4% 760 µL dH_2O 140 µL acrylamid (Rotiphorese® Gel 40) 10 µL bromophenol blue 5 µL APS 10% 0.5 µL TEMED
Electrophoresis buffer (×10)	30.3 g Tris base 144 g Glycine 10 g SDS Completing to 1L with dH_2O

Extraction buffer	1 mL RIPA buffer 20 mM sodium fluoride 1 mM sodium orthovandate 10 mM sodium pyrophosphate 1 mM phenylmethylsulfonyl fluoride 1 mM okadaic acid Completing with dH_2O to 10ml; adding one-tablet protease inhibitors and mixing well.
Laemmli ×4 buffer	5 mL 0,5 M Tris Hcl (pH 6,8) 0.6 mL SDS 10% 4g Glycerol 0.4 mL bromophenol blue 1% Completing with dH_2O to 10 mL Final buffer: 800 µL of the previous mixture + 200 µL β-mercaptoethanol
Resolving gel (8%)	1 mL 1.875 M Tris Hcl (pH 8.8) + SDS 0.4% 1.4 mL acrylamid (Rotiphorese® Gel 40) 2.6 mL dH_2O 25 µL APS 10% 2 µL TEMED
Transfer buffer (x10)	Stock solution: 30.3 g tris + 144 g glycine Completing to 1L with dH_2O Working solution: 70 mL stock solution + 140 mL methanol + 490 mL H_2O
Tris buffered saline (TBS)	8 g NaCl + 20 mL 1 M Tric Hcl (pH 7.6) Diluting to 1L with dH_2O
Wash and diluent buffer (TBST)TBS-Tween	0.1% Tween-20 in TBS

3-3-5. Treatment of the cells with different materials and cell lysate preparation:

Detroit 532 cells (passage 14) were seeded at density (2-4×10.000) cells/cm^2 in 75 cm^2 culture flasks containing vitamin-rich medium and incubated at 37°C and 5% CO_2 in moist atmosphere. Cells, at 70-80% confluency, were trypsinized and splited at a ratio 1:2 every four to seven days until passage 23.

Cells at passage 23 were harvested and seeded at density 3000 cells/cm^2 in petri dishes using 7 mL culture medium (vitamin-rich or vitamin-free medium). After 24 hours, the culture medium was sucked and replaced with a fresh medium without FBS. Then we added different materials at different concentrations [Hcy: 50, 100, or 150 μM; SAH: 50, 100, or 150 μM; SAM: 100, 200, or 300 μM; DZA: 50, 100, or 150 μM; lovastatin: 5, 10, or 20 μM]. In each run, we left double dishes without adding any material to be used as controls and double dishes of each concentration were prepared. In trials, where β- or γ-secretase inhibitors were used, they were added at the same time with the additive drug using 30 nM of each. After 24 hours medium was discarded. Cells were washed twice with ice-cold PBS. 250 μL of ice-cold lysis buffer were added and cells were scraped off the dish. Cell suspension was gently transferred into an Eppendorf tube and placed on ice. Cell lysates were sonicated on ice for 20 seconds five times with 5 seconds intervals. Lysates were centrifuged at 14000 g and 4°C for 15 minutes. Aliquots of the supernatant were immediately stored at –70°C.

Cell viability was tested in both control and treated cells using trypan blue and calculated as following:

$$\text{Cell viabilty \%} = \frac{\text{number of viable cells}}{\text{number of total cells}} \times \text{dilution rate} \times 100$$

We found no difference between cell viability in treated cells and in control cells.

3-3-6. Total protein assay (Bicinchoninic acid method):

This is a two-step assay, in which Cu^{2+} is first reduced to Cu forming a complex with protein amide bonds. In the next step, bicinchoninic acid (BCA) forms a purple complex with Cu which is detectable at 562 nm. The color intensity is directly proportional to the amount of protein. A 7-point standard curve (1000, 800, 600, 500, 400, 250, and 125 μg/mL) was prepared by using a stock solution of BSA (1 mg/mL). 10 μL of samples, standards, and blanks (dH_2O) were then mixed with 200 mL working solution (BCA, 4 % CuSO4-5H_2O 50:1 v/v). These mixtures were then incubated for 30 min in a water bath at 37°C, cooled to room

temperature, centrifuged for 30 seconds, and then applied to a 96 well plate. The absorption was measured at 562 nm using spectrophotometer.

3-3-7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE):

Cell lysates were mixed with Laemmli ×4 buffer (7:1). This mixture was heated for 5 min at 95°C, sonicated, and centrifuged briefly.

The resolving gel and collecting gel were prepared as described before and they were used immediately or stored in moist atmosphere in a refrigerator. A fresh electrophoresis buffer was used. Samples containing 5 µg protein and 10 µL of a protein marker were loaded into the gel. Electrophoresis was performed for 90 min at 200 V; next blotting was done using freshly prepared transfer buffer. The blotting, using PVDF, was run for one hour at 350 mA and 100 V. Blots were used immediately or stored in a desiccators at 2-8°C for a few days.

3-3-8. Westernblot

Membranes were blocked in a blocking buffer (1% BSA in TBST) for one hour at room temperature to prevent non-specific binding. The membrane was briefly rinsed twice with TBST and incubated with the primary antibody diluted with TBST as follows:

Mouse anti –Alzheimer precursor protein A4: 1:1500

Polyclonal antibody to amyloid precursor protein/APP: 1:1000

Beta actin antibody: 1:6000

The incubation was made overnight at 4°C, then the membrane was washed three times for 5 min each with TBST at room temperature. Next, it was incubated with HRP labeled secondary antibody diluted with (1% BSA in TBST) as follows:

Polyclonal rabbit anti-mouse immunoglobulin's: 1:1500

Polyclonal antibody to rabbit IgG (H&L): 1:5000

Goat polyclonal to rabbit IgG-H&L: 1:5000

Subsequently membrane was washed three times for 5 min each with TBST at room temperature.

3-3-9. ECL Plus detection:

ECL Plus TM Western Blotting Reagents (GE Healthcare® formerly Amersham Biosciences) utilizes chemiluminescence for the detection of target proteins. It consists of a lumigen PS-3 acridian substrate, which is converted to an acridinium ester intermediate when catalyzed by HRP (horseradish pyroxidase). The ester intermediate reacts with peroxide in alkaline

conditions and emits light, which can be detected by autoradiography (film). The kit includes Solution A (substrate solution containing tris buffer) and Solution B (acridian substrate solution in dioxane and ethanol).

Washed membranes were incubated in the detection buffer [solutions A and B 40:1(v/v)] for 5 min at room temperature. Autoradiography films of the drained membranes were made in a dark room. Photo films were developed immediately and proceeded to test bands by densitometry with Image Labscan (Amersham Biosciences). Bands density was measured using ImageQuant TL program (Amersham Biosciences). Intensity of APP band was normalized for the corresponding beta-actin band.

3-3-10. Human Aβ (1-42) ELISA assay:

Aβ (1-42) was detected in extracts of cells cultured in the vitamin-free medium and treated with SAH or SAM. This kit is constructed as a sandwich ELISA format with two kinds of antibodies. The monoclonal antibody BAN50, which epitope is human Aβ (1-16), is coated on 96 well surfaces of separable microplate and acts as a capture antibody for N-terminal portion of human Aβ (1-42). Captured human Aβ (1-42) is recognized by another antibody, BC05 (Fab' fragment), which specifically detects C-terminal portion of Aβ (1-42), labeled with HRP. After addition of TMB solution, positive samples will develop a blue color. The reaction is terminated by the addition of a stop solution, which produces a yellow color. The absorbance is then measured at 450 nm.

4- RESULTS

4-1. <u>Study (1): Markers of methylation are related to that of neurodegeneration:</u>

This part of the study included 117 patients of whom 31 had multiple sclerosis (MS), 19 had stroke, 31 had dementia (8 had Alzheimer disease), and 36 had peripheral neuropathy. The control group consisted of 65 subjects with different neurological disorders presented in Table 6.

Table 6: Neurological disorders of the control group patients

Diagnosis	Number	Diagnosis	Number
Peripheral facial pareses	4	Recurrent transient ischemic attack	1
Chronic fatigue syndrome	1	Ataxia (cerebral/hereditary)	1/1
Chronic pain syndrome	4	Meralgia paresthetica	1
Pseudo-tumour	5	Spatial spinal paralysis	1
Migraine	4	Parkinson (untreated at diagnosis)	4
Meningitis (basal, viral)	2/4	Cervical myelopathy	1
Myasthenia gravis	1	Somatisation disorder	1
Atypical face pain	1	No pathological findings	7
Cerebral or focal convulsions	5	Unknown diagnosis	8
Arterial hypertension	8		

Data summerized in Table 7 showed that stroke, dementia, and peripheral neuropathy patients were significantly older than control patients. In addition, patients with peripheral neuropathy had higher concentrations of blood markers (tHcy, Cys, SAH, and SAM) and CSF markers (tHcy and SAH) in addition to lower median concentration of CSF-folate compared to the controls. Nevertheless, these results seem to be related to the older age of patients because these differences were no more significant after adjusting for age.

Patients with MS had higher serum concentrations of holoTC in addition to a lower concentration of CSF-SAH and a higher CSF-SAM/SAH ratio compared to the controls.

Despite the marked age differences, patients with stroke showed no significant differences in any blood or CSF marker as compared to the controls.

As patients with dementia were much older than the control patients, comparison between CSF and blood markers was not performed, because adjustment for age was not possible in this case.

Table 7: Concentrations of serum/plasma and CSF vitamins and methylation markers according to disease status						
	All (n=182)	Controls (n=65)	MS (n=31)	Stroke (n=19)	Peripheral neuropathy (n=36)	Dementia (n=31)
Age, years		46 (22-68)	49 (31-63)	63 (38-79)*	66 (36-74)*	72 (55-81)*
Plasma/serum markers						
tHcy, µM	10.7 (7.4-18.0)	9.4 (7.3-16.2)	10.7 (7.6-14.4)	10.2 (7.0-17.4)	11.9 (7.0-19.1)*†	12.9 (8.2-28.2)
Cys, nM	372 (180-886)	329 (150-661)	355 (157-1146)	420 (154-874)	415 (199-1038)*†	491 (247-1764)
MMA, nM	197 (128-394)	186 (109-300)	197 (116-366)	176 (144-510)	189 (142-340)	293 (149-681)
Total B12, pM	249 (166-419)	242 (158-403)	266 (181-402)	253 (162-485)	270 (183-630)	195 (145-364)
Folate, nM	19.6 (9.9-41.1)	19.3 (11.4-42.2)	21.1 (9.5-41.6)	20.3 (12.6-42.0)	22.3 (9.9-43.8)	16.1 (5.4-27.1)
Vitamin B6, nM	36.9 (13.8-100.8)	37.9 (17.8-90.6)	10.6 (16.1-168.4)	33.3 (12.2-115.6)	42.0 (15.3-199.7)	19.5 (8.0-45.4)
HoloTC, pM	70 (32-136)	63 (32-128)	89 (35-156)*	64 (32-154)	81 (44-143)	58 (20-133)
SAM, nM	123 (89-218)	116 (87-171)	114 (74-236)	130 (93-206)	138 (88-228)*†	159 (95-369)
SAH, nM	18.1 (10.0-37.9)	16.2 (9.3-27.4)	13.5 (9.9-51.5)	18.2 (10.9-48.2)	19.6 (11.0-37.2)*†	25.1 (11.1-61.7)
SAM/SAH ratio	7.0 (3.8-13.0)	6.8 (3.8-13.8)	8.2 (3.0-14.4)	7.7 (3.4-11.9)	6.7 (4.1-10.1)	6.9 (1.6-12.3)
CSF markers						
tHcy, µM	0.10 (0.06-0.17)	0.09 (0.06-0.16)	0.08 (0.05-0.16)	0.08 (0.06-0.15)	0.11 (0.07-0.18)*†	0.10 (0.06-0.32)
Cys, nM	49 (22-98)	54 (17-108)	42 (22-105)	48 (18-105)	34 (18-75)	47 (25-125)
MMA, nM	359 (267-552)	359 (266-574)	367 (281-525)	426 (333-600)	326 (214-511)	419 (267-746)
Folate, nM	19.5 (13.9-26.6)	20.7 (14.1-27.7)	20.5 (14.6-29.5)	19.2 (14.2-26.4)	18.6 (12.9-23.5)*†	18.4 (12.3-27.4)
HoloTC, pM	16 (4-27)	16 (6-23)	16 (3-26)	17 (4-73)	17 (7-39)	11 (4-23)
SAM, nM	267 (180-356)	268 (197-355)	305 (151-389)	273 (172-339)	268 (187-385)	250 (180-356)
SAH, nM	13.5 (8.4-24.1)	13.2 (7.7-24.0)	10.2 (7.4-16.7)*	13.5 (7.1-24.8)	15.5 (9.1-22.5)*†	15.2 (9.7-30.0)
SAM/SAH ratio	19 (10-34)	20 (11-39)	28 (13-42)*	22 (10-30)	18 (12-24)	17 (7-29)
Aβ (1-42) pg/mL	651 (374-1060)	739 (444-1092)	552 (340-737)*	587 (327-919)	606 (520-1000)	666 (290-1090)

Data are median (10^{th}-90^{th}) percentiles. * $p<0.05$ compared to the control group (ANOVA and post hoc Tamhane-T tests).
† Differences are no more significant after adjusting for age and CSF/serum albumin.

