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同胱胺酸與血管病之危險性

Homocysteine and the Risk of Vascular Diseases

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中文摘要

關鍵詞： 同胱胺酸;大腦白質病變;中風;心血管疾病;失智症

背景：

同胱胺酸是否是血管病的獨立危險因子，我們提出一些可能的假說:同胱胺酸有可能造成血流動力學的改變而減緩流速，進而造成血管動脈硬化;可能造成腦部大腦白質病變，進而引發中風及失智症;同胱胺酸是血管病的危險因子;高血清同胱胺酸與失智症有關，如果降低血清同胱胺酸，可能可以改善智力，或阻止智力惡化。

研究方法：

針對以上的假設，我們執行以下計劃：1.觀察性研究：觀察不同濃度的同胱胺酸對頸動脈與椎動脈的血流動力影響;2.病例對照研究：有大腦白質病變者其同胱胺酸濃度是否與無病變者不同;3.長期追蹤研究：同胱胺酸的濃度是否為未來發生腦中風及心臟病的預測因子;4.隨機雙盲研究：維他命療法對降同胱胺酸及智力改善或減緩智力退化是否有臨床效用。

結果：

同胱胺酸並不影響大血管之血流動力學。同胱胺酸為大腦白質病變之危險因子，每增加 1 μmol/L 同胱胺酸，其發生白質病變之相對危險值為 1.15 (95% CI, 1.01-1.31)。在長期世代追蹤研究，平均追蹤時間 11.95 年，同胱胺酸高的族群，其發生腦中風的危險並無顯著增加，但發生心血管疾病之危險性及死亡率有顯著增加。我們定出臨床上最適當的切點，發現同胱胺酸大於 9.47 μmol/L 者，發生心血管病之危險為小於此值的人的 2.3 倍 (95% CI,1.24-4.18)，大於 11.84 μmol/L 者，死亡之危險為 2.4 倍(95%CI,1.76-3.32)。降同胱胺酸維他命療法之

隨機雙盲實驗，治療半年後，維他命組，血清中葉酸及 B12 濃度明顯高於用安慰劑組，且同胱胺酸濃度在治療組也明顯低於對照組，然而此二組智能之變化並沒有因用維他命治療而有不同。

結論：

同胱胺酸並不影響血管的血流動力，但與微小血管產生的大腦白質病變有關。長期追蹤無症狀之成人，同胱胺酸並不會增加發生腦中風的危險，但卻是心血管病及死亡之危險因子。以降低同胱胺酸之維他命療法，對阿茲海默症患者之智能改善，沒有明顯的幫助。

Abstract

Key words: homocysteine; cerebral white matter lesion; stroke; coronary heart disease; dementia

Background

The relationship between elevated plasma homocysteine (Hcy) and vascular disease is stronger in retrospective than in prospective studies. We proposed the following 4 hypotheses: 1. Hcy may influence the hemodynamic flow of cerebral arteries and then may further induce atherosclerotic change; 2. Hcy may induce microangiopathy and lead to cerebral white matter change which may be related to future stroke and dementia; 3. Baseline Hcy may be related to future vascular event; 4. Hcy-lowering therapy with vitamin supplementation might be benefit for persons with dementia.

Material and methods

We conducted a cross-sectional study to explore the relationship between Hcy and the hemodynamic status of carotid and vertebral artery; a case-control study for Hcy and cerebral white matter lesions; a cohort study for Hcy and long-term vascular events; an experimental randomized control trial study for Hcy-lowering therapy on dementia.

Results

Hcy was not associated with the hemodynamic change on the extracranial cerebral arteries. However, Hcy is an independent risk factor for cerebral white matter change (multivariate RR 1.15, 95% CI 1.01-1.31). In the prospective cohort study with median 11.95 years of follow-up, participants with Hcy more than 9.47 μmol/L had a 2.3-fold risk for cardiovascular events (95% CI, 1.24-4.18, p=0.008), and participants with Hcy more than 11.84 μmol/L had a 2.4 fold risk for death (95% CI, 1.76-3.32, p<0.0001). Multivitamin supplements significantly elevated the concentration of vitamin B12

($p<0.0001$) and folic acid ($p<0.0001$) and lowered the plasma homocysteine concentration ($p=0.004$) after 26 weeks' treatment. However, no significant differences between the vitamin and placebo groups in the scores of cognition and activities of daily living were found.

Conclusions

Hcy was not associated with the hemodynamic change on the large extracranial cerebral arteries. The effects of Hcy on the brain may be related to cerebral microangiopathy. Homocysteine was significantly related to the cardiovascular events and all-cause death, with optimal cutpoint values as 9.47μmol/L and 11.84μmol/L respectively. Oral supplements by over-the-counter multi-vitamins containing B6, B12, and folic acid decreased Hcy concentration in patients with mild to moderate Alzheimer's dementia. However, there were no statistically significant beneficial effects on cognition and function for daily living.

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Chapter 1. Introduction and literature review

This research is specifically devoted to homocysteine (Hcy) and its relationship to vascular disease and dementia[1, 2]. Hcy plays a central role in folate and methionine metabolism, is now regarded by many as a potentially major risk factor for cardiovascular and cerebrovascular diseases[3, 4]. Hcy is also reported as a possible risk factor for Alzheimer's disease[2]. However, observational studies about the relationship between Hcy and vascular diseases are not consistent with the prospective studies[1]. Furthermore, the clinical benefit for the Hcy-lowering therapy on the prevention of vascular disease or Alzheimer's dementia is still unclear [5-7]. This research aimed to explore the role of Hcy on the brain vessels regarding cerebral hemodynamic status; cerebral microangiopathy related white matter lesions; long term vascular events in baseline healthy subjects; as well as the Hcy-lowering therapy on cognitive function in patients with dementia.

1.1 Historic aspects of homocysteine and vascular disease

Recognition of an association between the sulfur-containing amino acid Hcy and vascular disease had its origin in the 1960s [8]. It followed the identification of a new inborn error of metabolism in which a large quantity of Hcy was excreted in the urine[8] and shown to be associated with precocious vascular disease [9]. It was soon established that the disorder was due to cystathionine β-synthase deficiency [10]. The identification of other cases soon followed and in 1964 there was the first documentation of the pathological findings in homocysteinuria and the description of widespread of vascular changes and thrombosis [9]. In1969, it was first suggested by McCully that among the complex metabolic changes occurring in cystathionine β-synthase deficiency and the factor mediating the vascular changes was a greatly elevated concentration of homocysteine [11]. McCully noted that hyperhomocysteinemia arising from different

metabolic defects was associated with premature arteriosclerosis.

1.2 Biochemistry, metabolism and determinants of serum homocysteine

1.2.1 Source of homocysteine

Homocysteine is derived primarily from methione in dietary protein. Foods contain only trace amounts of homocysteine, which maintained at low concentration in both animal and plant cells. But the abundance of methione, the proximal precursor of homocysteine, varies widely according to the source of food proteins. Ingestion of a meal rich in protein elevate plasma homocysteine [12].

1.2.2 Methionine cycle (Figure 1)

Homocysteine is a metabolite of the methione degradation cycle. Methionine is metabolized via-S-adenosyl methionine and S-adenocyl-homocysteine to homocysteine in the course of producing methyl groups for use in synthetic process. On a normal diet about 50% of the homocysteine formed is metabolized via the transsulfuration pathway. The first step involves the enzyme cystathionine β-synthase for which pyridoxine (B6) is the co-factor and deficiencies of this enzyme result in the usual form of homocysteinuria. The remaining 50% of formed homocysteine is remethylated to methinone and requires 5, 10-methyltetrahydrofolate as substrate, and methylcobalamine as a co-factor. Remethylation by trimethylglycine may also occur via a separate pathway. The methionine cycle is complete when homocysteine in remethylated back to methionine [13].

1.2.3 Determinants of serum homocysteine

There are many variables that influence serum Hcy concentration. Age and sex are two of the stronger determinants of fasting Hcy levels [14]. Serum Hcy increases throughout life in both sex and greater in men than in women. Life style and diet are also correlated with Hcy. Smokers have higher levels of Hcy than non-smokers [15]. Hcy levels are

inversely correlated with vitamin intake [16]. Folate and cobalamin deficiency are common cause of moderate to severe hyperhomocysteinemia. Several diseases also influence the Hcy levels. Renal failure is the clinical status most often responsible for elevation of Hcy [14]. Hcy is moderately elevated in hypothyroidism and low in hyperthyroidism [17]. This may be related to the influence of thyroid status on riboflavin or folate function, glomerular infiltration rate, or creatinine synthesis. A variety of drugs affect Hcy levels. They act via different mechanisms, including inhibition of vitamin (folate, cobalamin or vitamin B6) function, by affecting Hcy production and interfering with renal function [14]. Genetic defects in the enzymes that metabolize Hcy can contribute to mild, moderate and severe hyperhomocystenemia, depending on the nature of the gene product and the level of residual enzyme activity [14]. The most common enzyme defect associated with moderately raised Hcy is due to a single, common, polymorphic variant of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene.

1.3 Vascular pathology and pathophysiology of hyperhomocysteinemia

1.3.1 Vascular pathology of hyperhomocysteinemia

The findings of vascular damage, characterized by fibrin deposition, focal necorsis of artery wall, swelling of endothelial cells, and microthrombi suggest a common pathophysiological atherogenic process in hereditary hyperhomocysteinemia [9]. The development of arteriosclerotic plaques in homozygous homocysteinria closely parallels the pathology of human atherosclerosis.

1.3.2 Plausible mechanism of vascular damage by hyperhomocysteinemia

In vitro evidence exists for effects of Hcy on atherogenesis and thrombogenesis. Experimental studies have shown that homocysteine may be harmful to vascular smooth muscles cells and promote proliferation of vascular smooth muscle cells. Hcy may also

have other possibly detrimental effects [18-24]. However, most of the studies showing these effects have been undertaken with supra-physiological concentrations of nonphysiological forms of Hcy [25]. Thus, the results of many studies [19-24] may not be generalisable to humans with mild and moderate hyperhomocysteinuria.

1.4 Homocysteine as a risk factor for vascular diseases

1.4.1 Homocysteine, hemodynamics and atherosclerosis

Hemodynamic status of slow flow and low shear stress related vascular remodeling is one of the pathogenesis of atherosclerosis [26-28]. Hemodynamic data obtained by ultrasonography include blood flow velocity, flow resistance and flow volume [29, 30]. Hcy has been reported to be higher in those with slow coronary artery flow than in those with normal flow velocity [31]. Hcy is an important factor for atherosclerosis in the large cerebral arteries [32]. In 1,041 Framingham residents who had Hcy measurement and carotid sonography. The adjusted odds ratio (OR) for stenosis ≥ 25% was 2.0 (95% confidence interval, CI, 1.4-2.9) for subjects with Hcy levels in the highest compared to the lowest quartile [32]. In the Atherosclerosis Risk in Communities (ARIC) Study, subjects with thickened intima-medial carotid walls (≥ 90th percentile) were more likely to have elevated Hcy levels compared to those without thickened walls (<70th percentile) [33]. Hcy may possibly slow the blood flow velocity, and further induce atherosclerosis with reducing the brain flow volume. However, the reports investigating the relationship between serum Hcy and the cerebral hemodynamic status were limited.

1.4.2 Homocysteine, microangiopathy and cerebral white matter lesions (WML)

Sclerosis of small cerebral arteries and arterioles is responsible for the diffuse periventricular white matter abnormalities and the central lacunar lesions evidenced in computed or magnetic resonance tomography [34]. The severity of cerebral white matter

lesions (WML) increases with age and the presence of arterial hypertension [35-37]. Although the clinical significance of these lesions remains to be fully understood, WML have been associated with dementia, depression, stroke and mortality [38-41]. Patients with Alzheimer's disease (AD), vascular dementia, and depression have more severe WML than control [38, 39].

It is well known that hyperhomocysteinemia is an independent risk factor for vascular disease. The pathophysiologic mechanism by which Hcy induces angiopathy remains unclear, although various hypotheses have been proposed [42-44]. A prospective study performed in Japanese showed significant association between Hcy and risk of lacunar infarcts [45]. This finding indicated that Hcy may be related to small vessel disease. Silent brain lacunar infarcts and WML are thought have a small vessel disease origin and are frequently seen in neurologically asymptomatic elderly people [35, 36, 46] and are common among persons with cognitive impairment and dementia [38, 39]. Studies have determined the association between Hcy and vascular lesions as well as Hcy and dementia [2, 47-51]. In view of aforementioned report, we propose a hypothesis that Hcy may induce the risk of cerebral arteriole angiopathy and further induce WML and lacunar infarcts; and then further cause cognitive impairment. A population-based study from Rotterdam demonstrated that Hcy is associated with cerebral WML [52]. However, studies about the association between Hcy and WML are limited among Asia population.

1.4.3 The association between total serum homocysteine and clinical vascular events

Epidemiological studies, using cross-sectional, case-control, and cohort designs, have examined the association between Hcy and cerebrovascular disease. Many case-control

and cohort studies have identified a strong, independent and dose-related association between moderately elevated Hcy and atherosclerotic vascular disease, including stroke [1]. Brattstrom et al., first reported a case-control study in 1984 that moderate hyperhomocysteinemia was a possible risk factor for atherosclerotic cerebrovascular diseases [53]. These investigators measured non-protein-bounded Hcy following methionine loading in 19 patients with TIA or minor stroke. Sixteen of these patients had Doppler or angiographic evidence of internal carotid artery stenosis or occlusion. In 1992 Brattstrom et al., in a large series of stroke patients, found that plasma Hcy concentrations were not only elevated in carotid artery disease and lacunar stroke but also in hemorrhagic and embolic stroke [47]. Coull and colleagues found similar elevations of Hcy in patients with acute stroke and TIA. Plasma Hcy concentration was moderately but significantly higher in patients than in control (P<0.0001)[48]. In a meta-analysis employing 27 studies relating Hcy to arteriosclerotic vascular in relation to the effects of folic acid on lowering Hcy concentration, Boushey et al., determined the effects of Hcy on the risk of three categories of vascular disease (coronary, cerebrovascular and peripheral artery diseases)[54]. Elevations of Hcy were considered an independent graded risk factor for arteriosclerotic vascular disease: a 5 μmole/L Hcy increment elevates risk by as much as a cholesterol increase of 0.5 mmole/L (20 mg/dL). The OR for cerebrovascular disease is 1.5 (95% CI, 1.3 to 1.9) [54].

However, not all reports have been consistent. Several prospective cohort studies have failed to demonstrate a positive association between Hcy and stroke [55, 56]. A Finnish study failed to show a significant association between hyperhomocysteinemia and stroke [55]. The relation of serum total Hcy with incidence of atherosclerotic disease was investigated among 7424 men and women aged 40-64 years free of atherosclerotic disease at baseline in 1977. During the 9-year follow-up, 134 male ad 131 female cases

with either myocardial infarction or stroke were identified. The mean serum Hcy concentration of male cases and controls was 9.99 μmole/L and 9.24 μmole/L at baseline and that of female cases and controls was 9.58 μmole/L and 9.24 μmole/L respectively. There was also no significant association between Hcy and atherosclerotic disease, myocardial infarction or stroke in logistic regression analyses. The odds ratios varied from 1.00 to 1.26 for Hcy. The results of this prospective population-based study do not support the hypotheses that serum Hcy is a risk factors for atherosclerotic disease. Furthermore, two large randomized placebo-controlled trials of Hcy-lowering therapy, Vitamins to Prevent Stroke Study (VITATOPS) for patients with patients with a recent transient ischemic attack or stroke, as well as the Vitamins to Prevent Stroke (VISP) study for patients with a first ever non-disabling stroke, failed to show the clinical benefit on stroke prevention [57, 58].

1.5 The points favor or not favor on a causal relationship between Hcy and vascular diseases or cognitive impairment

In view of the literatures, we can summarize some following findings and conclusions about this issue.

1.5.1 The points in favor of a causal relationship between Hcy and vascular diseases

(1) An association between elevated Hcy and atherosclerotic vascular disease is biologically plausible; experimental studies have shown elevated Hcy to be atherogenic and thrombogenic [19-23].

(2) Systematic review of epidemiological studies [59, 60] and meta-analysis study [4] have revealed a reasonably strong, independent, dose-related relationship between higher plasma concentration Hcy and the occurrence of cerebral, coronary, and peripheral vascular disease.

(3) High Hcy has been associated with dementia [49-51, 61]. The influence of elevated Hcy on the intracranial small vessels disease was thought to be the cause of cognitive impairment [62].

(4) Reducing plasma Hcy by means of multivitamin therapy produces favorable effects on surrogate markers of vascular disease, such as endothelial function [63-65], and carotid artery intima thickness [66].

1.5.2 The points that cast doubt on a causal relationship between Hcy and vascular diseases

(1) Most experimental studies that have shown hyperhomocysteinemia to be atherogenic and thrombogenic have been undertaken with supraphysiological concentrations of Hcy [19-25].

(2) There have been inconsistencies in the results of epidemiological studies obtained by different methods; stronger associations have been found in retrospective cross-sectional, or case-control studies, and small or no associations have been found in prospective cohort studies [55, 60].

(3) The temporal relationship between the onset of elevated Hcy and the onset of white matter lesions, cognitive impairment, stroke and other vascular events is unclear [52, 67, 68].

(4) There is no reliable evidence homocysteine lowering therapy can prevent vascular events [5, 6, 57, 58, 69]. Two large randomized placebo-controlled trials of Hcy-lowering therapy, Vitamins to Prevent Stroke Study (VITATOPS) for patients with a recent transient ischemic attack or stroke, as well as the Vitamins to Prevent Stroke (VISP) study for patients with a first ever non-disabling stroke, failed to show the clinical benefit on stroke prevention [57, 58].

Importantly, it is necessary to reassess the role of Hcy on the vascular diseases.

1.6 Homocysteine as a risk factor for dementia

Subjects with cardiovascular risk factors and a history of stroke have an increased risk of both vascular dementia and Alzheimer's disease [70-72]. Plasma total Hcy has recently emerged as a vascular risk factor. And vascular factor of hypoperfusion is related to AD [73-75], hyperhomocysteinemia may play a role in developing AD. Besides, it is generally known that deficiency of certain vitamins, particularly vitamin B_{12}, can cause cognitive impairment, which can be reversed by correcting the deficiency[76-79]. Methionine synthesis from homocysteine requires folic acid as a methyl donor, and methyl cobalamin (vitamin B12) as the intermediate methyl acceptor and donor. Vitamin B6 participates in the alternate homocysteine catabolic pathway leading to the production of cysteine. Thus vitamin deficiency can induce hyperhomocysteinemia [80, 81]. There is evidence that plasma homocysteine (Hcy) concentration may be a modifiable risk factor for cognitive decline [2]. Hcy concentration has been reported to be higher in persons with Alzheimer's dementia [82-84]. The results of several studies showed that Hcy was inversely associated with performance of cognitive function [85-87].

There is great interest in cheap and less harmful over-the-counter vitamin supplements that may retard the worsening of cognition in the elderly.

The inverse relation between plasma Hcy levels and blood concentrations of folic acid and vitamin B_{12} makes it difficult to distinguish the independent effects of each on cognitive function. There is no report of Hcy-lowering therapy for mild to moderate Alzheimer's dementia.

Chapter 2. Hypotheses and objectives

2.1 Hypotheses

(1) Hypotheses for the effects of Hcy on cerebrovascular disease

(a) Hcy may influence the hemodynamic flow of cerebral arteries with reducing the flow velocities and then further induce atherosclerotic change.

(b) Hcy may induce microangiopathy and resulted cerebral white matter change which are essential for future stroke and dementia.

(c) The impacts of elevated plasma Hcy are significant on vascular diseases

(2) Plasma Hcy levels are higher in patients with AD. Hcy induced vascular factors that may underlie AD. Therefore homocysteine lowering therapy with vitamin supplementation might be benefit for persons with AD.

2.2 Objectives

Our **aims** are as follows:

(1) To explore the influence of Hcy on the hemodynamics of the extracranial cerebral arteries – carotid and vertebral arteries;

(2) To evaluate the association between Hcy and the microangiopathy related cerebral white matter lesions;

(3) To evaluate the prediction of Hcy on vascular events among ethnic Chinese healthy people;

(4) To evaluate the benefit of Hcy-lowering therapy on cognitive performance in patients with AD.

Chapter 3. Subjects and Methods (Figure 2)

3.1 Impacts of homocysteine on hemodynamic status

3.1.1 Participants

The study participants were invited from the population presenting to En Chu Kong Hospital (Taipei, Taiwan) for a health physical check-up between 1999 and 2007. We recruited 542 participants (mean age ± SD, 55.2 ± 14.8 years, 56% males), who were free from history of stroke, transient ischemic attack, coronary heart disease, or intermittent claudication. All participants underwent detailed studies that included clinical questionnaires, physical examination that included measurements of height, body weight, body mass index, and blood pressure. Duplex ultrasonography on the carotid artery, vertebral artery and blood laboratory tests were performed.

3.1.2 Ultrasound procedure and hemodynamic measurements

Ultrasonography was performed with a Hewlett-Packard 5500 system equipped with a high-resolution broadband width linear array transducer (4-10 MHz) [88]. Participants were examined in the supine position. An experienced technologist who was kept unaware of the patient' clinical data made all ultrasound measurements. Images were obtained bilaterally from the proximal common carotid artery (CCA) to distal CCA, including bifurcation, internal carotid artery (ICA) and external carotid artery (ECA), as well as the vertebral artery (VA) obtained from C2 to C6 segments (Figure I-1, I-2).

We measured the flow velocity and resistance of the CCA, ICA, ECA and VA as the flow parameters [89]. We also calculated the flow volume of the VA. Regarding the flow velocity, we measured the peak systolic velocity, end-diastolic velocity, time average flow velocity, mean flow velocity. The following formula is used to calculate the mean flow velocity: (mean flow velocity = [peak systolic velocity + 2 end-diastolic velocity]/3) [90]. As for the resistance of the vessel, resistance index and pulsatility index

were measured. Resistance index is calculated with the formula: (resistance index = (peak systolic velocity - end-diastolic velocity)/ peak systolic velocity) [91]. Pulsatility index described the shape of the waveforms. The formula calculating pulsatility index is: [pulsatility index = (peak systolic velocity - end-diastolic velocity)/ mean flow velocity)] [92]. Pulsatility index and resistance index are believed as presumptive measures of downstream vascular resistance [90]. We measured diameter to calculate the flow volume. Color-coded flow image was used to measure the diameter. Flow volume is the product of flow velocity and the area in which this velocity occurs (Flow volume [cm^3/s] = flow velocity [cm/s] × area [cm^2]) [90].

3.1.3 Laboratory analysis

Blood samples were collected to determine the levels of Hcy, total cholesterol, triglycerides, high-density lipoprotein, low-density lipoprotein, blood glucose, hemoglobin, platelets, blood urea nitrogen, creatinine, and uric acid after an overnight fasting with standard techniques. Venous blood samples for Hcy, collected into tubes containing ethylenediaminetetraacetic acid, were centrifuged within 30 minutes at 2000 rpm for 10 minutes, and then separately stored at -70°C until analysis. Serum Hcy concentrations were measured by fluorescence polarization immunoassay (Abbott IMx System) [93, 94], which were correlated well to high performance liquid chromatography. The coefficients of variation were within 5.2% [95].

3.1.4 Statistical analysis

Populations were categorized according to Hcy quartiles. Continuous variables were presented as the mean (SD) or the median, while the categorical data were presented in contingency tables. All the flow parameters of the examined vessels in the statistical analyses were the mean values from bilateral measurements, including diameter, peak systolic velocity, end-diastolic velocity, time average flow velocity, mean flow velocity,

resistance index, pulsatility index and total flow volume. ANOVA and the χ^2 test were used to test the differences of the vascular risk factors and CCA and VA flow parameters between Hcy quartiles.