Concentrations of plasma/serum and CSF metabolites in our study seemed to be affected by age. To test this, we pooled the data from all study populations and divided our patients into 4 groups according to quartiles of age (Table 8). On the one hand, advanced age was associated with higher blood concentrations of tHcy, Cys, MMA, SAH, and SAM and lower concentrations of serum folate and holoTC. On the other hand, concentrations of CSF-tHcy and that of CSF-SAH increased with age. Moreover, levels of CSF-folate and CSF-MMA, and the ratio of SAM/SAH in CSF decreased with increasing age. CSF-holoTC and CSF-SAM did not differ significantly with age.

In addition, we found that CSF-folate was a stronger predictor (beta = -0.403) of CSF-tHcy than age (beta = 0.298).

Table 8: Blood and CSF markers according to age

Quartile of age	**Q1**	**Q2**	**Q3**	**Q4**	**P**
Age, years	**17-40**	**41-55**	**56-68**	**69-86**	
Plasma/serum markers					
tHcy, µM	9.0 (6.0-12.7)	10.2 (7.8-20.2)*	11.0 (7.9-15.0)*	14.0 (8.1-28.4)*	< 0.001
Cys, nM	246 (129-472)	388 (188-844)*	362 (197-877)*	510 (283-1950)*	< 0.001
MMA, nM	166 (90-294)	179 (133-299)	196 (147-370)*	253 (146-621)*	< 0.001
Total B12, pM	253 (165-433)	259 (173-435)	243 (162-424)	246 (162-422)	0.699
Folate, nM	18.8 (11.9-42.8)	21.9 (10.5-43.7)	21.3 (12.7-42.8)	15.2 (7.4-25.8)*	< 0.001
HoloTC, pM	70 (26-128)	71 (41-128)	73 (39-163)	57 (26-139)	0.077
SAM, nM	106 (73-143)	116 (89-179)	132 (97-219)*	164 (105-327)*	< 0.001
SAH, nM	12.8 (8.6-27.0)	16.4 (10.4-29.2)	19.8 (11.8-37.3)*	25.6 (13.8-79.7)*	< 0.001
SAM/SAH ratio	8.3 (3.4-14.1)	7.3 (4.1-12.2)	7.5 (3.8-12.7)	6.1 (2.8-11.2)	0.079
Creatinine, µM	66.3 (53.0-88.4)	70.7 (53.0-97.2)	79.6 (53-106.1)	88.4 (53.0-189.2)*	<0.001
CSF markers					
tHcy, µM	0.07 (0.05-0.12)	0.10 (0.06-0.17)	0.10 (0.06-0.14)	0.13 (0.09-0.24)*	< 0.001
Cys, nM	53 (17-110)	42 (19-97)	33 (18-70)	65 (27-131)	0.002
MMA, nM	392 (251-776)	359 (289-509)	348 (261-522)	359 (258-554)	0.188
Folate, nM	21.0 (16.3-28.9)	19.8 (12.3-27.4)	19.7 (15.3-23.1)	16.9 (11.9-21.8)*	< 0.001
HoloTC, pM	14 (5-25)	17 (3-23)	18 (6-38)	14 (4-29)	0.204
SAM, nM	254 (174-332)	270 (154-375)	259 (181-356)	274 (190-374)	0.464
SAH, nM	10.5 (6.5-14.4)	12.7 (7.9-20.2)	15.3 (9.1-25.4)*	16.7 (10.3-30.0)*	< 0.001
SAM/SAH ratio	26 (16-40)	19 (11-36)	16 (8-31)*	17 (8-29)*	< 0.001

Data are median (10^{th}-90^{th}) percentiles. * $p<0.05$ according to the post hoc Tamhane-T2 test compared to the first quartile.

To investigate the association between concentrations of CSF-SAH and that of other vitamin biomarkers we divided concentrations of CSF-SAH into quartiles (data from all patient groups) (Table 9).

In plasma/serum samples, higher concentrations of CSF-SAH were related to higher concentrations of tHcy, Cys, SAH, and SAM. Moreover, higher CSF-SAH was associated with lower concentrations of serum folate. CSF data showed that higher concentrations of SAH were associated with higher tHcy and Cys, lower folate, and lower SAM/SAH ratio. Consequently, CSF-SAH and concentrations of other vitamin biomarkers associated in CSF and in plasma/serum in the same way.

Table 9: Concentrations of plasma/serum and CSF biomarkers of B vitamins according to CSF-SAH

Quartile of CSF SAH	**Q1**	**Q2**	**Q3**	**Q4**	**P**
SAH, nM	4.7-10.1	10.2-13.4	15.5-17.4	17.5-39.3	-
Age, years	41 (16)	44 (18)	57 (15)*	64 (13)*	< 0.001
Plasma/serum markers					
tHcy, µM	10.2 (3.1)	10.0 (8.1)	12.1 (9.6)	12.6 (5.0)*	0.019
Cys, nM	308 (320)	388 (686)	451 (644)*	491 (571)*	0.007
MMA, nM	201 (117)	188 (94)	214 (138)	262 (256)	0.018
Total B12, pM	279 (1159)	280 (506)	274 (1111)	254 (90)	0.851
Folate, nM	22.3 (16.2)	21.4 (11.8)	17.8 (13.1)	16.5 (10.2)*	0.036
HoloTC, pM	68 (37)	56 (40)	62 (44)	69 (42)	0.349
SAM, nM	110 (34)	130 (128)	134 (62)	157 (101)*	0.002
SAH, nM	15.2 (6.8)	17.0 (11.8)	22.2 (41.7)*	24.4 (39.9)*	0.001
SAM/SAH ratio	7.3 (3.5)	7.7 (3.1)	6.1 (4.5)	6.4 (3.7)	0.162
Creatinine, µM	69.8 (14.4)	73.3 (16.9)	97.0 (94.3)	93.9 (56.3)	0.032
CSF markers					
tHcy, µM	0.08 (0.04)	0.10 (0.26)*	0.10 (0.03)*	0.14 (0.19)*	< 0.001
Cys, nM	42 (27.5)	46 (33)	46 (22)*	49 (35)*	0.666
MMA, nM	414 (318)	372 (130)	363 (111)	372 (127)	0.334
Folate, nM	20.8 (4.2)	20.2 (5.3)	18.4 (4.4)	17.1 (4.8)*	0.003
HoloTC, pM	11 (12)	13 (24)	13 (7)	14 (20)	0.431
SAM, nM	247 (71)	270 (58)	260 (61)	258 (72)	0.450
SAM/SAH ratio	29.6 (9.4)	23.3 (6.1)*	17.1 (4.3)*	11.1 (3.9)*	< 0.001
Aβ(1-42) pg/mL	606 (188)	566 (237)	630 (250)	702 (239)	0.135

Geometric mean (SD). * $p<0.05$ compared to the first quartile (post-hoc Tamhane-T2 test).

CSF-Aβ (1-42) concentration in all study populations decreased with advanced age but the association was not significant. We found no association between Aβ (1-42) and methylation markers, vitamins or tHcy in the total group or in patients with dementia. The median concentrations of CSF-Aβ (1-42) according to tertiles of SAM/SAH ratio in non-demented patients are shown in Figure 8. We found that subjects within the higher tertile of CSF-SAM/SAH ratio had lower concentrations of CSF-Aβ (1-42) comparing to subjects within the lower tertile. This association remained significant after adjustment for age.

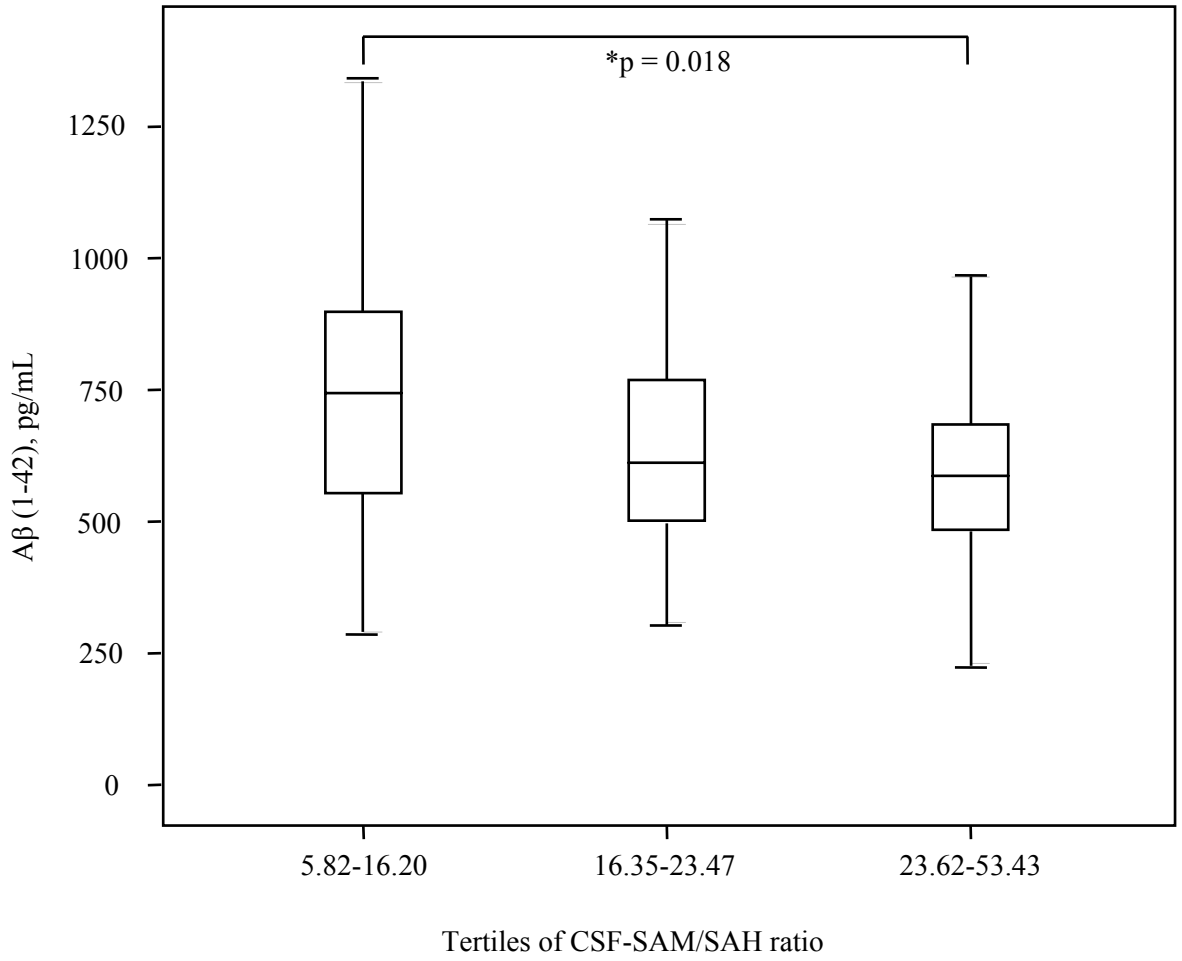


Figure 8: Concentrations of Aβ (1-42) accordiong to CSF-SAM/SAH ratio

In addition, in non-demented patients aged 30-60 years, higher concentrations of CSF-Aβ (1-42) associated with higher CSF concentrations of tHcy and SAH as well as lower concentrations of CSF-SAM (Figure 9).