Multiple linear regression models were used to examine the relationship between Hcy and the aforementioned flow parameters of the examined vessels. We estimated the means of the flow parameters with adjustment for age and sex and additionally for current smoking, systolic blood pressure, body mass index, serum creatinine, serum uric acid and high density lipoprotein. In above analyses, we modeled Hcy concentrations as quartiles to avoid the assumption of linearity and to reduce the effects of outliers. To test for linear trends across Hcy quartiles, we used median Hcy concentration for each category in the multivariate model. In addition, to estimate the effects of Hcy, we calculated the odds ratio (OR) and corresponding 95% confidence interval (CI) for the change of flow parameters of the CCA and VA according to one-SD increase of Hcy.

All statistical tests were 2-tailed and the P values <0.05 were considered statistically significant. Analyses were performed with SAS software (version 9.1; SAS Institute).

3.2 Homocysteine and cerebral white matter lesions (WML) on MRI

3.2.1 Participants

This study population was drawn from subjects presenting to En Chu Kong Hospital (Taipei, Taiwan) for a routine physical check-up between 1999 and 2007. Detailed physical examinations and questionnaires were performed and administered. MRI was performed on all the study subjects. The inclusion criteria for case subjects were as follows: healthy adults with no history of vascular disease including cerebrovascular disease, transient ischemic attack, and myocardial infarction; WML was

noted on the MRI check-up. A diagnosis of WML was made by MRI examination and by agreement between independent experienced neurologist and neuroradiologist. As for the control subjects, we selected healthy individuals, who were free from a recent or past history of cerebrovascular disease or of myocardial infarction, presenting at our hospital for a health examination, and were with normal brain MRI. Both case and control group were prospectively enrolled during the same period. Baseline demographic data and a history of conventional vascular risk factors were obtained from participants from both groups. Diagnosis of brain MRI study was made without knowledge of Hcy levels and presence of risk factors.

3.2.2 MRI protocol

WML were considered present if visible as hyperintense on FLAIR and T2 weighted images on the subcortical or periventricular area (Figure II-1), without prominent hypointensity on T1 weighted scans.

All examinations were performed using 1.5-T magnet system (Eclipse, Picker International, Inc., Highland Hts, Ohio). The image protocol consisted of T2-weighted spin echo for coronal slice (TR/TE = 3,600/112 msec), T1-weighted spin echo for sagittal slice (TR/TE=500/12.1 msec), FLAIR (TR/TE=6,000/96 msec, inversion time 2,100) for axial slice, diffusion weighted image and MR angiography.

3.2.3 Statistical analysis

To estimate the magnitude of the association between Hcy and WML, we used OR and 95% CI. For the crude analysis of baseline characteristic, we used the χ^2 test for categorical data, and the two sample *t*-test for continuous data. For the multivariate analysis, we used logistic regression to adjust for possible confounders, i.e., age, sex,

hypertension and diabetes mellitus. Logistic regression models were also used to estimate the OR of WML for quartiles of Hcy levels, to assess the relation between Hcy and other relevant risk factors. The relationship between Hcy levels and the presence of WML was also analyzed using multivariate regression models.

3.3 Homocysteine and long-term vascular events (Chin-Shan cohort)

3.3.1 Participants and study design

The participants were enrolled in the Chin-Shan Community Cardiovascular Study, a prospective community-based study of risk factors and cardiovascular consequences.[3, 96, 97] In brief, the study was started in 1990 with an initial cohort 3602 participants, who were recruited on the basis of official registrations. This study was approved by the institutional review boards of the National Taiwan University. The study collected information regarding medical history, the results of physical examination and laboratory tests, and an assessment of health status that included evidence of stroke and cardiovascular disease since baseline assessment and the follow-up period. Among the participants, 2117 subjects had homocysteine measurement during 1994 to 1995, in whom 2009 were free from history of stroke, cardiovascular disease and cancer. We collected detailed information about lifestyle factors including alcohol intake, smoking, and regular exercise, as well as data regarding socioeconomic status and family history of stroke and coronary heart disease (CHD). With regard to the follow-up schedule, we gathered information about stroke, cardiovascular events and deaths through monthly collections of official death certificate documents, by annual questionnaires, and by house-to-house visits.

3.3.2 Ascertainment of events

The study outcomes were stroke, CHD and all-cause death. Stroke was defined as a

sudden neurological deficit of vascular origin that lasted longer than 24 h and was proved by evidence from an imaging study [98]. Transient ischemic attacks were not included in this definition. Incident CHD cases were defined as non-fatal myocardial infarction, fatal CHD, and hospitalization for percutaneous coronary intervention and coronary artery bypass surgery. Fatal CHD was considered to have occurred if fatal myocardial infarction was confirmed by hospital records, if CHD was listed as the cause of death on the death certificate or was the underlying and most plausible cause of death [99]. Deaths from any cause were identified from official certificate documents and further verified by house-to-house visits.

3.3.3 Measurements of serum homocysteine and other biochemical variables

The procedures of blood sampling have been reported elsewhere [98, 100, 101]. In brief, all venous blood samples drawn after a 12-h overnight fast were immediately refrigerated and transported within 6 h to the National Taiwan University Hospital. Serum samples were then stored at -70 ºC until analysis. Serum Hcy were collected into tubes containing ethylenediaminetetraacetic acid and were measured by fluorescence polarization immunoassay (Abbott IMx System) [93, 94], which correlated well with high performance liquid chromatography. The coefficients of variation were within 5.2% [95].

3.3.4 Statistical methods

Participants were classified by quartiles of Hcy concentration. Continuous variables were presented as mean (SD) or median values, and categorical data were presented in contingency tables. ANOVA and the chi-square test were used to test differences between quartiles. Relationships between baseline Hcy concentrations and blood pressure, fasting glucose, body mass index and lipid profiles were evaluated with age- and sex-adjusted Spearman partial correlation coefficients.

Incidence rates for stroke, CHD and all-cause death were calculated for each Hcy

quartile by dividing the number of cases by the number of person-years of follow-up. We estimated the relative risk (RR) and 95% confidence interval (CI) by the multivariate Cox proportional hazards models. We specified 4 models for estimating the RRs of events in higher Hcy quartiles relative to the lowest quartile. In model 1, we estimated the univariate RR of Hcy with the first quartile as the reference. In model 2, we adjusted for age and sex variables. In model 3, we additionally adjusted for body mass index, educational level, occupation, alcohol intake, smoking, and regular exercise. In model 4, we adjusted for the presence/absence of hypertension and diabetes at baseline, family history of stroke and coronary heart disease, continuous variables of total cholesterol, triglyceride, HDL-C and LDL-C concentrations along with adjustments for the variables in model 3. We modeled Hcy concentrations as quartiles to avoid the assumption of linearity and to reduce the effects of outliers, and we used median Hcy concentrations for the categories to test for linear trends across quartiles. In secondary analyses, we constructed receiver-operating-characteristic (ROC) curves to generate the optimal cutoff point with highest Youden's index for the occurrence of stroke, CHD and death. We then calculated hazard ratios for stroke, CHD and death for high and low Hcy level using aforementioned cutpoints. The multivariate-adjusted hazard ratios were presented as the model 4.

To test the feasibility of the cutpoints of Hcy, ROC curves were further plotted again for full adjusted model (age, sex, body mass index, educational level, occupation, alcohol intake, smoking, regular exercise, hypertension, diabetes and family history of stroke and coronary heart disease) with Hcy and for that without Hcy. The discrimination of models with and without events were assessed by comparing the *c* statistics for CHD and death respectively, where Hcy level was treated as binary variable with aforementioned cutpoints. In addition, the increased discriminative value

of Hcy was further examined with the method described by Pencina et al.[102]. Two statistics, including, net reclassification improvement, and, integrated discrimination improvement, were presented with the priori meaningful risk categories set as follows: the 10-year estimated low, median and high risk rate were defined at 5%, 15% and 25% for stroke; 10%, 25% 30% for CHD ; and 5%, 10% 20% for death [103].

All statistical test were 2-tailed with type I error of 0.05, and *P* values <0.05 were considered statistically significant. Analyses were performed with SAS software (version 9.1; SAS Institute).

3.4 Homocysteine lowering clinical trial on Alzheimer's disease

3.4.1 Participants and study design

This is a 26-week, double-blind, placebo-controlled, randomized clinical trial conducted in En Chu Kong Hospital, Taiwan from July 2003 to March 2006. The inclusion criteria were history of cognitive decline that was gradual in onset and progressive over a period of more than 6 months; clinical diagnosis of mild to moderate AD based on criteria set forth by the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) [104]; a score of 10-26 on the Mini-Mental State Examination (MMSE) [105, 106]; and a screening score of 1-2 on Clinical Dementia Rating Scale (CDR) [107]. Exclusion criteria were the following: history of epilepsy; clinically significant hepatic, renal, pulmonary, metabolic or endocrine disturbances, or significant cardiovascular disease that would impede the subject's ability to complete the trial; vascular dementia or evidence of cerebrovascular disease; low serum levels of folic acid (<5 ng/ml) and vitamin B_{12} (<180 pg/ml); use of any vitamin supplements and all the agents for the treatment of dementia (approved, experimental, or over the counter) except for acetylcholine esterase inhibitors (AChEIs); cognitive impairment due to acute cerebral

trauma, hypoxic cerebral damage, infection, primary or metastatic cerebral neoplasm, significant endocrine or metabolic disease, or mental retardation. Computer tomography (CT) or Magnetic radioimaging (MRI) was performed on all study participants to rule out structural brain lesion (i.e. stroke, tumor, chronic subdural hematoma, etc.)

For ethical considerations, all of the 89 enrolled participants were also prescribed with AChEIs, the FDA approved treatment for AD patients. Except for one taking rivastigmin (® Exelone), all of the other patients were concurrently taking donepezil (® Aricept) in addition to our study regimens. All of the patients were then randomly allocated to receive the add-on therapy of either multi-vitamin or placebo according to a 1:1 ratio for six months. All of the study personnel and participants were blinded to treatment assignments and randomization was performed using a computer-generated randomization list. Efficacy measurements for the double-blinded phase were performed at baseline and week 26.

The study was conducted in accordance with Declaration of Helsinki and was approved by the hospital's Institution Review Board. Written informed consent was given by each patient or patient's representative or by the caregiver participating in this study.

3.4.2 Study regimens

The study regimens used mecobalamin preparation (trade name Methycobal) capsule (Eisai Co.) containing vitamin B12 0.5 mg with an active methyl base, and another over-the-counter vitamin named Pramet®FA (Abbott Laboratories Services Corp.) containing folic acid 1 mg, pyridoxine HCl 5 mg, iron ferrous 60 mg, nicotinamide 10 mg, calcium carbonate 250 mg, riboflavin 2 mg, thiamine mononitrate 3 mg, calcium pantothenate 1 mg, ascorbic acid 100 mcg, iodine 100 mcg, copper 150 mcg, vitamin B_{12} 3 mcg, vitamin A 4000 I.U., and vitamin D3 400 I.U. In Taiwan, Pramet®FA tablet

was originally used as a kind of nutritional supplement for pregnant women. The placebo of Pramet®FA and Methycobal were made by the Genovate company (the manufacturer for Abbott Corp. in Taiwan) and Eisai Pharmaceutics Company respectively and were identical in size and color to vitamin supplements.

3.4.3 Assessment and outcome measures

Patients were assessed at the beginning with detailed history taking, blood tests, neuro-imaging studies, and neuropsychological tests. Screening cognitive tests included the scale of CDR and MMSE. Baseline blood tests included standard fasting venous blood sample for blood chemistry (liver and renal function profile, fasting sugar), complete blood count (CBC), thyroid function (T3, TSH), syphilis blood test (VDRL, TPHA), and serum levels of B_{12}, folic acid, and homocysteine.

The study patients were enrolled and received baseline cognitive measures, including Cognitive Abilities Screening Instrument (CASI, Chinese version, C-2.0, Liu) [108-110], Alzheimer's Disease Assessment Scale (ADAS-Cog/11, Chinese version, Liu) [111-113], the Simplified Barthel Activities of Daily Living Index (ADL), and the Instrumental Activities of Daily Living Scale (IADL). The patients were then interviewed by the same neurologist, who was blinded to the patients' assigned study medication, at the outpatient department at weeks 2, 6, 10, 14, 18, 22, and 26 for physical and neurological examination. Any subjective or objective findings, as well as any favorite effects or adverse events (AEs) after treatment were recorded. At week 26, serum levels of B_{12}, folic acid, and Hcy were re-measured. Cognitive performance and daily living function were assessed by the scores of MMSE, CASI, ADAS-Cog/11, ADL, and IADL.

3.4.4 Outcome measures

The primary efficacy outcomes were the change from baseline to week 26 in

ADAS-Cog [113] for the cognition evaluation, which includes an assessment of 11 items on the cognitive subscale of the ADAS (spoken language, comprehension, recall of test instruction, word-finding difficulty, following commands, naming, constructions, ideational praxis, orientation, word recall, word recognition). Cognitive assessments were carried out at screening baseline and at week 26 by the same independent rater who was blinded to the patients' assigned study medication and other study information, such as adverse events.

Secondary efficacy outcomes included assessment of cognition changes from baseline to week 26 on activities by using the score of the CASI and MMSE. The CASI complete form (CASI-C) provides quantitative assessment (scoring from 0 to 100) of attention, concentration, orientation, short-term memory, long-term memory, language abilities, visual construction (copying two intersecting pentagons), list-generating fluency, abstraction, and judgment. These instruments were based on information collected independently from the subject and caregiver. Another secondary outcome measurement was an evaluation of the change from baseline to week 26 of activities of daily living function. The measurements included the ADL and the IADL. The ADL indices were measured using a 10-item for evaluating daily function (toilet, grooming, feeding, physical ambulation, dressing, bathing), while the IADL scale (based on the report of M. P. Lawton and E. M. Brody) [114] used an 8-item evaluation of the ability to use the telephone, shopping, food preparation, housekeeping, laundry, mode of transportation, responsibility for own medications, ability to handle finances. Secondary outcomes also included the change of plasma Hcy, serum vitamin $B_{12,}$ and folic acid levels.

3.4.5 Safety evaluations

Safety and tolerability were evaluated by comparing treatment groups with respect to

physical examination findings, changes in vital signs, laboratory test abnormalities, concomitant medication use, and compliance with study medication, as well as the monitoring of adverse events (AEs) throughout the study. An AE was defined as any undesirable effect experienced by a patient during the trial, whether or not related to treatment. A serious adverse event (SAE) was defined as any AE that was life threatening or resulted in death, hospitalization, prolongation of hospitalization, or significant disability.

3.4.6 Data analysis

To compare the effects of multi-vitamin therapy and placebo on the cognitive function assessed using the ADAS-cog scale between the time of enrollment and week 26, according to an intention-to-treat strategy, the initial assumption was that there were 2 points of differences in the ADAS-cog scale between these 2 groups, with 4 of common standard deviation. We estimated the sample size required for a two-tailed significance level of 0.05 and a power of 0.90. On this basis, 43 patients were needed in each group. Assuming the drop-out rate was 20%, the ideal number of total enrolled participants should be up to 108.

We first compared characteristic variables between the placebo and treatment groups, including baseline plasma levels of homocysteine, vitamin B_{12}, and folic acid, as well as cognitive screening tests using the *chi*-square test for categorical data and the Student's t test for continuous data. The changes in plasma concentrations of vitamin B_{12}, folic acid, and homocysteine were then compared, from baseline to 6 months, using non-parametric Wilcoxon two sample tests because the values of the variables were not normally distributed.

Paired t–test was used to evaluate the efficacy of the intervention on cognition (MMSE, CASI, ADAS- Cog) and daily living function (IADL and ADL) by comparing

the changes from baseline to 6 months between the placebo and treatment groups. All were based on an intention-to-treat analysis. A multiple linear model was performed to assess the association between the change of homocysteine and change of cognition. The ANCOVA test was used to compare the efficacy of intervention adjusting the effect of baseline cognition status, age, and sex variables. Linear regression was used to assess the association between the score change and the Hcy change after adjustment for baseline Hcy values, age, and sex. The number of adverse events between the placebo and treatment groups was also compared by using the *chi*-square test and Fish exact test if the expected number less than 5.

Chapter 4. Results

4.1 Homocysteine and the hemodynamic status of the extracranial large arteries (Table I-1 to Table I-7)

The median (interquartile range) and mean (± SD) of tHcy for the 542 study participants were 9.1 (7.4 to 11.3) and 9.9 μmol/L (± 4.9) respectively. The median (interquartile range) of tHcy was 7.1 (6.4 to 9.2) μmol/L in women compared with 10.1 (8.4 to 12.1) μmol/L in men (P <0.0001). The respective ranges for tHcy (μmol/L) quartiles were: first, 1.91-7.36; second, 7.37-9.07; third, 9.08-11.32; and fourth, 11.33-58.7. Baseline characteristics of the study population by tHcy quartiles were listed in Table I-1. Higher tHcy levels were associated with older age, current smoking, systolic blood pressure, body mass index, serum creatinine, and serum uric acid. With regards to flow parameters across tHcy quartiles, participants with higher tHcy levels were more likely to have slow flow velocity (systolic and diastolic flow velocity) in the CCA, ICA and VA (Table I-2, I-3). The resistance (including resistance index and pulsatility index) was increased in the CCA and decreased in the VA as Hcy increase (Table I-2, I-3). The diameter and flow volume of the vertebral artery were similar across tHcy quartiles.

After adjusting for demographics and risk factors, the least-squares mean values of the carotid artery and the VA flow parameters across quartiles of tHcy are shown in Table I-4, I-5. There was no significant difference on the flow velocity, resistance, VA diameter and flow volume between different levels of tHcy. When tHcy considered as a continuous variable, the tHcy levels were not significantly related to the aforementioned hemodynamic status of the CCA, ICA and VA (Table I-6, I-7). The results were unchanged if we calculated the parameters of the examined vessels from left and right side separately (data not shown).

4.2 Homocysteine and microangiopathy related cerebral WML (Table II-1 to Table II-2)

We conducted a case control study in a total of 258 asymptomatic adults (mean age 51 years) which were classified into case group with microangiopathy related cerebral white matter lesions noted on the MRI and control group with normal brain MRI study. The mean serum Hcy levels was 9.1 μmol/L in the 65 participants of case group and 8.9 μmol/L in control group (p=0.83). The demographic and laboratory data between theses two groups were shown in Table II-1. However, after fully adjustment of the confounding variables such as age, sex, blood pressure and other vascular factors, Hcy remained an independent risk factor for cerebral white matter lesions. Every 1 μmol/L increase of Hcy, the relative risk for white matter change was 1.15 (95% CI 1.01-1.31, P=0.03) (Table II-2).

4.3 Homocysteine and long-term clinical vascular events (Chin-Shan cohort) (Table III-1 to Table III-4)

Baseline characteristics of the participants are shown in Table III-1. Participants in the highest Hcy quartile were less likely to be female and be in professional business job, compared with those in the lowest quartile. They were likely to smoke, and drink alcohol and had a higher prevalence of taking regular exercise and had hypertension at baseline. A higher Hcy concentration was associated with an old age, higher systolic blood pressure, higher triglyceride and lower HDL-C concentrations.

Over a median 11.95 years' follow-up among the 2009 participants, we documented 114 cases of stroke (including 92 ischemic and 22 hemorrhagic strokes), 95 cases of CHD and 380 deaths (including 50 cardiovascular mortality cases). The

incidence rates for each event increased appreciably with Hcy quartile for stroke, CHD and all-cause death (Table III-2). The RRs for individuals in the highest quartile of Hcy, compared with those in the lowest quartile, were 4.40 (95% CI, 2.43-7.94; *P* for trend <0.0001) for stroke, 13.90 (95% CI, 5.00-38.65; *P* for trend <0.0001) for CHD and 5.59 (95% CI, 3.99-7.84, *P* for trend <0.0001). After fully adjustment for risk factors (model 4), the RRs for individuals in the highest quartile of Hcy compared with those in the lowest quartile were 1.0 (95% CI, 0.49-2.45; *P* for trend, 0.67) for stroke, 3.73 (95% CI, 1.22-11.40; *P* for trend, 0.04) for CHD and 1.35 (95% CI, 0.90-2.03, *P* for trend, 0.001) for all-cause of death. A significant linear trend for an increased RRs of CHD and death with each quartile of Hcy levels was found (Table III-2, Figure III-1, III-2, III-3).

We then performed subgroup analyses in the type of stroke and death. We analyzed the association of Hcy level with subtype of stroke including ischemic and hemorrhagic stroke, and the association of Hcy with cardiovascular mortality and non-cardiovascular death. High Hcy was not significantly associated with stroke including ischemia and hemorrhage: the multivariate RRs across Hcy quartiles were similar as shown in Table III-3. For the risk of death, a significant linear trend on the cardiovascular death (P=0.0002) as well as on the non-cardiovascular death (P=0.017) with risk increased as serum Hcy elevation. We found that Hcy effect on the cardiovascular death was more prominent (P=0.002) (Table III-3).

Secondary analyses

To determine the optimal cutpoint of Hcy in predicting the main events, we performed a secondary analysis by constructing a receiver-operating characteristic (ROC) curve for the events. The optimal cutpoints with highest Youden's index (sum of sensitivity and specificity -1) were 9.7, 9.47 and 11.84 for stroke, CHD and death respectively. We then used these cutpoints of Hcy in multivariate Cox proportional

hazard model. The RRs were 0.94 (95% CI, 0.57-1.54, *P* <0.79) for stroke, 2.3 (95% CI, 1.24-4.18, *P*=0.008) for CHD and 2.4 (95% CI, 1.76-3.32, *P* <0.0001) for death (Table III-4). The *c* statistic was 0.803 in stroke. However, we found that the *c* statistic remained the same value after adding Hcy in multivariate model. For predicting CHD, the *c* statistic of the fully adjusted model without Hcy was 0.749, and it increased to 0.763 after adding Hcy. The *c* statistic also increased from 0.839 to 0.845 when Hcy added to this full model for the prediction of death. The discriminative ability with additional use of the threshold of Hcy was not significant in prediction of stroke: the net reclassification improvement was estimated at 0.0016 (P=0.36) and the integrated discrimination improvement (IDI) was estimated as 0.000028. (P=0.45) for the risk of stroke. For CHD events, the integrated discrimination improvement increased to 0.01 (p=0.008) for CHD. For predicting death, the integrated discrimination improvement was 0.028 (p=0.00002). The ROC curve of fully model (variables including age, sex, BMI, education, job, smoking, drinking, exercise habit, hypertension, diabetes mellitus, family history of vascular event, serum fasting glucose, total cholesterol, triglyceride, HDL, LDL) with and without Hcy for predicting CHD was shown in Figure III-4 while ROC for predicting death was shown in Figure III-5.

4.4 Homocysteine lowering clinical trials on dementia (Table IV-1 to Table IV-4)

4.4.1 Efficacy of homocysteine –lowering vitamin therapy

Of the 154 patients who were screened, 65 were excluded because they did not want to enter the trial or they had been taking vitamin supplements or they did not completely meet the inclusion criteria. The remaining 89 patients (mean age: 75 ± 7.3 years) with mild-to-moderate AD were randomized, with 45 assigned to the vitamin group and 44 to

the placebo group. Seven participants in the vitamin group and 10 in the placebo group were lost to follow-up. Five participants in the vitamin group and 4 in the placebo group withdrew due to adverse events. Of the total 63 study participants left, 54 completed the tests of cognition (27 in each group) and were included in the final analysis (Figure IV-1). The characteristics of the two groups at baseline are shown in Table IV-1.

The characteristics including sex, age, baseline level of homocysteine, B_{12}, folic acid, MMSE, CASI results were not different between the two groups. The mean baseline MMSE score was around 18 in both groups.