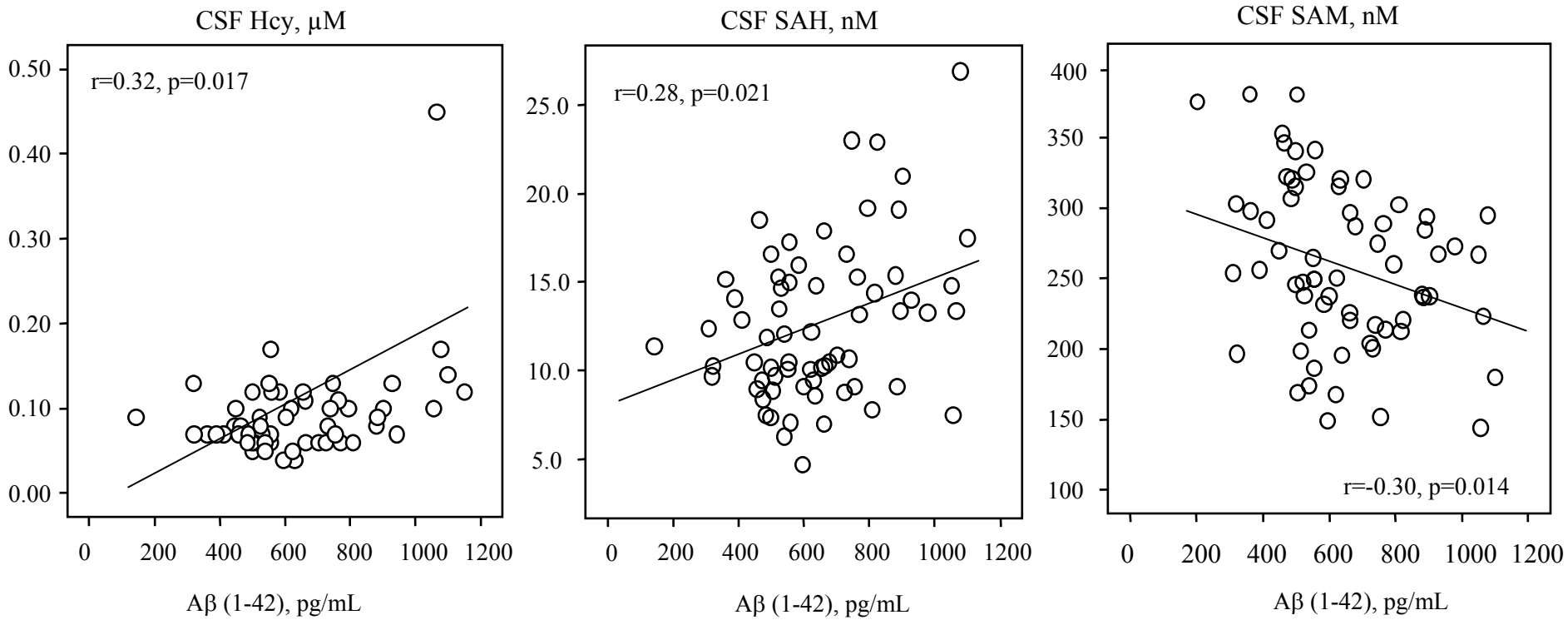


Figure 9: The correlation between concentrations of Aβ (1-42) and that of Hcy, SAH, and SAM in CSF of non-demented patients

4-2. <u>Study (2): B vitamins may improve cognitive function in elderly:</u>

This part of the study included 69 subjects (mean age 78 years, 62 females), who were allocated to receive a vitamin supplements or a placebo.

We have tested the correlation between different markers at baseline. We found that higher concentrations of tHcy were associated with lower SAM/SAH ratio (Figure 10.A) and MMSE scores were not related to SAM/SAH ratio (Figure 10.B). In addition, we found that SAM concentrations were positively correlated with orientation test scores (Figure 10.D) and scores indicating intellectual abilities (abstract thinking, judgement) were negatively related to serum concentrations of MMA at start

(Figure 10.C).

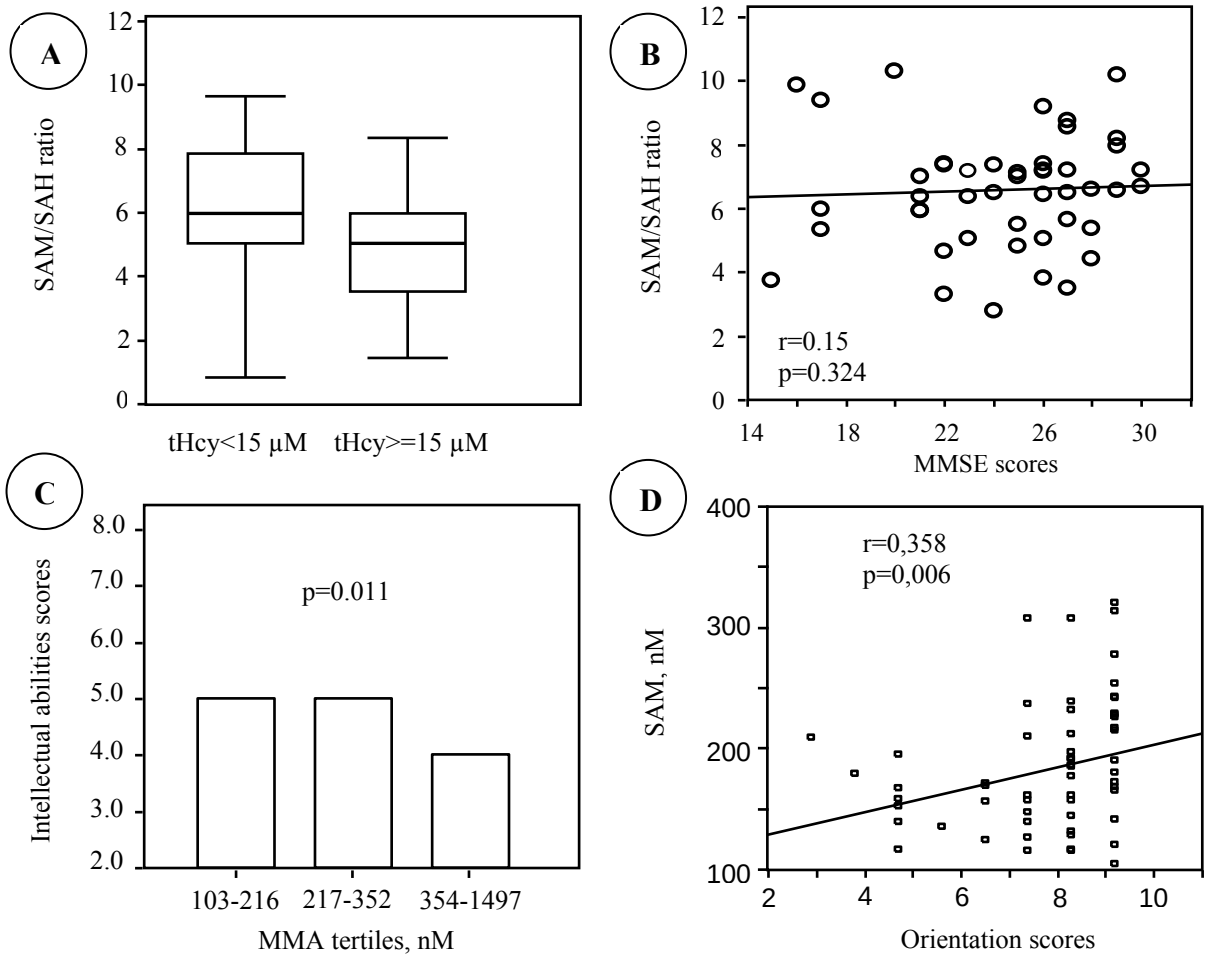


Figure 10: The correlation between different markers at baseline

A: Median SAM/SAH ratio according to tHcy concentrations in serum.
B: The correlation between MMSE scores and plasma SAM/SAH ratio.
C: The relation between serum MMA tertiles and intellectual abilities scores.
D: The correlation between plasma SAM concentrations and orientation scores.

Data summurized in Table 10 shows serum concentrations of different metabolites at baseline, day 21, and day 45 in both placebo and vitamin groups.

At baseline, no significant differences in serum concentrations of tHcy or MMA were detected between the two treatment arms. Cys concentrations were higher in the treatment group as compared to the placebo group. At the end of the intervention, concentrations of tHcy, Cys, and MMA were lower in the vitamin treated group compared to the placebo group.

Table 10: Serum concentrations of the metabolites at baseline, day 21, and day 45 according to treatment allocation

	Placebo	**Vitamin**	***p**
at start			
tHcy, µM	17.5 (8.3)	18.4 (6.6)	0.273
Cys, nM	534 (804)	669 (407)	0.007
MMA, nM	336 (192)	323 (237)	0.442
SAH, nM	25 (12)	30 (17)	0.186
SAM, nM	170 (54)	184 (54)	0.361
SAM/SAH ratio	6.7 (1.9)	6.1 (1.8)	0.253
at day 21			
tHcy, µM	18.0 (5.8)	9.1 (2.3)	<0.001
Cys, nM	471 (386)	367 (206)	0.257
MMA, nM	429 (247)	237 (77)	<0.001
SAH, nM	28 (23)	34 (22)	0.202
SAM, nM	155 (68)	186 (61)	0.064
SAM/SAH ratio	6.4 (1.8)	5.6 (2.2)	0.849
at day 45			
tHcy, µM	18.4 (7.0)	9.9 (2.5)	<0.001
Cys, nM	654 (799)	410 (222)	0.037
MMA, nM	529 (531)	248 (93)	0.001

Data are mean (SD); * p values are according to Mann-Whitney test.

The SIDAM tests were done at baseline, at day 21, and at day 45. At day 21, both of the treatment arms showed improvement in total SIDAM scores (Figure 11.B). Nevertheless, scores indicating higher cortical functions (aphasia, apraxia, and agnosis) improved significantly only in the vitamin group (Figure 11.A). Furthermore, in the vitamin group, subjects who showed improved intellectual function abilities (n=9) had higher baseline concentrations of MMA and higher reduction in MMA (Δ-MMA) and tHcy (Δ-tHcy) compared to subjects who showed no improvement (Table 11).