4.4.2 End points

As compared to baseline levels, the Hcy level was reduced while B_{12} and folic acid were increased after the intervention. The mean plasma homocysteine concentration was 3.02 μmole per liter lower in the vitamin group than in placebo group (p=0.008). The values of plasma concentrations of Hcy, folic acid, and vitamin B_{12} were not normally distributed at baseline and at six months. The changes of plasma concentration of Hcy, folic acid, and vitamin B_{12} concentrations of each individual were checked from baseline to week 26 and were compared between the placebo and vitamin groups using non-parametric Wilcoxon two-sample tests. They were significantly different between these 2 groups (Table IV-2). The median changes were a 9.9 ng/mL increase for folic acid and a 237 pg/mL increase for B_{12} in the vitamin group, while the median changes were decreased for folic acid and B_{12} in the placebo group.

The intervention effects on cognition and daily function was assessed by reviewing the change of scores on the MMSE, CASI, ADAS-cog, ADL, and IADL for each individual participant from baseline to 6 months. The increase of score in the tests of MMSE, CASI or ADL indicates improvement of cognition and daily function. However, higher score of the tests of ADAS-cog and IADL suggested deterioration of cognitive

ability. Table IV-3 listed the changes of scores at each assessment scale. There were no significant differences between the vitamin and placebo groups. The mean change of MMSE score was 0.15 in vitamin group and 0.41 in placebo group (p=0.79). The mean change of ADAS-cog score was 0.67 in the vitamin group and -0.9 in placebo group (p=0.34). For the CASI test, there was a 1.7 decrease in the vitamin group and a 0.12 increase in the placebo group (p=0.51). Changes in ADL and IADL scores were also not different between these two groups, with p values of 0.7 and 0.89, respectively.

The age and gender were stratified to see if these factors influenced the effects of vitamin on cognitive function. We defined the increase of score in MMSE, CASI and the decrease of score in ADAS-cog after intervention as improvement, and then calculated the odds ratio of treatment in either group of patients with age ≥ 75 years or < 75 years old. Both groups had no significant treatment effects in every assessment scale. The results after stratification of gender also showed no effect of intervention. The age and sex adjusted odds ratio (95% confidence interval and p value) for IADL, ADAS, CASI and MMSE were 0.77 (0.32 to 1.86 , p=0.56), 1.32 (0.50 to 3.46, p=0.57), 1.20 (1.47 to 3.07, p=0.69) and 1.90 (0.69 to 5.24, p=0.21), respectively (Figure IV-2).

The Hcy values were assessed from baseline to week 26 in each participant and classified the patients into the Hcy-increase group and Hcy-decrease group, regardless of treatment regimen received. The mean change and the 95% limit in each cognition scale were evaluated. Figure IV-3 showed that there was no significant change of score in each cognition scale and between the Hcy-increase and Hcy-decrease groups. The p values were 0.3, 0.2, and 0.9 for the MMSE, CASI, and ADAS scales, respectively, but all were not significant.

4.4.3 Safety

The analysis of safety and tolerability were based on a total of 89 patients (45 in vitamin

and 44 in placebo group). All had been concurrently taking AChEIs (one took rivastigmin and all other took donepezil) and both treatment groups had similar compliance rates. Study withdrawals included 12 of the 45 (27%) in the vitamin group and 14 of the 44 (32%) in the placebo group (Figure IV-1). Each group had 3 patients who withdrew due to AE. The proportion of patients who experienced at least one AE during the study period (46.7% and 31.8% in the vitamin and placebo groups, respectively) was not significantly different in both groups (p=0.1).

Muscle pain (11.1% in vitamin and 6.8% in placebo) and insomnia (8.9% in vitamin and 9.1% in placebo) were the two common causes of AE (Table IV-4). More patients in vitamin group experienced delirium (8.9%). The majority of AEs were rated as mild to moderate. As for the SAE, three patients were hospitalized in the study period. Two patients in the vitamin group were admitted due to stroke and Parkinson's disease with rigidity worsening. In the placebo group, one was hospitalized due to coronary artery disease.

Chapter 5. Discussion

5.1 Homocysteine and cerebral hemodynamic status

Our results from the health adults showed that Hcy concentrations were not significantly associated with the flow velocity, resistance and total flow volume of the carotid and vertebral artery. To our knowledge, this study is the first to investigate the effect of homocysteine on the flow velocity and volume on the brain vessels.

Homocysteinemia plays an important role for the risk of atherosclerosis and arterial stenosis on the carotid and coronary arteries [56, 115]. Some recent studies reported the effects of Hcy on the hemodynamic status [31, 116-120]. The relationship between hemodynamic status and vascular atherosclerosis had been established [26-28]. Zarins et al. reported quantitative correlation of plaque localization with flow velocity profiles and wall shear stress [26]. In viewing of the previous studies about Hcy and the hemodynamic status, significant inverse correlation between Hcy and the flow velocity were reported on the coronary artery and ophthalmic artery [31, 121]. High serum Hcy concentrations were associated with the slow flow phenomenon in non-stenotic coronary artery. Barutcu and colleagues first investigated the relation between Hcy and the coronary slow flow phenomenon [122]. Other researchers also found elevated levels of plasma Hcy in patients who have angiography proven normal coronary artery with slow flow in symptomatic [31, 121] and asymptomatic adults [118]. Hyperhomocysteinemia impairs coronary flow velocity reserve in experimental study [116]. The mechanisms of coronary slow flow are still not clear. Serum Hcy related endothelial dysfunction and oxidative stress were suggested to be the causes of coronary slow flow [31, 116]. Although there were increasing evidence of the Hcy effect on the slow flow, our study demonstrated that such association does not exist on the carotid and vertebral artery in asymptomatic adults.

In viewing the study design among the reports about Hcy and flow hemodynamic status, the aforementioned studies were small case control study [31, 121]. The largest study among previous reports had 50 participants in control group and 53 in case group [31, 116]. Our study measured the flow velocities and serum Hcy levels in a total of 542 people. The larger sample size in our study made a greater statistic power. The results that showed no significant association between levels of Hcy concentrations and flow velocity was found after fully adjustment for age and sex and other vascular confounding factors. These results remained consistent in either statistic method with Hcy analyzed as quartile categories or as a continuous variable. One experienced technician, who was blind to the clinical data, performed all the duplex examination on all the participants in the study without bias of interobserver reliability.

The possible reasons why our study results were different from those in previous reports are discussed as following. First, the study population was different between the studies. Participants of previous reports were selected among those who were suspected to have coronary artery disease while our study subjects were free from symptoms of vascular diseases. Compared with our asymptomatic population, people with coronary symptom may have different life style, risk factors and medications, which may change the effects of Hcy on the blood flow. The effects of Hcy in different category of population may not be the same. Second, the measurements of the flow velocity on the carotid artery or VA in this study and coronary artery on the other studies are quite different. The coronary flow was measured by angiography [31, 116-118, 122] while flow of the carotid artery or VA in our study was measured by duplex ultrasonography. The absolute values of flow velocity might be different from various methods of measurements and that possibly made our results different form other studies. As compared to ultrasonography, angiography was more possibly to underestimate the

presence of atherosclerotic plaques [31]. Besides, flow volume and flow resistance can not be measured in aforementioned coronary studies. Intravascular ultrasonography, which could overcome this limitation, was not performed in these studies [31, 116-118, 122]. Our study performed by ultrasonography provided more hemodynamic data than previous reports.

Another possible reason for explaining our results different from others is that our target vessels are the arteries to the brain not to the heart. The hemodynamic patterns of the brain and heart vessels are different because of the different physiological mechanisms [30]. The flow patterns on the duplex of the peripheral vessels, including the heart arteries, are triphasic waveforms with high resistance while the carotid artery and vertebral artery show low resistance flow in order to give adequate blood supply to the brain and the brain has richer collateral circulation than any other organ [30]. Besides, the blood flow in the brain is more constant than in the heart among healthy people. This is due to the brain autoregulation system which can prevent people form syncope while the blood pressure fluctuating in daily living [123]. The differences of collateral circulation on the brain and the heart might influence the effects of Hcy on the blood flow on these two vital organs. So clinically, the effects of Hcy on the cardiac and cerebral vascular disease might not be the same [7, 124].

The study limitations are as following. First, the arteries we sampled were extracranial carotid and vertebral arteries which may not completely indicate the hemodynamic status of the intracranial cerebral blood flow. The results of our study might not be extrapolated to the whole brain circulation. Second, as compared to the carotid artery which the intima-media thickness and plaques can be easily visualized by duplex study, the examination of the VA is rather difficult, because the image is interrupted by transverse process of the vertebra body [125]. Some of our sampled VA may

have plaques hidden behind the transverse process canal. This technical problem can not be overcome by sonography. The hemodynamic patterns may change with plaque formation. In this study, we could not perform subgroup analyses on the flow parameters of the VA with and without plaques.

5.2 Homocysteine and cerebral white matter lesions (WML)

In the present study, we found that Hcy was significantly associated with WML. After adjustment for known cardiovascular risk factors, including age, sex, hypertension, diabetes mellitus and smoking, Hcy and age remained as independent risk factors for WML. These findings suggest that Hcy had clinical utility in terms of identifying patients at increased risk of WML.

The strong association observed in our study between Hcy levels and risk of WML is concordant with previous studies that Hcy levels were higher in patients with silent brain infarction [67, 126]. Silent brain infarcts and WML are thought to have a common vascular origin. Both involve the cerebromicorvascular system. Cerebral small vessel disease is a major cause of vascular cognitive impairment and dementia [127]. Baseline WML and lacunes predicted both WML progression and new lacunes [128]. Postmortem studies have shown that WML correspond to heterogeneous pathological substrates with varying degrees of demyelination, arteriosclerosis, and gliosis representing "incomplete" infarction, but also tissue degeneration [129, 130]. Silent brain lacunar infarction, although "silent" in clinical manifestation, is a "complete" infarction. Lacunes are small cavities located in the white matter or subcortical gray matter. They have been regarded as small ischemic infarcts, but several pathogenetic mechanisms may exist [131]. The MR images of lacunes are different from that of WML. Infarcts were defined as focal hyperintensities on T2 weighted images and had to have corresponding prominent hypointensities on T1 weighted images in order to distinguish them from cerebral WML. WML were considered present if visible as hyperintense on proton density and T2 weighted images, without prominent hypointensity on T1 weighted scans [132]. Previous studies performed in Japan and Korea focused on the association

between Hcy and silent brain infarcts without investigating the association with WML [67, 126]. To the best of our knowledge, this is the first study to demonstrate an association between Hcy and risk of WML among Asian population. The present study demonstrated the effect of Hcy on the incomplete infarction of WML. The findings obtained in this study were consistent with the results from the Rotterdam study [52]. In the Rotterdam Scan Study, periventricular WML were rated semiquantitatively (grade range 0-9) and subcortical WML were measured by total volume [52]. Elevated serum Hcy was associated with severe grade of WML and this study was performed in elderly (aged 60-90 years). In our study, the severity of WML of case group was varying from mild to severe grade. And our participants were younger than Rotterdam's study, with mean age 50.9 years. Our study confirmed the association of Hcy with the risk of WML even in young asymptomatic adults.

The mechanisms through which elevated Hcy levels might cause vascular damage to the brain are unclear. Elevated Hcy may promote atherosclerosis by damaging the vascular wall [1] or by its direct toxic effect on nerve cells [133]. The hypotheses of atherothrombosis by Hcy could not explain the effect of Hcy on the risk of WML. Results of present study were consistent with Fassbender's finding that Hcy was associated with small vessel disease [62]. Further histopathological studies are needed to examine the association with microvascular disease.

In conclusion, we found a relation between serum Hcy and cerebral WML. However, this is a cross-sectional study and results should be confirmed by prospective longitudinal studies.

5.3 Homocysteine and clinical vascular events

Our data suggest serum total Hcy concentration was significantly associated with the risk of CHD and death among ethnic Chinese adults but not significantly related with stroke. Furthermore, we determined the optimal cutpoints of Hcy by selecting the values of highest sensitivity and specificity. The use of cutpoints of Hcy added appreciably to the overall prediction of CHD and death risk. These findings highlight the strength of using new definition of hyperhomocysteinemia for the prediction of future cardiovascular risk and death in asymptomatic persons.