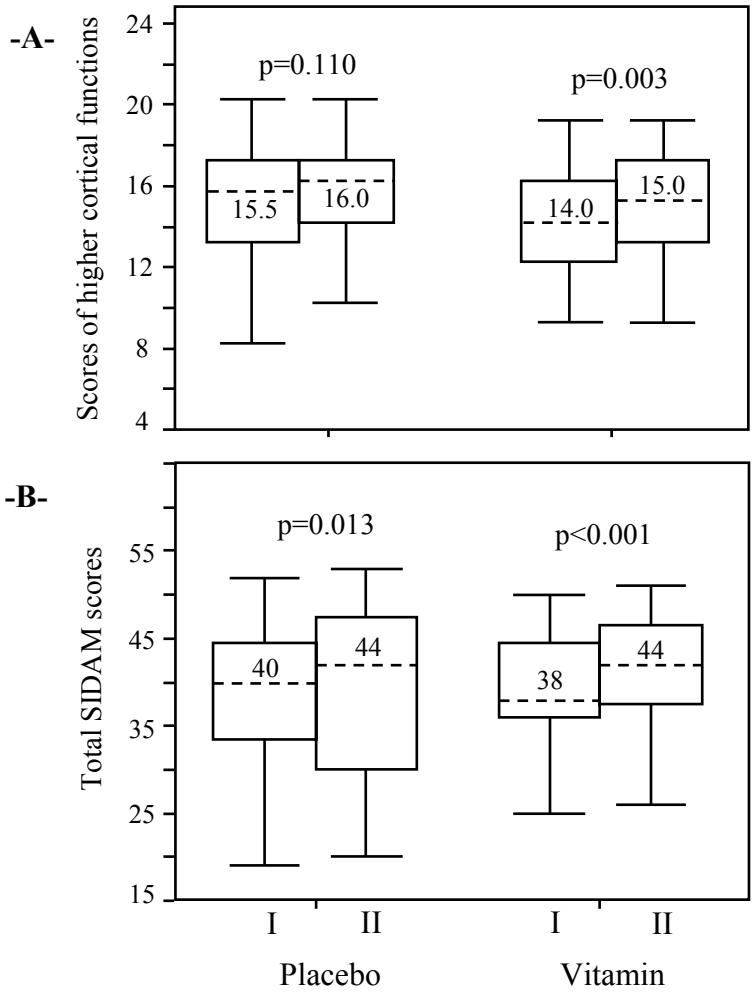


Figure 11: Scores of total SIDAM and higher cortical function tests in placebo and vitamin groups (I: at baseline, II: at day 21)

Table 11: Intellectual function correlation to MMA and tHcy in the vitamin group

	Intellectual function improved (n=9)	Intellectual function did not improve (n=24)	P*
Baseline intellectual function scores	4.3 (1.2)	4.9 (0.3)	<0.001
Intellectual function scores (day 21)	4.7 (0.5)	4.9 (0.3)	0.174
Baseline tHcy, µM	21.7 (7.7)	17.7 (5.7)	0.157
Δ-tHcy, µM	12.2 (7.7)	8.9 (4.8)	0.306
Baseline MMA, nM	326 (244)	262 (113)	0.016
Δ-MMA, nM	216 (365)	52 (66)	0.057

Data are mean (SD); * p values are according to Mann-Whitney test.

At the end of the intervention, both of the treatment arms showed improvement in total SIDAM scores (Table 12).

Table 12: Scores of different dementia tests at baseline and at day 45 in both placebo and vitamin groups

		baseline (0)	day 45	p
MMSE	Placebo	26 (17-29)	27.5 (22-30)	0.001**
	Vitamin	24 (17-28)	27 (20.5-29.5)	0.001**
Orientation	Placebo	9 (5-10)	10 (7-10)	0.006**
	Vitamin	9 (6.5-10)	9.5 (7.5-10)	0.045*
Memory function	Placebo	14 (10.3-18)	16.5 (12.6-19.7)	0.001**
	Vitamin	11.5 (9-16.5)	15 (11.5-17)	0.008**
Intellectual abilities	Placebo	5 (4-5)	5 (5-5)	0.186
	Vitamin	5 (3-5)	5 (4.5-5)	0.328
Higher cortical function	Placebo	16 (11-19)	16 (13-19)	0.050
	Vitamin	14.5 (9-17.5)	15.5 (10.5-19)	0.178
Total SIDAM scores	Placebo	44 (31-51)	48 (38-54)	0.001**
	Vitamin	39 (29-48)	45 (37-50.5)	0.002**

Data are median (10^{th}-90^{th}) percentiles. * $p<0.05$, ** $p<0.01$ (ANOVA).

4-3. Study (3): One-carbon cycle metabolites might affect APP level and/or its processing:

4-3-1. Incubation in a vitamin-rich or in a vitamin-free medium:

We tested the effect of Hcy, SAM, and SAH on full length APP in DS fibroblasts grown in a vitamin-rich or in a vitamin-free medium. Figure 12.B shows that in both mediums, the three different concentrations of Hcy (50, 100, 150 μM) did not significantly affect the full length APP. We used concentrations of Hcy similar to those found in moderate HHcy or in patients with homocysteinuria. Within the range of Hcy concentrations used in this study, there was no sufficient evidence supporting a dose-dependent effect on APP expression.

The effect of SAM (100, 200, 300 μM) on APP in fibroblasts grown in a vitamin-rich or in a vitamin-free medium is shown in figure 13.B. SAM caused no significant change in full length APP in cells grown in a vitamin-rich medium. In contrast, SAM significantly lowered total APP in cells grown in a medium free of the B vitamins compared to those grown in the vitamin-rich medium.

We further tested whether the postulated neurotoxic effect of Hcy can be related to its toxic hydrolysis byproduct, SAH. Fibroblasts grown in a vitamin-free medium and then incubated for 24 hours with 3 different concentrations of SAH, showed a significant increase in the immunoreactivity of full length APP (Figure 14.B). This effect seemed to occur in a non-concentration-dependent manner. In contrast to this, fibroblasts incubated with SAH in a vitamin-rich medium showed rather lowered total APP.

Compared to cells incubated with SAM or Hcy, those incubated with SAH showed a significant increase in the immunoreactivity of APP in a vitamin-free medium.

4-3-2. Effect of Hcy, SAH, and SAM on C99 protein in cells incubated in a vitamin-free medium:

While Hcy did not change full length APP as mentioned before, it caused a significant increase in C99 immunoreactivity that was not dose-dependent. In contrast, SAH, which enhanced the accumulation of APP, caused rather lower C99 protein expression. SAM markedly lowered full length APP, but had no remarkable effect on C99 (Figure 15.B).

A

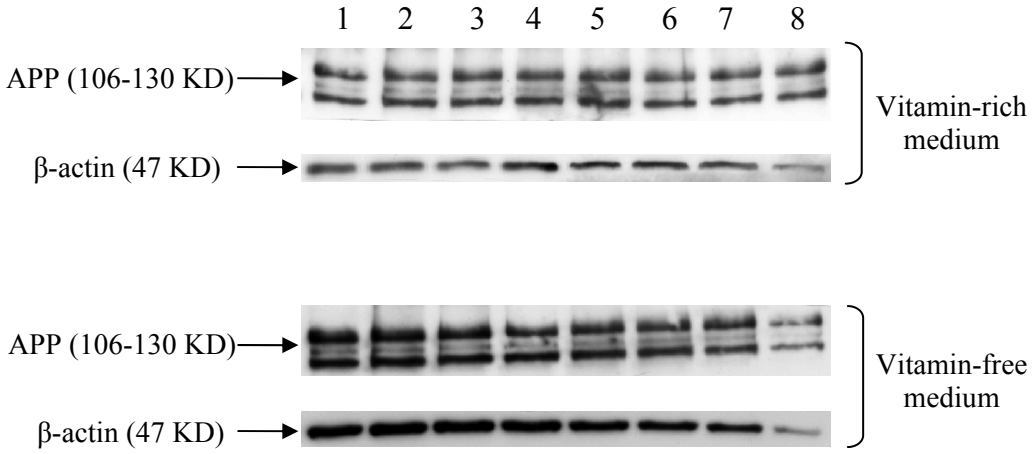


Figure 12.A: Westernblot of APP and β-actin proteins in fibroblast cell extracts treated with Hcy in either vitamin-rich medium or vitamin-free medium for 24 hours. Panels (1-2) are from control samples (cells not treated with Hcy). Panels (3-4) are cell extracts treated with Hcy 50 µM. Panels (5-6) are cell extracts treated with Hcy 100 µM. Panels (7-8) are cell extracts treated with Hcy 150 µM.

B

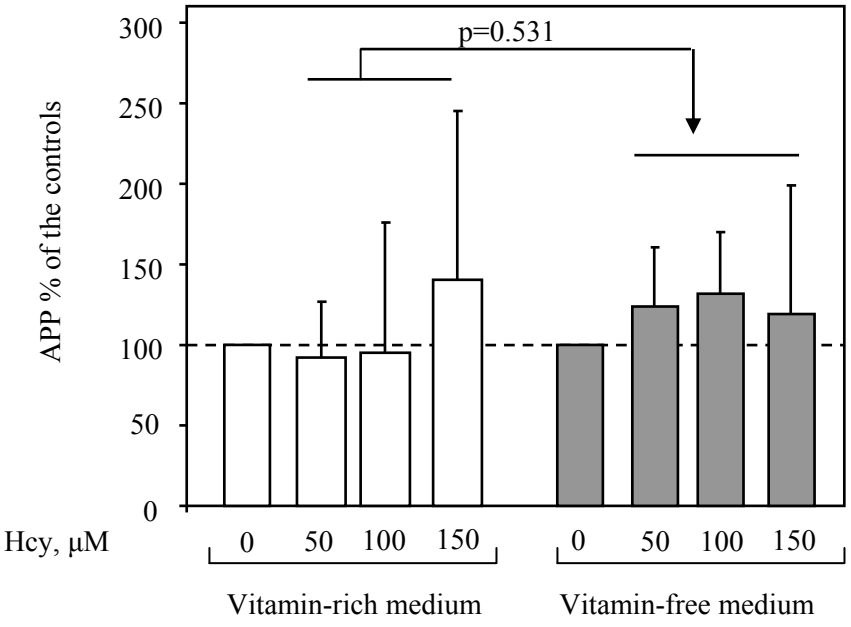


Figure 12.B: The effects of Hcy on APP expression in DS fibroblasts incubated in a vitamin-rich or in a vitamin-free medium. Total number of experiments ranged between 4 and 6 for each concentration. Results are presented as average and standard deviation.

Figure 12: Effect of Hcy on APP expression

A

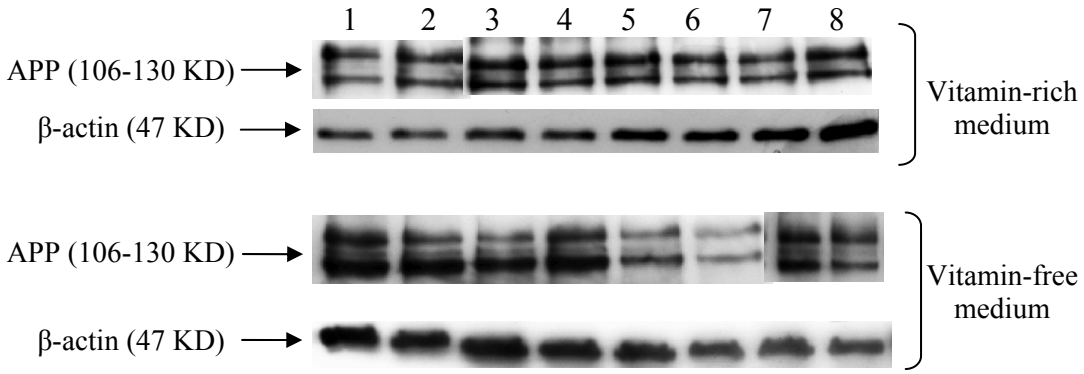


Figure 13.A: Westernblot of APP and β-actin proteins in fibroblast cell extracts treated with SAM in either vitamin-rich medium or vitamin-free medium for 24 hours. Panels (1-2) are from control samples (cells not treated with SAM). Panels (3-4) are cell extracts treated with SAM 100 µM. Panels (5-6) are cell extracts treated with SAM 200 µM. Panels (7-8) are cell extracts treated with SAM 300 µM.