The association between Hcy and stroke was inconclusive. Two large prospective studies demonstrated significant linear trend for an increased relative risk of incident stroke with each quartile of Hcy levels compared with lowest quartile among elderly people [134, 135]. Nonetheless, in the Physicians' Health Study, baseline serum Hcy for 109 participants who developed ischemic stroke during 5 years of follow-up were compared with those of 427 controls matched for age, smoking status, and length of follow-up [136]. There was no significant difference in mean Hcy at baseline. The results of some prospective studies are also conflicting [2]. It remains to be determined whether the association is causal or whether the association is confounded due to factors associated with high Hcy, such as vitamin deficiency, diabetes, age, male sex, hypertension, smoking, social class and preexisting vascular disease [137, 138]. Besides, the underlying pathogenetic mechanism in stroke is complex. Effects of Hcy on different subtype of stroke such as embolic, large artery, small artery and other etiologies may be different [139, 140].

In contrast with the negative association between Hcy and stroke, our findings demonstrates clearly the association of Hcy with the risk of incident CHD and death,

which was consistent with results of studies performed in healthy women [141], postmenopausal women [142] and in high risk participants [143-146]. Compatible with some previous prospective cohort studies [142-147], top quartile of Hcy independently increases 2-3 times the risk of future CHD as compared with the lowest level of Hcy and our results showed the graded relationship between serum Hcy concentrations and risk of coronary events. Our data also showed positive association of high Hcy with not only cardiovascular death but also non-cardiovascular mortality. Previous study revealed that hyperhomocysteinemia can predict total and cardiovascular mortality in high risk subjects [145], and this study, furthermore, demonstrated the predictive ability of Hcy in community based asymptomatic population without preexisting vascular disease.

We are interested in defining hyperhomocysteinemia for the use of Hcy screening in primary prevention. However, there is no general agreement on the optimal value of Hcy that confers the vascular risk. Clarke and colleagues first establish the diagnostic criterion for hyperhomocysteinemia by comparing post methionine loading Hcy levels between those with cystathionine β-synthase deficiency and 27 age- and sex-matched normal subjects. A level of 24.0 μmol per liter or more of Hcy was 92% sensitive and 100% specificity in distinguishing the two groups [148]. The Rotterdam Study results among the elderly showed the risk of CHD increased (OR, 3.0; 95%CI, 1.5-6.1) in particular above 85th percentile (≧18.6 μmol/L). In another prospective study of 21,520 men aged 35 to 64, the risk of ischemic heart disease in highest quartile (15.17 μmol/L) of Hcy level was 3.7 (95% CI, 1.8-4.7) as compared with the lowest quartile [149]. According to a statement by the American Heart Association [150], a serum level of 15 μmol/L, corresponding to the 90th percentile of the distribution, was chosen as the cutoff point for an elevated level. This Hcy value was significantly correlated to CHD among patients with type 2 diabetes mellitus [146]. A prospective study among postmenopausal

women found the adjusted RRs of myocardial infarction or stroke in Hcy level ≧ 50th (11.19 μmol/L), 75th (13.26 μmol/L) , 90th (17.29 μmol/L), and 95th (20.70 μmol/L) were 1.9 (95%CI, 1.0-3.4), 2.2 (1.2-4.0), 1.9 (0.9-4.3) and 4.6 (1.7-12.3) respectively [142]. Another long-term cohort of women population in Sweden found the fifth quintile of Hcy (≧14.18 μmol/L) had the relative risk as 1.86 (95% CI, 1.06-3.26) for myocardial infarction events [141]. Our data showed the participants with Hcy level of 9.47 μmol/L or more had highest specificity and sensitivity rate in association with the risk of future CHD. Furthermore, we observed people with high Hcy level had higher total and cardiovascular mortality. We determined that the Hcy value of 11.84 μmol/L may be an optimal cutpoint with better specificity and sensitivity rate for prediction of all-cause of death. As compared with previous studies [141-143, 145], our data showed mild or moderate elevation, not extremely high, of Hcy is significantly related to the risk of CHD and death in the future.

Optimal cutpoints of Hcy were selected across possible values with the range of test results. The value of maximal Youden's index was determined to be the optimal value. However, this data-driven selection of optimal cutpoints is prone to bias, meaning that it can systematically lead to overestimation of the sensitivity and specificity [151]. The amount of bias in sensitivity and specificity predominantly depended on the sample size. Small sample size (<200) may lead to overly optimistic estimate of sensitivity and specificity in studies. Nonetheless, such bias can be lowered in study with big sample size as in this data [151].

The *c* statistic is the most commonly used method of determining model discrimination. In this study, the *c* statistics were calculated to compare the additional value of Hcy in predicting the risk of vascular events and death in the model fully adjusted for all the confounders. The c statistics increased with Hcy adding in the model

of CHD and death, but not of stroke. Two new measures, net reclassification improvement (NRI) and integrated discrimination improvement (IDI), were used on the comparison of predicted values from two models. Tests for the IDI were based on t-tests comparing differences in predictions between two models. The majority of individuals in this cohort may be at low risk, and changes in their predicted probabilities are small and lead to a low overall IDI. Nonetheless, the differences in predictive probabilities between the model of conventional risk factors without Hcy and that with Hcy were statistically significant in both the models for CHD and death respectively.

Our study had several potential limitations. First, our study lacked information on some determinants of total Hcy levels such as dietary patterns, food fortification and vitamin supplements. The use of a single Hcy measurement to classify persons may also have underestimated the strength of any association because of regression dilution [152]. Second, stroke was classified into ischemic, hemorrhagic and mixed type. Without definite image documentation, misclassification on the stroke type may develop. In addition, the subtypes of ischemic stroke, such as large vessel atherosclerotic infarcts, lacunar infarcts, embolic infarcts, and ischemic stroke of unknown or other origin, were not specified and Hcy may have different impacts on different subtype of stroke [139, 140].

5.4 Homocysteine-lowering vitamin therapy on dementia

In this randomized, double-blind, placebo controlled, 26-week trial of subjects with mild to moderate AD, the daily use of over-the-counter supplements containing folic acid, vitamin B_{12}, and B_6 caused plasma homocysteine concentrations to decrease by 3.02 μmole per liter in the vitamin group than in placebo group. However, there were no significant differences in the change of score of cognition and daily living function between these two groups, even after stratification by age and gender. There was no

significant cognitive improvement in each group and no significant association between the change of cognition score and the change of Hcy concentration in either treatment group.

As for the Hcy-lowering therapy, different dosage and combinations have been studied and recommended in several reports. B_6, B_{12}, and folic acid supplementation has been successfully used in selected settings to reduce moderate hyperhomocysteinemia in patients with normal or abnormal B_{12} concentration [153-157]. Among the three vitamins, however, only folic acid seems to have a universal potential for reducing fasting plasma levels of Hcy [158]. Daily supplementation with both 0.5-5 mg folic acid and about 0.5 mg vitamin B_{12} should be efficient [159]. Additional combination with 5 to 10 mg of vitamin B_6 has been reported to be safe and tolerable in reducing Hcy concentration[159]. In viewing the entire over-the-counter vitamin supplements in Taiwan, the combined use of Methycobal capsule (Eisai Co.), containing methyl based vitamin B_{12} 0.5 mg, and a nutritional regimen registered for pregnant women named Pramet®FA (Abbott Laboratories Services Corp.), containing folic acid 1 mg and pyridoxine HCl 5mg, may meet the requirement of Hcy-lowering vitamin regimens. These regimens were also accessible and affordable over-the-counter daily supplements.

Although observational studies suggest an inverse association between Hcy concentration and cognitive performance [112-114], a Cochrane review of randomized controlled trials of folic acid with or without vitamin B_{12} [160], as well as another review for vitamin B_6 supplement [161], concluded that there was no benefit for healthy but cognitively impaired people. The participants in those previous studies had cognitive impairment but were not all diagnosed to have Alzheimer's dementia. Besides, some studies did not mention the subjects' serum level of B_6, folic acid, or vitamin B_{12}, which could influence the results of vitamin therapy. Moreover, the intervention periods were

no more than 12 weeks. A two-year, controlled trial of homocysteine lowering therapy showed no improvement for healthy older people [162]. B_{12} or folic acid deficiency can be reversible factors in cognition performance, but most of the aforementioned studies did not clarify the vitamin status in the included subjects [153-155]. A randomized controlled trial with homocysteine-lowering therapy in patients with mild to moderate AD and with normal B_{12} and folic acid status has never been reported before in reviewed literatures. This study was specifically designed to address the diagnosis of Alzheimer's dementia as an inclusion criterion. We not only assessed the cognition performance but also the daily living function, as well as the homocysteine concentration change after 6 months of intervention.

Our study showed significant effects on lowering of the homocysteine concentration by these over-the-counter multi-vitamin supplements as compared to the placebo group. Though there were evidences that plasma Hcy is associated with cognition function and Alzheimer's disease, the Hcy-lowering therapy had no significant clinical beneficial effects on cognition and daily living function in these AD patients. These results were similar to other clinical trials on healthy elderly or patients with vascular disease [163, 164]. In terms of homocysteine association with atherosclerotic vascular disease [32, 165, 166], several randomized controlled trials also showed that the Hcy-lowering therapy had no consistent effects on the incidence of vascular events [167-172]. The possible explanations for the lack of statistic significance of the Hcy-lowering therapy for cognition function in this study are as follows: 1. although there was no significant effect in our study, there still showed trend of benefit favoring vitamin treatment (Fig IV-2). Our sample size may be too small for a significant statistic power. 2. The treatment duration may be too short to have significant effects. It might take a longer duration to improve the cognition function by Hcy-lowering therapy. 3.

The pathogeneses of Alzheimer's dementia are complicated and are related to amyloid deposition in addition to vascular factor [173]. Controlling one risk factor such as Hcy may not be sufficient to obtain the clinical benefit. 4. Our study participants were in the mild or moderate stage of dementia. The clinical effects of vitamin therapy may be little or not obvious on such irreversible and significant damage of brain. Further study with selection of target participants with mild cognition impairment and very early stage of dementia may be indicated for further exploring the efficacy of Hcy-lowering therapy on Alzheimer's dementia.

Our study had several limitations. The duration was relatively short and thus, longer cognitive benefits from Hcy-lowering in AD patients cannot be excluded. Some of the participants also suffered from symptoms of gastric upset, nausea, and vomiting, which were often related to AChEIs [174]. Some subjects who dropped out were possibly due to these adverse effects, causing a smaller-than-desired final sample size. Further large randomized controlled trials with longer periods are needed.

Chapter 6. Summary, Conclusions and Future direction

6.1 Summary of the findings in these serial studies

Study 1:

In the first cross-sectional, observational study, total of 542 asymptomatic adults (mean age 55 years old) were enrolled. Carotid duplex studies were performed in all the participants to measure the flow velocities, resistance or volume in the common, internal and external carotid arteries (CCA, ECA and ICA) and the vertebral arteries (VA). Subjects with higher serum Hcy had slower velocities on the CCA, ICA and VA. After adjustment of vascular risk factors of age, sex, smoking habit, body mass index and serum lipid profile, the effect of Hcy on the hemodynamic status of the extracranial cerebral arteries.

Study 2:

We conducted a case control study in a total of 258 asymptomatic adults (mean age 51 years old) which were classified into case group with microangiopathy related cerebral white matter change noted on the MRI and control group with normal brain MRI study. The mean serum Hcy levels was 9.1 μmol/L in the 65 participants of case group and 8.9 μmol/L in control group (p=0.83). After fully adjustment of the confounding variables such as age, sex, blood pressure and other vascular factors, Hcy was an independent risk factor for cerebral white matter change. Every 1 μmol/L increase of Hcy, the relative risk for having cerebral white matter lesions was 1.15 (95% CI 1.01-1.31, P=0.03).