B

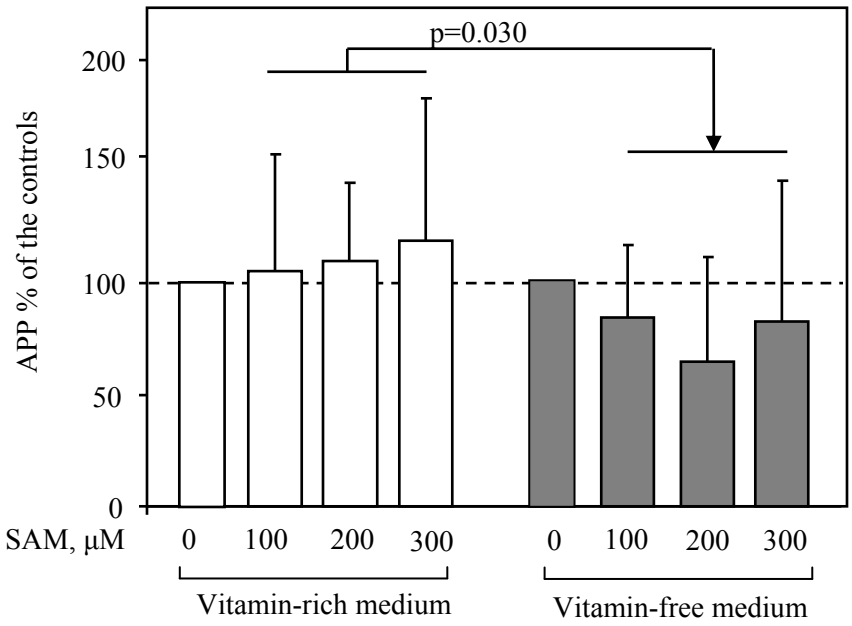


Figure 13.B: The effects of SAM on APP expression in DS fibroblasts incubated in a vitamin-rich or in a vitamin-free medium. Total number of experiments ranged between 4 and 6 for each concentration. Results are presented as average and standard deviation.

Figure 13: Effect of SAM on APP expression

A

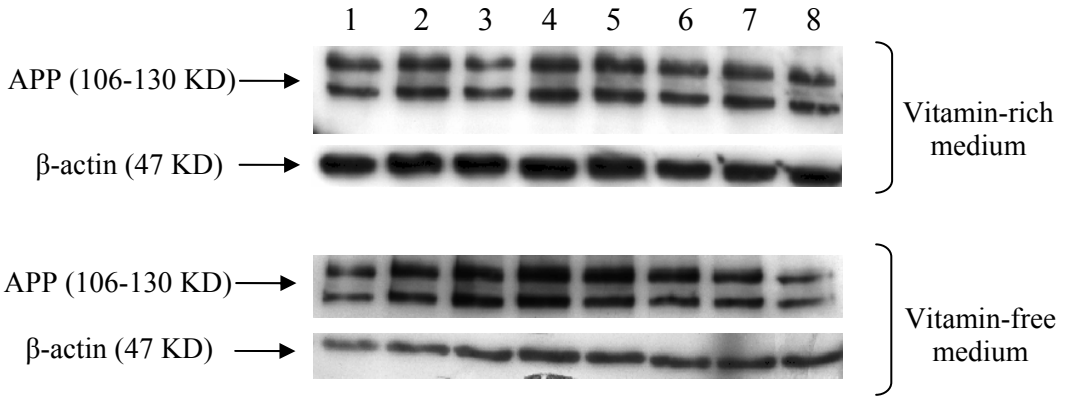


Figure 14.A: Westernblot of APP and β-actin proteins in fibroblast cell extracts treated with SAH in either vitamin-rich medium or in a vitamin-free medium for 24 hours. Panels (1-2) are from control samples (cells not treated with SAH). Panels (3-4) are cell extracts treated with SAH 50 μM. Panels (5-6) are cell extracts treated with SAH 100 μM. Panels (7-8) are cell extracts treated with SAH 150 μM.

B

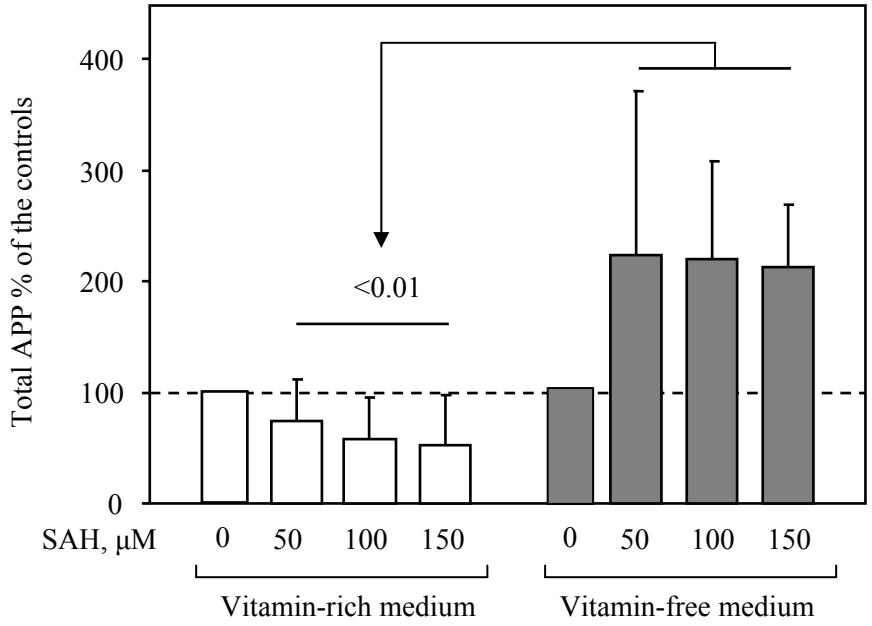


Figure 14.B: The effects of SAH on APP expression in DS fibroblasts incubated in a vitamin-rich or a vitamin-free medium. Total number of experiments ranged between 4 and 6 for each concentration. Results are presented as average and standard deviation.

Figure 14: Effect of SAH on APP expression

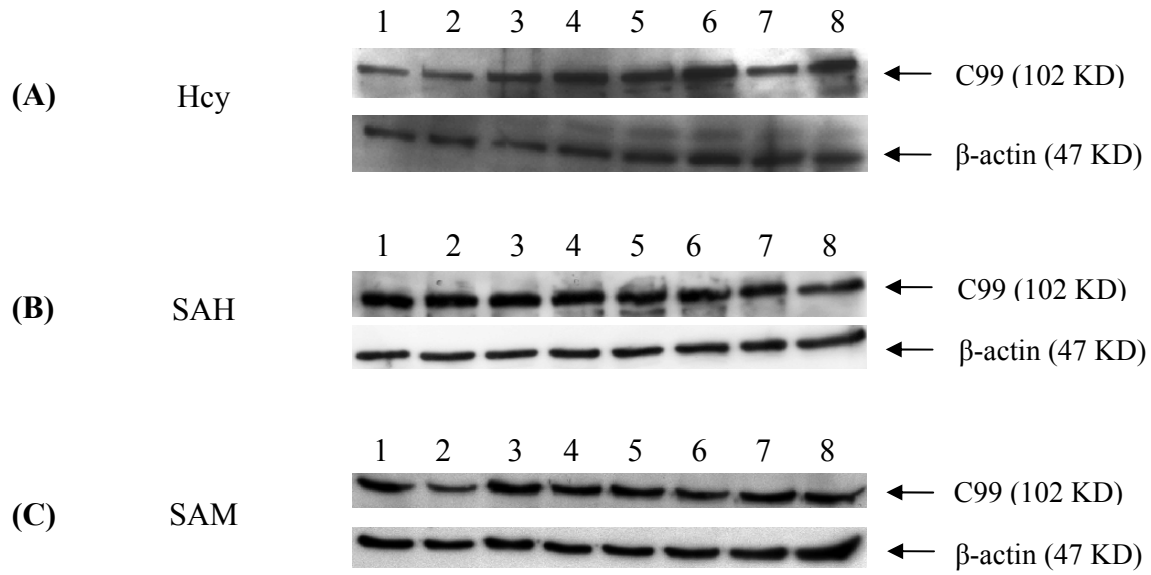


Figure 15.A: Western blot of C99 and β-actin in Down syndrome fibroblasts incubated in a vitamin-free medium and treated with different concentrations of Hcy, SAH, or SAM

Figure	Cells treated with	Panel 1	Panel 2	Panel 3	Panel 4	Panel 5	Panel 6	Panel 7	Panel 8
(A)	Hcy	C	C	50 µM	50 µM	100 µM	100 µM	150 µM	150 µM
(B)	SAH	C	C	50 µM	50 µM	100 µM	100 µM	150 µM	150 µM
C)	SAM	C	C	100 µM	100 µM	200 µM	200 µM	300 µM	300 µM

C: Control samples (Untreated cells).

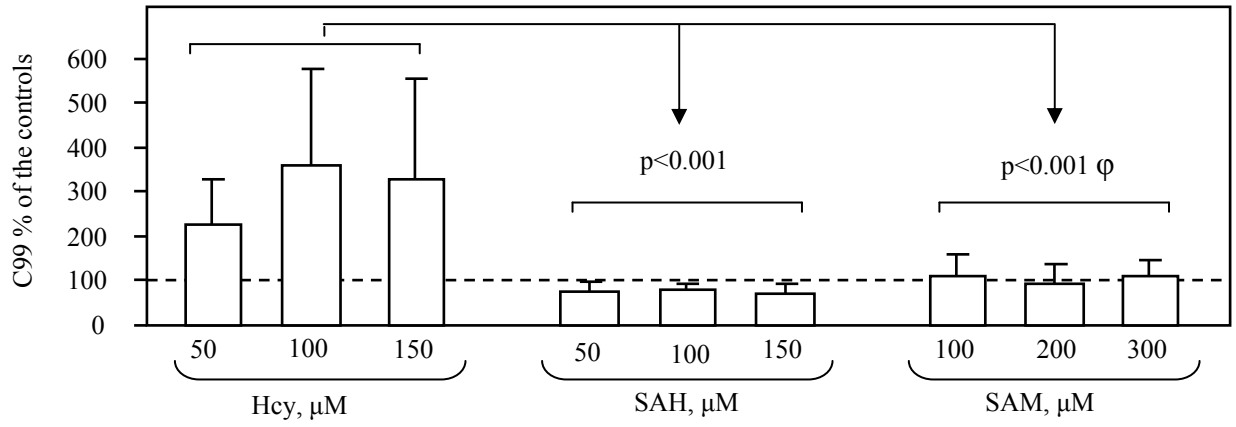


Figure 15.B: The effects of Hcy, SAH, and SAM on APP expression in DS fibroblasts incubated in a vitamin-free medium. Total number of experiments ranged between 5 and 6 for each concentration. Results are presented as average and standard deviation. φ: p=0.027 compared to SAH.

Figure 15: Effect of Hcy, SAH, and SAM on C99 expression

4-3-3. Effect of adding β-or γ-secreatse inhibitors on protein levels of APP and C99:

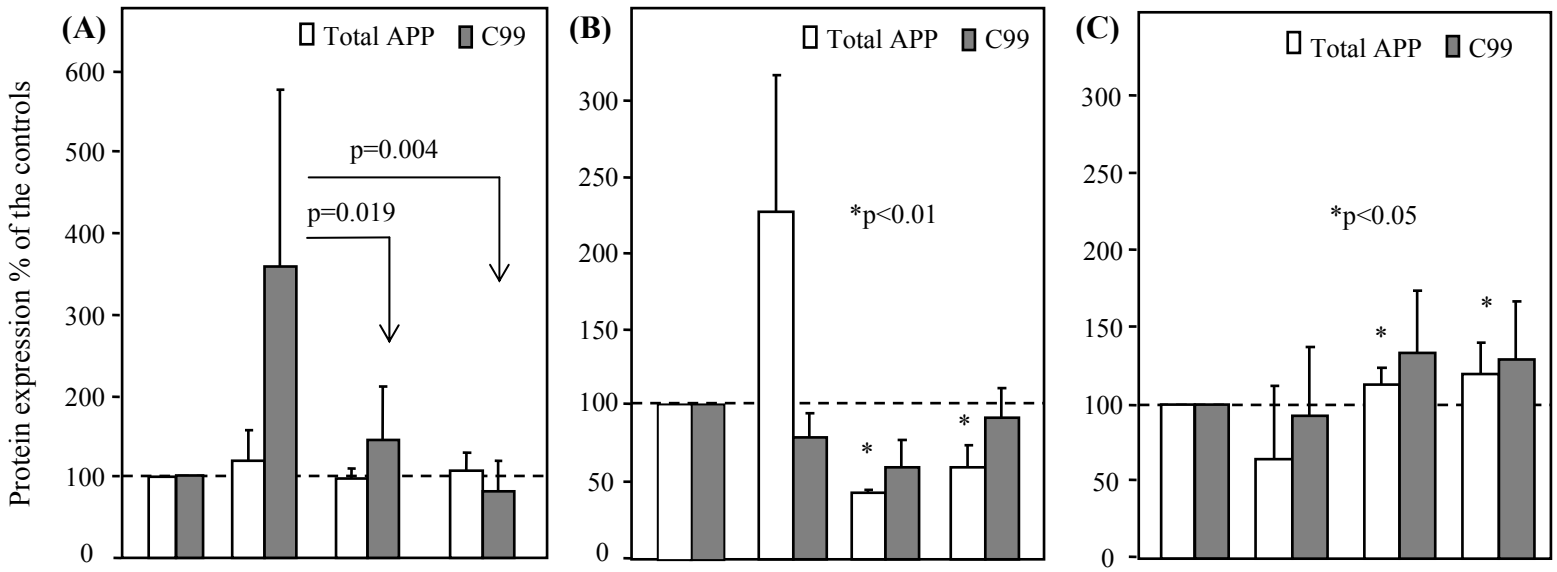
We were further interested to test whether the effect on total APP was related to an effect on APP processing by means of β-or γ-secretases that process APP via the amyloidogenic pathway. For this purpose, we used β-or γ-secretase inhibitors to try to antagonize the effect of the added substances. The following experiments were all conducted in a vitamin-free medium.

As the effects of Hcy, SAH, and SAM on APP and C99 levels were not dose-dependent as shown before, the possible role of β-or γ-secretase inhibitors on full length APP and C99 was tested using only one concentration of each substance. We observed that either β- or γ-secretase inhibitors antagonized the effect of Hcy (100 μM) on C99. No change in the protein expression of total APP was obtained under these conditions (Figure 16.A).

The effect of SAH (100 μM) on total APP was prevented by the addition of either β-or γ-secretase inhibitors (Figure 16.B). Furthermore, protein expression of APP and C99 in the presence of SAM (200 μM) and β-or γ-secretase inhibitors seemed to be in contrast to that caused by adding SAH. Both inhibitors reversed the effect of SAM on total APP and C99 (Figure 16.C).

4-3-4. Effect of SAH and SAM on the intra-cellular concentrations of Aβ (1-42):

Concentrations of the final product of the amyloidogenic pathway, Aβ (1-42), were tested in extracts of cells grown in a vitamin-free medium and treated with either SAM or SAH. At least three independent experiments of each condition were tested. The results were adjusted for total protein content. We found that the intracellular concentrations of Aβ (1-42) were decreased by 62% in the presence of SAM (200 μM) and increased by 22% in the presence of SAH (100 μM) comparing to cells incubated only in a vitamin-free medium.



Vitamin free medium	+	+	+	+	+	+	+	+	+	+	+	+
Hcy, 100 µM	-	+	+	+	-	-	-	-	-	-	-	-
SAM, 200 µM	-	-	-	-	-	-	-	-	-	+	+	+
SAH, 100 µM	-	-	-	-	-	+	+	+	-	-	-	-
β-secretase inhibitor	-	-	+	-	-	-	+	-	-	-	+	-
γ-secretase inhibitor	-	-	-	+	-	-	-	+	-	-	-	+

Figure 16: The effect of Hcy (100 µM) (A), SAH (100 µM) (B), and SAM (200 µM) (C) on total APP and C99 expression in DS fibroblasts incubated in a vitamin-free medium in the presence or absence of β-or γ-secretase inhibitors. Total numbers of experiments were 6 for each applied condition. Results are presented as average and standard deviation.

4-3-5. Effect of adding lovastatin on protein expression of APP and C99 in DS fibroblasts in the absence and presence of β-or γ-secretase inhibitors:

DS fibroblasts incubated with 3 concentrations of lovastatin (5, 10, 20 μM), in either a vitamin-rich or a vitamin-free medium, showed less immunoreactivity of full length APP compared to the control cells incubated in the same medium without lovastatin. However, the reduction of APP in cells incubated with lovastatin in a vitamin-rich medium was significantly higher than that in cells incubated with the drug in a vitamin-free medium (Figure 17.A). Moreover, the APP-lowering effect of lovastatin in both mediums occurred in a dose-dependent manner.

In addition, cells grown in a vitamin-free medium and treated with lovastatin, showed reduced immunoreactivity of C99 protein comparing to control cells (Figure 17.B).

The effect of lovastatin (20 μM) on both APP and C99 protein levels was reversed in the presence of β-or γ-secretase inhibitors (Figure 17.C).

4-3-6. Effect of adding DZA on protein expression of APP and C99 in DS fibroblasts:

We tested the effect of three different concentrations of DZA (50, 100, 150 μM) on the immunoreactivity of full length APP in DS fibroblasts grown in either a vitamin-rich or a vitamin-free medium. In a vitamin-rich medium, DZA reduced APP immunoreactivity in a dose-dependent manner (Figure 18). In a vitamin-free medium, DZA did not cause any significant change in either APP or C99 levels comparing to control cells (Figure 19). However, in the presence of 150 μM DZA, the immunoreactivity of full length APP in cells grown in a vitamin-rich medium was significantly lower than that in cells grown in a vitamin-free medium ($p=0.014$).

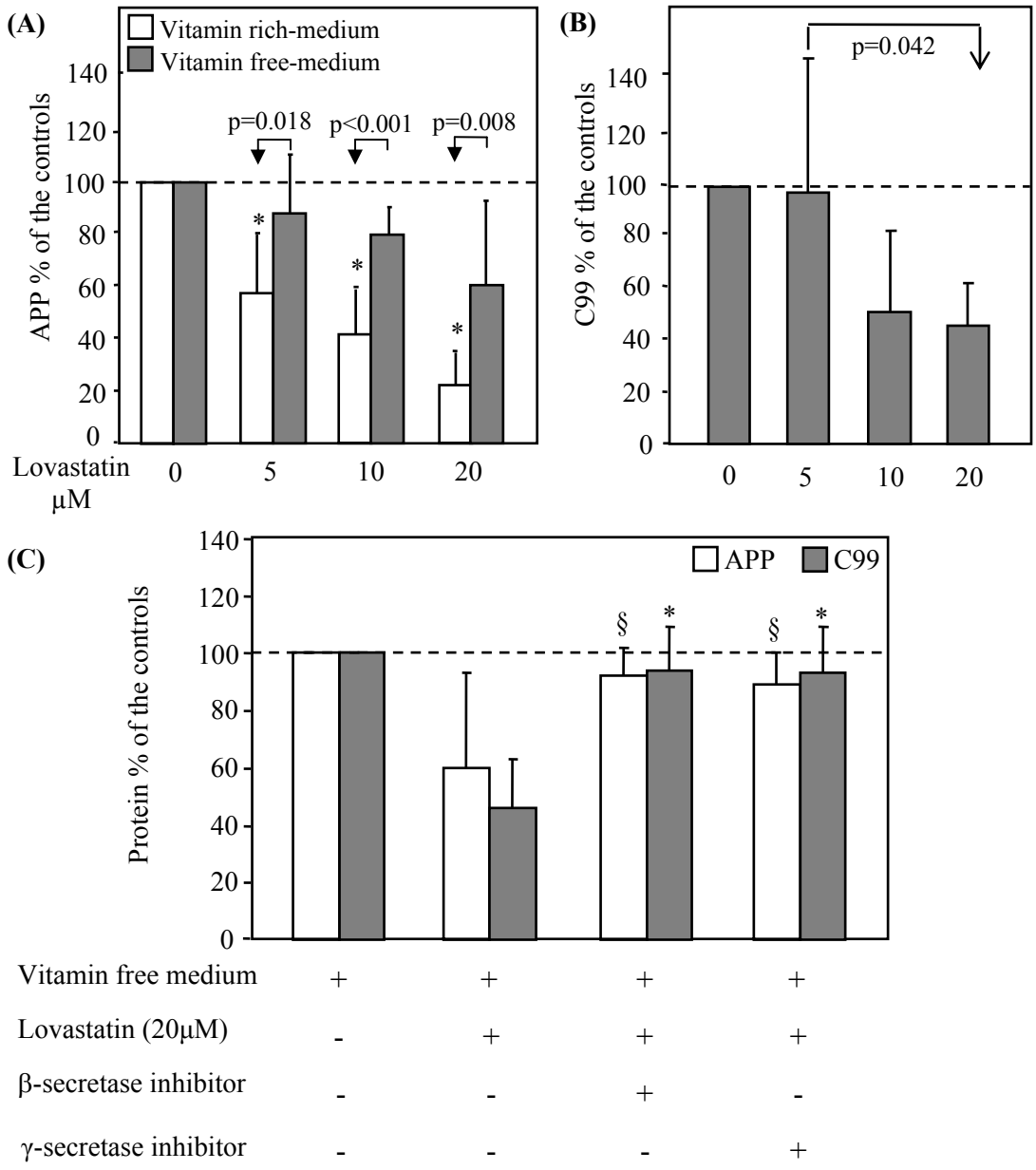


Figure 17: Effect of lovastatin on protein expression of APP and C99 in DS fibroblasts
(A): Effect of lovastatin on protein expression of APP in cells incubated in a vitamin-free or in a vitamin-rich medium. Total number of experiments ranged between 8 and 11 for each concentration and condition. * $p<0.05$ compared to vitamin free medium.
(B): Effect of lovastatin on protein expression of C99 in cells incubated in a vitamin-free medium. Total number of experiments ranged between 5 and 6 for each concentration.
(C): Effect of lovastatin (20 µM) on protein expression of APP and C99 in cells incubated in a vitamin-free medium and in the absence or the presence of β- or γ-secreatase inhibitors. Total number of experiments ranged between 5 and 11 for each concentration and condition. * $p<0.005$ and § $p\leq0,05$ compared to cells treated only with lovastatin. Results are presented as average and standard deviation.

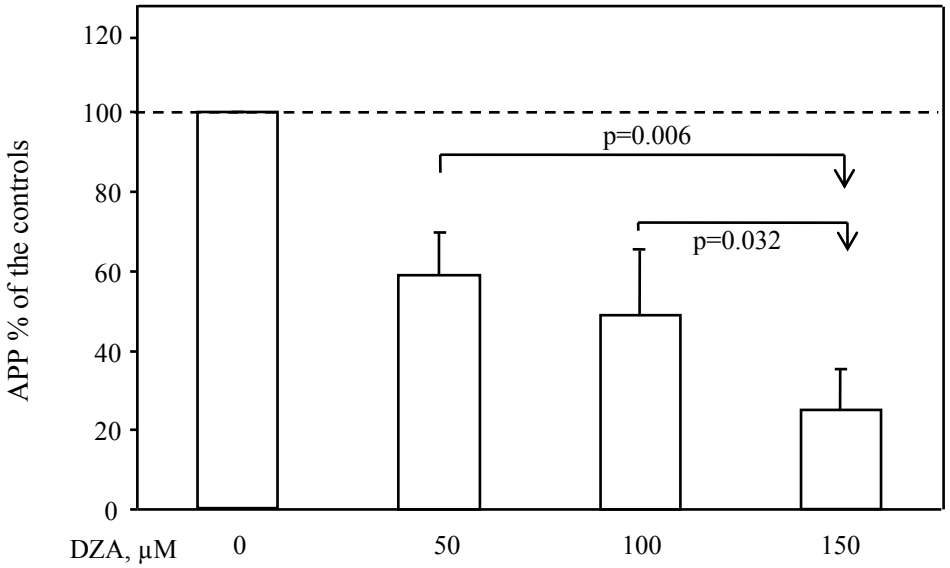


Figure 18: The effect of DZA on APP expression in DS fibroblasts incubated in a vitamin-rich medium.
Total number of experiments ranged between 5 and 6 for each concentration. Results are presented as average and standard deviation.