Study 3:

We conducted a community-based prospective cohort study of 2009 participants (56% women; age range, 38-91 years), who were free from stroke, coronary heart disease (CHD) and cancer at baseline in 1994, and were followed up to 2007 (median 11.95

years). We documented 114 cases of stroke, 95 cases of CHD and 380 deaths. Cox proportional hazard model was used to examine the association between Hcy and the incidence of stroke, CHD, and all-cause death. The receiver-operating characteristic curve was preformed to identify the best Youden's index for determining the cutpoint of Hcy in risk prediction. Homocysteine levels remained significantly associated with the cardiovascular events and death in fully adjusted models. Participants with Hcy more than 9.47 μmol/L (sensitivity 81.1%, specificity 54.3%) had a 2.3-fold risk for cardiovascular events (95% CI, 1.24-4.18, *P*=0.008), and participants with Hcy more than 11.84 μmol/L (sensitivity 49.7%, specificity 84.0%) had a 2.4 fold risk for death (95% CI, 1.76-3.32, *P*<0.0001).

Study 4:

A total of 89 patients (44 women and 45 men; mean age: 75 ± 7.3 years) were randomized to receive multi-vitamin or placebo for 26 weeks. Homocysteine-lowering therapy was a daily supplement containing folic acid 1 mg, pyridoxine 5 mg and mecobalamin (B_{12}) 500mcg. Plasma levels of homocysteine and tests of cognition were conducted at baseline and after 26 weeks of treatment. In vitamin group, the concentration of vitamin B12 and folic acid were significantly elevated and the mean plasma homocysteine concentration was 3.02 μmole per liter lower than in the placebo group (p=0.008) after 26 weeks' treatment. Overall, there were no significant differences between the vitamin and placebo groups in the scores of cognition and activities of daily living. The proportion of adverse events was not significant different in both groups (p=0.1).

6.2 Strength and limitations

Study 1.

Strength:

Previous reports showed high serum Hcy levels were associated with the slow flow phenomenon in non-stenotic coronary arteries. But there was no study exploring the relationship between Hcy and hemodynamics status of the brain. To the best of our knowledge, our study was the first to investigate the effect of Hcy on the flow velocity of brain vessels. Our results demonstrated that the Hcy induced slow flow phenomenon in coronary artery does not exist for the cerebral arteries after fully adjustment for known cardiovascular risk factors. Besides, our sample size were much bigger than previous studies on the coronary flow and this made for greater statistical power.

Limitations:

The study limitations are as following. First, the arteries we sampled were extracranial carotid and vertebral arteries which may not completely indicate the hemodynamic status of the intracranial cerebral blood flow. The results of our study might not be extrapolated to the whole brain circulation. Second, as compared to the carotid artery which the intima-media thickness and plaques can be easily visualized by duplex study, the examination of the VA is rather difficult, because the image is interrupted by transverse process of the vertebra body. Some of our sampled VA may have plaques hidden behind the transverse process canal. This technical problem can not be overcome by sonography. The hemodynamic patterns may change with plaque formation. In this study, we could not perform subgroup analyses on the flow parameters of the VA with and without plaques.

Study 2.

Strength:

In the present study, we found that Hcy was significantly associated with cerebral white matter lesions (WML) after fully adjustment for known cardiovascular risk factors. This was the first report on this association among Asia population. These findings suggest that Hcy had clinical utility in terms of identifying patients at increased risk of WML. Our study confirmed the association of Hcy with the risk of WML even in young asymptomatic adults.

Limitations:

The severity of WML was not graded in present study. The varying degree of WML may have different clinical implication and prognosis. This is a cross-sectional study and these findings should be confirmed by prospective longitudinal studies.

Study 3.

Strength:

This prospective, community-based, large sample-sized, long-term follow-up (12 years) cohort study not only confirmed the association of serum total Hcy concentration with the risk of CHD and death among ethnic Chinese adults but also determined the optimal cutpoints of Hcy by selecting the values of highest sensitivity and specificity. The use of cutpoints of Hcy added appreciably to the overall prediction of CHD and death risk. These findings highlight the strength of using new definition of hyperhomocysteinemia for the prediction of future cardiovascular risk and death in asymptomatic persons.

Limitations:

First, our study lacked information on some determinants of total Hcy levels such as dietary patterns, food fortification and vitamin supplements. The use of a single Hcy measurement to classify persons may also have underestimated the strength of any

association because of regression dilution. Second, stroke was classified into ischemic, hemorrhagic and mixed type. Without definite image documentation, misclassification on the stroke type may develop. In addition, the subtypes of ischemic stroke, such as large vessel atherosclerotic infarcts, lacunar infarcts, embolic infarcts, and ischemic stroke of unknown or other origin, were not specified and Hcy may have different impacts on different subtype of stroke.

Study 4.

Strength:

Our study showed significant effects on lowering of the homocysteine concentration by these over-the-counter multi-vitamin supplements as compared to the placebo group. Though there were evidences that plasma Hcy is associated with cognition function and Alzheimer's disease, the Hcy-lowering therapy had no significant clinical beneficial effects on cognition and daily living function in these AD patients. These results were similar to other clinical trials on healthy elderly or patients with vascular disease. In reports of homocysteine-lowering therapy, this was the first specifically designed to address the diagnosis of Alzheimer's dementia as an inclusion criterion; cognition performance and daily living function were assessed, as well as the homocysteine concentration change after 26 weeks of intervention.

Limitations:

The duration was relatively short and thus, longer cognitive benefits from Hcy-lowering in AD patients cannot be excluded. Some of the participants also suffered from symptoms of gastric upset, nausea, and vomiting, which were often related to concomitant use of AChEIs. Some subjects who dropped out were possibly due to these adverse effects, causing a smaller-than-desired final sample size. Further large

randomized controlled trials with longer periods are needed.

6.3 Clinical implications

Our study provided the evidence that Hcy may be associated to small vessels disease and may influence microvascular system related perfusion in the brain. The decrease of cerebral perfusion not only leaded to white matter lesions but also contributed to cognitive impairment. Those findings confirmed the previous reports about the inverse relationship between Hcy and cognitive function. Hcy-lowering therapy, though was not effective to the cognitive function in patients with Alzheimer's dementia, but it may be beneficial to vascular dementia.

Lack of association of Hcy and stroke may be related to the different pathogenesis from different subtypes of ischemic stroke and different effects of Hcy on subtypes of stroke. Our study supported the relationship of Hcy with small vessel disease and this finding was consistent with Japanese reports. People with ischemic stroke from small vessels or lacunar infarction or with massive white matter lesions need to be evaluated the serum levels of Hcy. Further dietary suggestion or Hcy-lowering therapy may be indicated for prevention of recurrent stroke.

In the prospective cohort study, we confirmed the relationship of Hcy with risk of CHD and mortality, especially cardiovascular death. Besides, we also provided optimal cutpoints of homocysteine in clinical prediction, which has never been reported before. Subjects with serum Hcy level more than 9.5 μmol/L may be at high risk of CHD, while people with serum Hcy more than 11.8 μmol/L may have higher risk of mortality in the future years. We proposed a new definition of "hyperhomocysteinemia" for clinical practice. With such new definition, we can highlight the high risk population, and advise Hcy-lowering food or therapy for primary prevention from vascular disease and mortality.

6.4 Conclusions

In conclusion, we found that the Hcy had no significant effect on the hemodynamic change on the large extracranial cerebral arteries. Such findings are different from the Hcy effects on the coronary flow. Investigating on the intracranial cerebral artery and further prospective studies are needed to confirm the role of Hcy on the hemodynamic flow of the brain.

We found that Hcy was significantly associated with cerebral white matter lesions (WML). After adjustment for known cardiovascular risk factors, Hcy and age remained as independent risk factors for the risk of WML. These findings suggest that Hcy had clinical utility in terms of identifying patients at increased risk of WML. This findings indicated that the effects of Hcy on the brain may be related to cerebral microangiopathy and resulting to cerebral WML, and then further related to future cerebral vascular insults and cognitive impairment.

Individuals with higher level of Hcy had a significant higher risk of future CHD and all-cause of death. The optimal cutpoints of Hcy were 9.47 μmol/L for CHD and 11.84 μmol/L for death. Adding Hcy to the model that included the established risk factors improved the prediction for CHD and death among asymptomatic ethnic Chinese. The feasibility of incorporating Hcy in clinical screening for primary prevention warrants further research.

The randomized controlled trial demonstrates that multi-vitamin supplements can decrease plasma homocysteine concentration in patients with mild to moderate Alzheimer's disease and with normal serum levels of vitamin B_{12} and folic acid. However, the study did not support the hypothesis that a reduction of plasma homocysteine level can improve the cognitive function. Furthermore, multi-vitamin supplements had no statistically significant beneficial effects on both cognition and

daily living function.

6.5 Future research directions

(1) In addition to study on the hemodynamic status, research evaluating the relationship between Hcy and the hemorheology factor such as blood viscosity may be indicated.

(2) Our studies demonstrated the effect of Hcy on the brain may be mediated through microvascular system, further brain perfusion study such as perfusion CT with calculating the mean transit time of small cerebral vessels may be indicated for further exploring the effect Hcy on the intracranial perfusion and white matter lesions.

(3) Further grading on the WML in our study participants is indicated for comparison the relationship between Hcy levels and severity of cerebral WML.

(4) Longitudinal study for comparing the development of WML in subjects with high and low levels of Hcy is important since WML plays a role in future stroke and cognitive performance.

(5) The strong association of Hcy with the risk of future CHD and death indicated that Hcy still play an important role on the vascular disease. In further prospective studies, the subtypes of ischemic stroke, such as large vessel atherosclerotic infarcts, lacunar infarcts, embolic infarcts, and ischemic stroke of unknown or other origin should be specified since Hcy may have different impacts on different subtype of stroke.

(6) Since the mechanisms of stroke are heterogeneous and complicated, further randomized controlled trials of homocysteine-lowering therapy are needed in prevention of specified subtype of stroke such as small vessel infarcts or lacunar infarcts with leukoaraiosis.

(7) Further large randomized controlled trials with longer periods of homocysteine-lowering therapy are needed for people with vascular dementia.

(8) Our study participants were in the mild or moderate stage of dementia. The clinical effects of vitamin therapy may be little or not obvious on such irreversible and significant damage of brain. Further study with selection of target participants with mild cognition impairment and very early stage of dementia may be indicated for further exploring the efficacy of Hcy-lowering therapy on Alzheimer's dementia.