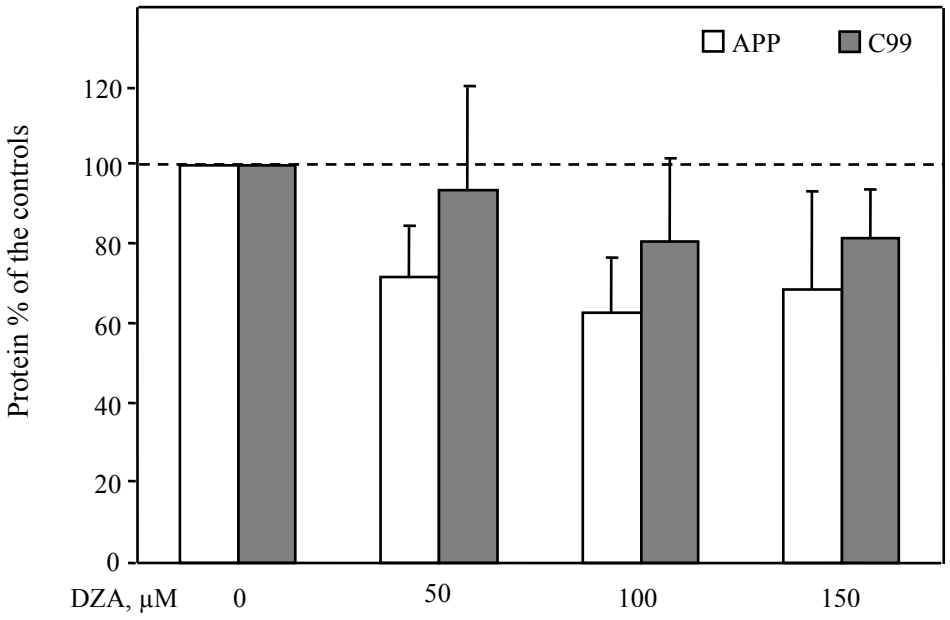


Figure 19: The effect of DZA on APP and C99 expression in DS fibroblasts incubated in a vitamin-free medium.
Total number of experiments ranged between 6 and 8 for each concentration. Results are presented as average and standard deviation.

4- DISCUSSION

4-1. Study (1): Markers of methylation are related to that of neurodegeneration:

There is body of evidence in the literature that markers of methylation are related to neurodegenerative diseases. Previous reports documented higher serum or plasma concentrations of tHcy and MMA in patients with dementia when compared to non-demented subjects (Clarke et al., 1998). Folate deficiency is associated with elevated levels of Hcy, cerebrovascular and neurological diseases, and mood disorders (D'Anci and Rosenberg, 2004). In the adult, epidemiological studies have linked lack of folate to neurodegenerative and neuropsychiatric diseases, including stroke, PD, dementia, and depression (Reynolds, 2002;He et al., 2004;Lamberti et al., 2005). Our study has shown that patients with peripheral neuropathy had higher concentrations of blood markers (tHcy, Cys, SAH, and SAM) and CSF markers (tHcy and SAH) in addition to lower median concentration of CSF-folate compared to the controls. However, these differences were no more significant after adjusting for age.

Concentrations of tHcy or related biomarkers in CSF have been tested in only a few studies (Hansson et al., 2006;Regland et al., 2004). Human studies demonstrated a lowered SAM or an increased tHcy or SAH in brains or CSF from patients with certain disorders of the CNS (Bottiglieri et al., 1990;Surtees and Hyland, 1990). Moreover, lower concentrations of CSF-folate were observed in late-onset dementia when compared to non-demented patients (Serot et al., 2001). We have shown that concentrations of folate decrease and that of tHcy increase in CSF with age (Table 8). This is in line with Bottiglieri et al. findings where highly significant decline in CSF folate with advanced age was shown (Bottiglieri et al., 2000b). In addition, we found that CSF-folate was a stronger predictor (beta = -0.403) of CSF-tHcy than age (beta = 0.298). The positive correlation between plasma and CSF concentrations of tHcy has been confirmed by a previous study (Selley et al., 2002). Therefore, elevation of tHcy in the blood may indicate its elevation in the brain where tHcy can have many neurotoxic effects.

Disturbed methyl group metabolism in the brain might be closely related to age and to methyl group metabolism in other parts of the body. As suggested by our results, elevation of CSF-SAH with age might be related to increased concentrations of tHcy in the plasma or in the CSF (Table 8). Hcy is converted into SAH via SAH hydrolase. The reaction in the SAH direction is favoured

in case of excess tHcy. This is in line with results on animals showing that dL-Hcy administration led to increased brain SAH (Gharib et al., 1983). Because SAH is a potent inhibitor of many transmethylases, the lower ratio of SAM/SAH in the CSF indicates a hypomethylation state in the brain and may affect several important biological pathways. It is of interest, that aging was related to higher CSF concentrations of tHcy and SAH, in addition to a lower folate and SAM/SAH ratio in the CSF (Table.8). These metabolic changes with age may be very important factors that play a paramount role in the genesis of age-related-disorders. In addition to the role of age as a significant modulator of CSF-SAH, our data demonstrated that a lower folate status (in the circulation or in the CSF) is related to an increased concentration of SAH in the CSF (Table 9). It is plausible that aging is associated with a higher concentration of SAH in CSF via lowering CSF-folate.

In AD brains, the intracerebral deposition of Aβ (mainly 1-42) is the most important pathologic process leading to dementia. In contrast to Aβ (1-40), Aβ (1-42) is insoluble and can accumulate in the plaques. Concentrations of Aβ (1-42) are lower in CSF from patients with dementia when compared to non-demented patients. The accumulation of Aβ is a long-term process that is thought to start at a young age. We found no association between Aβ (1-42) and methylation markers, vitamins, or tHcy in the total group or in patients with dementia. However, in non-demented patients, aged 30-60 years, our results showed that higher CSF-Aβ (1-42) concentrations associated with higher CSF concentrations of Hcy and SAH as well as with lower concentrations of CSF-SAM (Figure 9). In addition, subjects within the higher CSF tertile of SAM/SAH ratio had lower concentrations of CSF-Aβ (1-42) than subjects within the lower tertile. Our patients, other than those with dementia, were not tested for cognitive performance and we cannot exclude that some of elderly people were slightly demented which might affect concentrations of CSF-Aβ (1-42).

Available in-vitro evidence has suggested that tHcy accelerates dementia by stimulating Aβ deposition in the brain (Fuso et al., 2005). In line with this, a positive association between plasma concentrations of tHcy and that of Aβ has been documented in patients with neurodegenerative disease (Irizarry et al., 2005). Another clinical study demonstrated that folate treatment lowered concentrations of plasma Aβ (1-40) (Flicker et al., 2007).

The effect of SAM or B vitamin treatments on the insoluble Aβ (1-42) protein in CSF has not been tested. SAM treatment protects the neurons from degeneration by several mechanisms (Rao et al., 1997;Villalobos et al., 2000). The protective effect of SAM was suppressed by simultaneous administration of SAH (Sato et al., 1988).
These results in addition to our current one suggest that an increased SAH, rather than a reduced SAM may be a more important mediator in neurodegeneration. Because we demonstrated that CSF-tHcy is an important determinant of CSF-SAH (regression analysis), keeping tHcy and SAH at a low level may be important for preventing or slowing the deposition of Aβ.

In summary, our current study demonstrated that Hcy metabolism in the circulation is closely related to that in the brain. This relation seems to extend beyond a simple exchange of tHcy, SAH, SAM, or the vitamins across the blood brain barrier. Higher SAH can cause Aβ accumulation thus increasing the risk for neurodegenerative diseases. Testing the response of β-amyloid to B vitamin treatment in deficient subjects needs to be investigated.

4-2. <u>Study (2): B vitamins may improve cognitive function in elderly:</u>

In our study, at baseline, we have shown that higher concentrations of SAM were significantly associated with higher orientation scores. In addition, higher concentrations of MMA were significantly associated with lower scores of intellectual abilities (Figure 10). This results are inline with Lewerin and collegues results that showed that tHcy and MMA correlated independently with movement and cognitive performance in elderly subjects (Lewerin et al., 2005). HHcy is associated with an increased risk of cognitive dysfunction. In the Framingham Study, subjects with elevated concentrations of tHcy at baseline were more likely to develop dementia after several years compared with subjects with normal concentrations of tHcy (Seshadri et al., 2002).

As the association between cognitive function and tHcy and B vitamins is supported by more and more evidence, several teams are studying the effects of supplementation. In our study, we found that at day 21, total SIDAM scores were improved in both placebo and vitamin groups but scores indicating higher cortical function and intellectual function were improved only in the vitamin group (Figure 11) and this improvement was related to higher reduction in MMA and tHcy

concentrations (Table 11). At day 45, both of treatment arms showed improvement in total SIDAM scores (Table 12). We assume that the study patients, during hospitalization for the first three weeks, were subjected to more social interaction; enrolling in different cognitive tests and social events, consequently their cognitive status was improved. Additionally, between day 21 and 45, patients were at home and it might be possible that they did not correctly apply the supplementation course. Moreover, the short period time of B vitamins supplementation in our study might be not sufficient to obtain significant improvement in the cognitive status of the vitamin group in comparing to the placebo group.

On one hand, examinations of association between single nutrients and cognitive outcomes have found improvement in domains of cognitive function that tend to decline with age after administration of folic acid for 3 years (Durga et al., 2007). Other studies have investigated the effect of combined administration of folate and B vitamins on the cognitive status. Clinical improvement in all cases and cognitive scores improvement in 5 cases were found in 7 tested cases after administration of B vitamins and N-acetylcysteine (McCaddon, 2006). Bryan and colleagues found improvement in some measures of memory function in older women but no effect on mood after 35 days of folate and B vitamins administration (Bryan et al., 2002). Both of placebo and vitamin groups showed improved memory function.

On the other hand, several vitamin intervention studies document no improvement in measures of cognitive function. In a systematic review of randomized trials, Balk and colleagues analysed 14 trials and found that three trials of vitamin B6 and six of vitamin B12 found no effect overall in a variety of doses, route of administration, and population. One of three trials of folic acid found a benefit in cognitive function in people with cognitive impairment and low baseline serum folate levels. Six trials of combinations of the B vitamins all concluded that the supplementation had no effect on cognitive function (Balk et al., 2007). Likewise, another study found no improvement in cognitive function in elderly people with vascular events who were treated for 1 year with folic acid plus vitamin B12, even though the treatment effectively lowered plasma tHcy concentrations (Stott et al., 2005). However, major limitations of these studies and others preclude a firm conclusion that B vitamins supplementation has no effect on the cognitive performance.

Long-term duration, large participant's number, standardized cognitive tests that distinguish different cognitive domains, adjusting for demographic factors such as age and gender, doses and forms of the vitamins, and start of the treatments are all critical factors that have to be considered for judging available results or for future studies. One fact that should also be recognized is that the turnover of cells in the nervous system is negligible, whereas blood cells divide very rapidly. Therefore, vitamin treatment is known to improve hematological symptoms, while neurological symptoms may take longer to improve and may be only partially reversible. This is unsurprising given that the progression of dementia may extend over several decades. Therefore, it is currently believed that ensuring sufficient B vitamin intake might be more effective in disease prevention rather than in disease treatment.

4-3. Study (3): One-carbon cycle metabolites might affect APP level and/or its processing:

DS is the most common genetic cause of human mental retardation (Krivchenia et al., 1993). The excessive synthesis of multiple gene products derived from overexpression of the genes present on chromosome 21, such as APP (Glenner and Wong, 1984), superoxide dismutase, CβS, and β-secretase (BACE2) genes, is thought to underlie both the dysmorphic features and the pathogenesis of the neurological abnormalities that are characteristic of DS. A 157% increase in CβS enzyme activity has been previously documented in individuals with DS (Chadefaux et al., 1985). Cultured skin fibroblasts have been used successfully to elucidate the molecular and biochemical basics of many inborn errors of metabolism that cause neurological disease. Therefore, we used DS fibroblasts in our study as a model representing hypomethylation, oxidative stress, and neurodegeneration conditions.

HHcy is a recognized risk factor for neurodegenerative diseases. Elevated concentration of tHcy or low concentrations of folate are strongly associated with an increased risk of AD and cognitive dysfunction (Seshadri et al., 2002;Quadri et al., 2004). Furthermore, disorders of the transmethylation pathway have been observed in patients with DS (Pogribna et al., 2001). Low SAM/SAH ratio is a metabolic milieu that is associated with the accumulation of several neurodegenerative proteins (Obeid et al., 2007a).

One possible mechanism by which Hcy plays as neurotoxin is potentiating neurotoxicity induced by Aβ. Elevated plasma Aβ in DS patients has been reported by recent studies (Mehta et al.,

2007). Folate deprivation induced a marked increase in Hcy and ROS and increased Aβ-induced apoptosis, while folate supplementation prevented the generation of ROS by Aβ (Ho et al., 2003). In addition, folate deficiency was found to increase APP catabolism via the amyloidgenic pathway. One of the mechanisms tested was DNA-hypomethylation causing overexpression of a major γ-secretase, PS-1 (Fuso et al., 2005).

In the current study, we have shown that while SAM caused decreased protein expression of APP in cells incubated in a vitamin-free medium compared to cells incubated in a vitamin-rich medium, SAH enhanced the expression of APP in cells incubated in a medium free of B vitamins. The expression of C99 protein also seemed to be differently affected by SAH and SAM. SAH enhanced APP degradation into Aβ via C99 thus causing lowered protein expression of C99 and 22% increase of the intracellular concentrations of Aβ (1-42). The later was reduced by 62% in the presence of SAM (200 μM) comparing to cells incubated only in a vitamin-free medium.

To understand more the previous effects of SAH or SAM on APP immunoreactivity, we were further interested to test whether these effects were related to an effect on APP processing via the amyloidgenic pathway by means of β- and/or γ-secretases. For this purpose, we tested the effects of β- and γ-secretase inhibitors on APP and C99 expression in DS fibroblasts cultured in a vitamin-free medium and treated with SAH (100 μM) or SAM (200 μM). We found that the presence of β- or γ-secretase inhibitors reversed the effects of SAM and SAH on APP processing suggesting that while SAH increased Aβ accumulation via enhancing the enzymatic activity of the secretases affecting the amyloidgeneic pathway, SAM inhibits these secretases causing less production and accumulation of Aβ. These results seem in accordance with the study of Cavallaro and collegues, in which SAM has been shown to silence the PS-1 gene thus reducing Aβ (Cavallaro et al., 2006). One mechanism that might link methylation to APP processing is APP phosphorylation at Thr668 by means of PP2A that facilitates its cleavage (Lee et al., 2003;Pierrot et al., 2006).

Recent findings in neurons suggest that inhibition of PP2A promotes the axonal accumulation of β-CTF APP fragments by inducing microtubule destabilization and deficits in APP transport (Yoon et al., 2006). The formation of active PP2A depends on the methylation of its B subunit that is controlled by a specific SAM-dependent methyltransferase (Lee and Stock, 1993). PP2A

mediates the de-phosphorylation of tau protein affecting its accumulation. It has been reported that exogenous Hcy and folate deficiency in cultured neurons can increase SAH and/or decrease SAM levels and promote tau phosphorylation (Ho et al., 2003). Vafai and Stock provided experimental proof for the hypothesis that PP2A methylation links Hcy metabolism with tau hyperphosphorylation in AD (Vafai and Stock, 2002). Incubation of neuroblastoma cells with SAH caused reduced methylation of PP2A, a major brain Ser/Thr phosphatase, and this enhanced accumulation of phosphorylated APP and increased production of β-secretases-cleaved APP fragments and Aβ (Sontag et al., 2007). Therfore, one can spectulate that cells incubated with SAH might have shown reduced PP2A activity, enhanced phosphorylated forms of APP and thereby increased APP processing into Aβ.

Hcy in the concentrations used in this study showed no effect on APP expression in cells grown in a vitamin-rich or a vitamin-free medium suggesting that either the exposure time or the concentrations used were not sufficient to show this effect. However, Hcy caused enhanced protein expresion of C99 in cells grown in a vitamin-free medium. This effect was reversed using β- or γ-secretase inhibitors. Moreover, the mild effect of Hcy comparing to that of SAH might be due to increased catabolism of Hcy in DS fibroblasts expressing an additional copy of CβS. Our results suggest that Hcy enhances both β- and γ-secretases and therefore stimulating APP processing to C99. This suggestion corresponds with a recent observation that Hcy can induce mRNA and protein level of PS-1 and folate and vitamin B12 injection antagonized this elevation suggesting that HHcy can affect several key steps in APP processing (Zhang et al., 2009). In addition, our results can be related to the fact that DS subjects have enhanced amount of BACE2 (expressed on chromosome 21) that catabolize APP to C99. HHcy and B vitamins deprivation enhanced gene and protein expression of β- and γ-secretases and Aβ production in mice brain (Fuso et al., 2009). Our findings show that the effect of Hcy on C99 was reversed in the presence of γ-secretase inhibitors suggesting that Hcy enhanced PS-1 activity.

HHcy and B vitamins deficiency have been recognized as risk factors for neurodegenerative diseases. The hypothesis that lowering Hcy concentrations would improve cognitive performance has been widely tested and inconclusive results were obtained. Hcy concentrations could be reduced via manipulation of the methionine cycle with different factors such as B vitamins, SAM, and DZA. Combined folate, B12, and B6 dietary deficiency induced Aβ overproduction

via up-regulation of enzymes that mediate APP processing in the amyloidgenic pathway (Fuso et al., 2008). Folate deprivation induced Hcy accumulation, while DZA addition prevented this effect in addition to the increased generation of ROS that normally accompanies folate deprivation (Ho et al., 2003).

In the current study, we showed that DZA reduced protein expression of APP in DS fibroblasts incubated in a vitamin-rich medium in a dose-dependent manner. This suggests that vitamin availability contributed into the mechanism by which DZA may affect APP generation and/or processing. Furthermore, we showed that in vitamin-free conditions, DZA had no effect on the immunoreactvity of full length APP and C99. However, in the presence of the highest concentration of DZA (150 μM), a significant difference between the reduction of APP in a vitamin-rich medium and that free of vitamins was observed ($p=0.014$). We assume that higher concentrations of DZA are necessary to show its effect in vitamin-free conditions. In addition, the effects obtained in cells grown in a vitamin-rich medium support the hypothesis that DZA might affect the neurodegeneration process probably via mechanisms related to its role as anti-oxidants (Shea et al., 2004;Ho et al., 2003).

Oxidative stress is an early event in the development of neurodegeneration diseases (Nunomura et al., 2001). Oxidants including the aldehydic end product of lipid peroxidation, 4-hydroxynonenal, found to up-regulate BACE-1 expression and activity in differentiated neuronal NT_2 cells (Tamagno et al., 2002). A number of studies have shown that antioxidants, both endogenous and dietary, can protect nervous tissue from damage by oxidative stress (Behl, 1999;Zandi et al., 2004). The molecular mechanisms by which antioxidants might protect the nervous system from damage caused by ROS are however not clear. In one study, the antioxidant N-acetyl-L-cystein was found to lower gene expression of APP in human neuroblastoma cells (Studer et al., 2001). The nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is a protein complex that controls the transcrition of DNA (Mattson et al., 2000) was found to be activated by Aβ-mediated oxidative stress (Schreck et al., 1991). Animal studies have shown that folate and vitamin E deficiency led to increased PS-1 expression that was later attenuated by apple juice concentrate administration in a genetic model of AD (Chan and Shea, 2006).

Disturbaces in one-carbon metabolism might show interaction with other risk factors like hypercholesterolemia. Epidemiological studies indicate that high serum cholesterol levels increase the risk of AD, and it has been proposed that the homeostatic regulation of cholesterol

metabolism may be altered in AD (Knebl et al., 1994). Moreover, recent reports show a significant reduction in AD risk for patients treated with statins [competitive inhibitors of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase], the enzyme that catalyzes the rate limiting step in cholesterol biosynthesis. Taken together, these suggest that the reduction of cholesterol levels may inhibit AD pathogenesis.

In our study, we found that adding lovastatin to DS fibroblasts lowered APP expression in cells cultured in a vitamin-rich medium or in a vitamin-free medium. The reduction in the presence of vitamins was significantly higher than it in the absence of vitamins (Figure 17.A). This difference might be related to an effect of lovastatin on the ratio of methylated and non-methylated phospholipids that determine the fluidity of the cellular membrane and thereby APP processing. In addition, in a vitamin-free medium, we found that lovastatin lowered C99 expression in a dose-dependent manner (Figure 17.B). Moreover, in cells grown in a vitamin-free medium, the addition of β- or γ-secretase inhibitors reversed the effect of lovastatin on both APP and C99 expression. Therefore, we assume that lovastatin inhibits the activity of both β- and γ-secretases enhancing APP processing in the non-amyloidgenic pathway.

Studies on the effect of statins on amyloid processing are not consistent. For example, lovastatin lowered Aβ (1-42) by approximately 70% in mixed rat cortical neurons (Fassbender et al., 2001). Additionally, (1-10 μM) lovastatin caused increased cellular APP level in extracts of primary hippocampal neurons, but lowered APP in low-density lipid rafts (Won et al., 2008). The authors suggested that lovastatin might act via mechanisms not related to cholesterol lowering.

Over the last decade, it has been established an increasing insight into the molecular mechanisms involved in the role of cholesterol as a highly potent regulator for Aβ generation. The study of Grimm and collegues analyzed the cellular mechanism by which cellular cholesterol levels influence intracellular Aβ production and found that cholesterol depletion results in a parallel and additive inhibition of the β- and γ-secretase activities and this inhibition can be partly separated from each other and therefore appears to be independent of each other (Grimm et al., 2008). Other studies have shown increased α-secretase activity because of cholesterol depletion (Kojro et al., 2001).

One hypothesis of the postulated statin-induced shift from β- to α-cleavage is that the inhibition of HMG CoA reduction by statins results in reduction in mevalonate and isoprenylation of small GTPases, which is essential for internalization and sorting of early endosomes (de et al., 2003). β -secretase activity depends on endocytic membrane recycling and the endosomal system (Cataldo

et al., 2000). Therefore, reduction of endosomal re-internalization is expected to reduce Aβ formation in favour of α-cleavage. The other hypothesis is the effect of statins directly on cholesterol transport among endocellular compartments and cholesterol content within the plasma lemma. The cellular membrane contains many different kinds of lipids, including phospholipids, sphingomyelin, and cholesterol. The distribution of these components of any given part of the membrane is not uniform. Some patches of membrane, termed lipid rafts, contain high amounts of sphingolipids, which are orderly packed by cholesterol molecules. Since cholesterol is a rigid molecule and lipid rafts have high content of cholesterol, lipid rafts are considered to be regions of low membrane fluidity. Increasing evidence suggests that the enzymes that generate Aβ, particularly β-secretase, function best in a high-cholesterol environment (Fassbender et al., 2001). In contrast, Kojro and collegues showed that sites of APPsα production occur in membrane regions with low cholesterol content and high fluidity (Kojro et al., 2001).

In summary, our investigations on DS fibroblasts showed that disorders in the methylation status could affect APP production or degradation. DZA may improve cognitive function via mechanisms not related to its role as SAH hydrolase inhibitor and the proposed protective effect of lovastatin against dementia seems to depend on methylation status. In-vivo studies are warranted.

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Publications

Folate and methylation status in relation to phosphorylated tau protein(181P) and beta-amyloid(1-42) in cerebrospinal fluid.
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Posters

Protein expression of amyloid precursor protein (APP) in Down syndrome fibroblasts is modified by methyl group metabolism.
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