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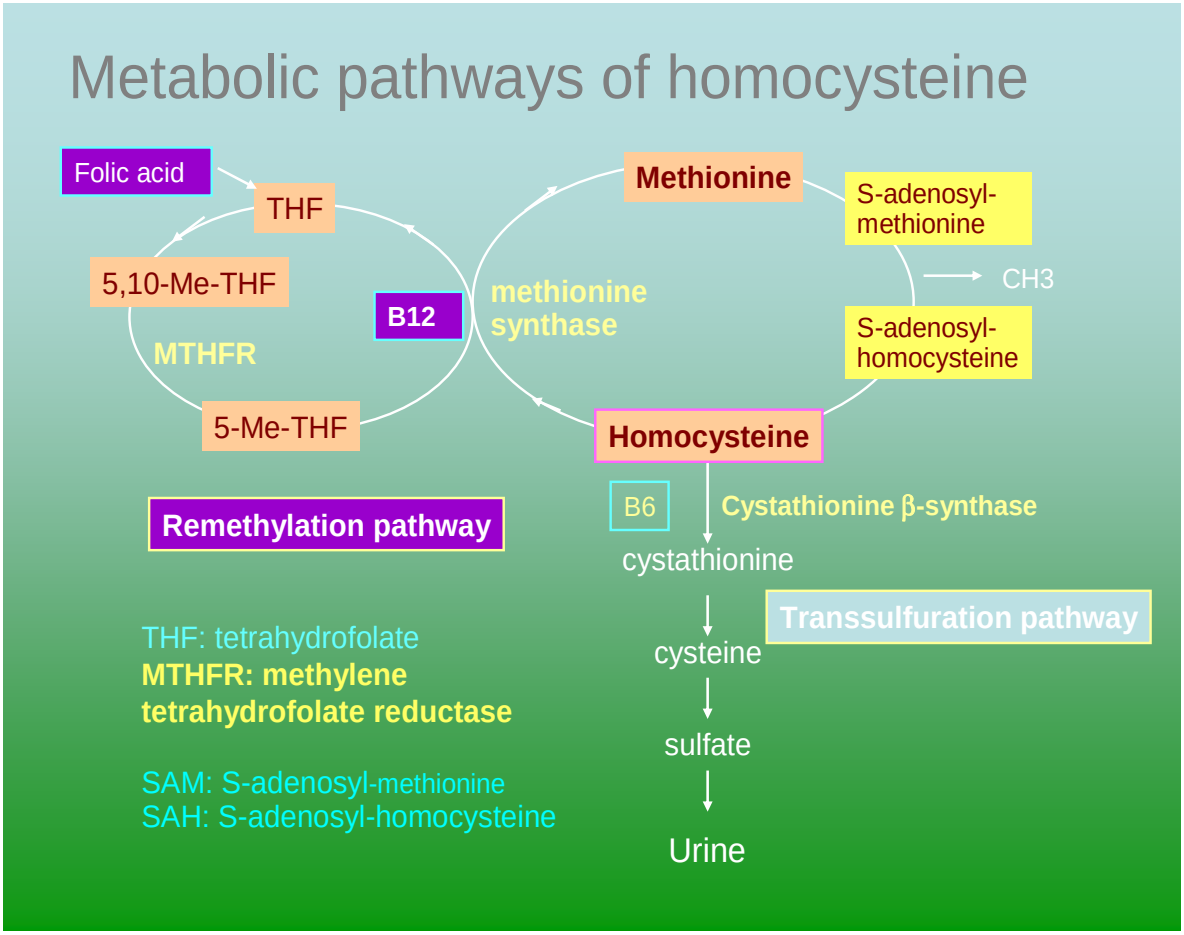
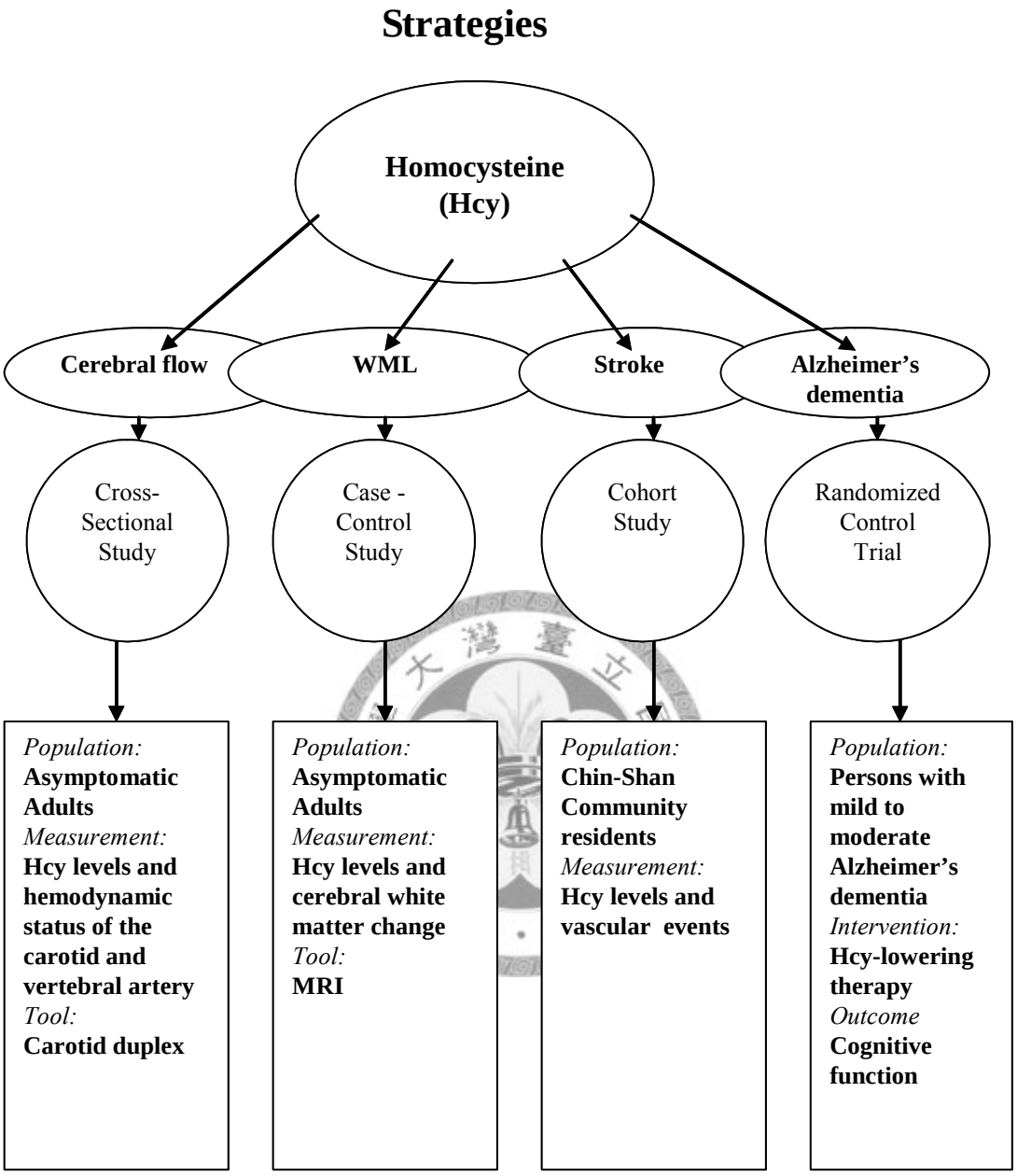
Figures

Figure 1. Metabolic pathway of homocysteine



The methione degradation pathway. Methione is metabolized via S-adenosyl methione and S-adenosyl-homocysteine to homocysteine in the course of producing methyl groups for use in synthetic process. The first step involves the enzyme cystathionine β-synthase for which pyridoxine (B6) is the co-factor and deficiencies of this enzyme resulted in the usual form of homocysteinuria. The remaining 50% of formed homocysteine is remethylated to methionine and requires 5, 10-methyltetrahydrofolate as substrate, and methylcobalamin as a co-factor.

Figure 2. Study design of four serial studies



Figures for homocysteine and cerebral hemodynamic status

Figure I-1. Duplex image (including B-mode and Doppler) of the common carotid artery

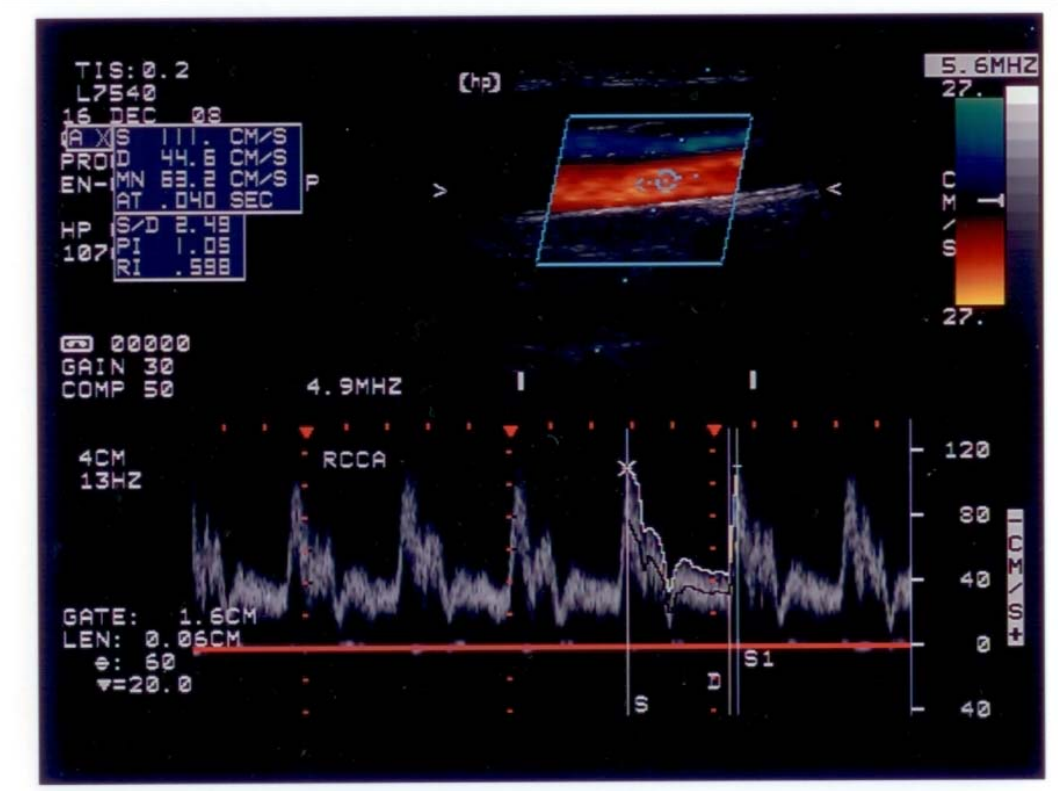
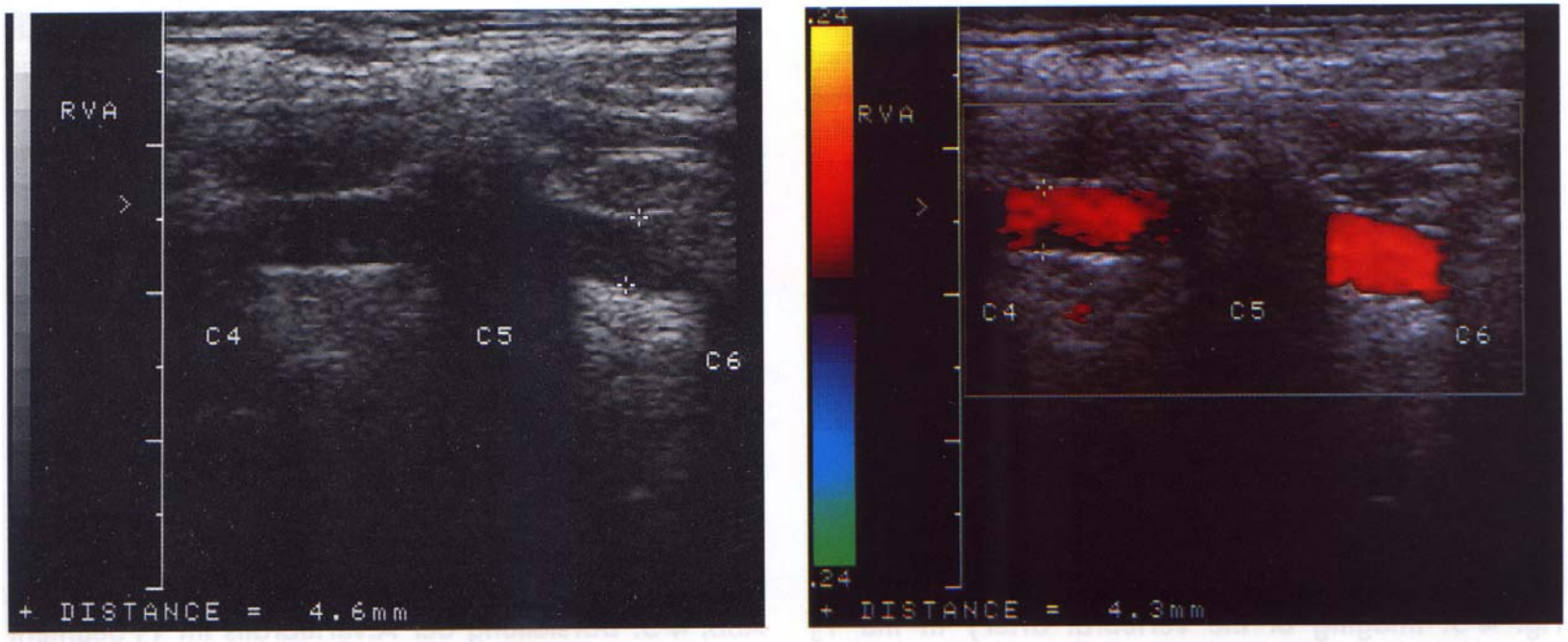
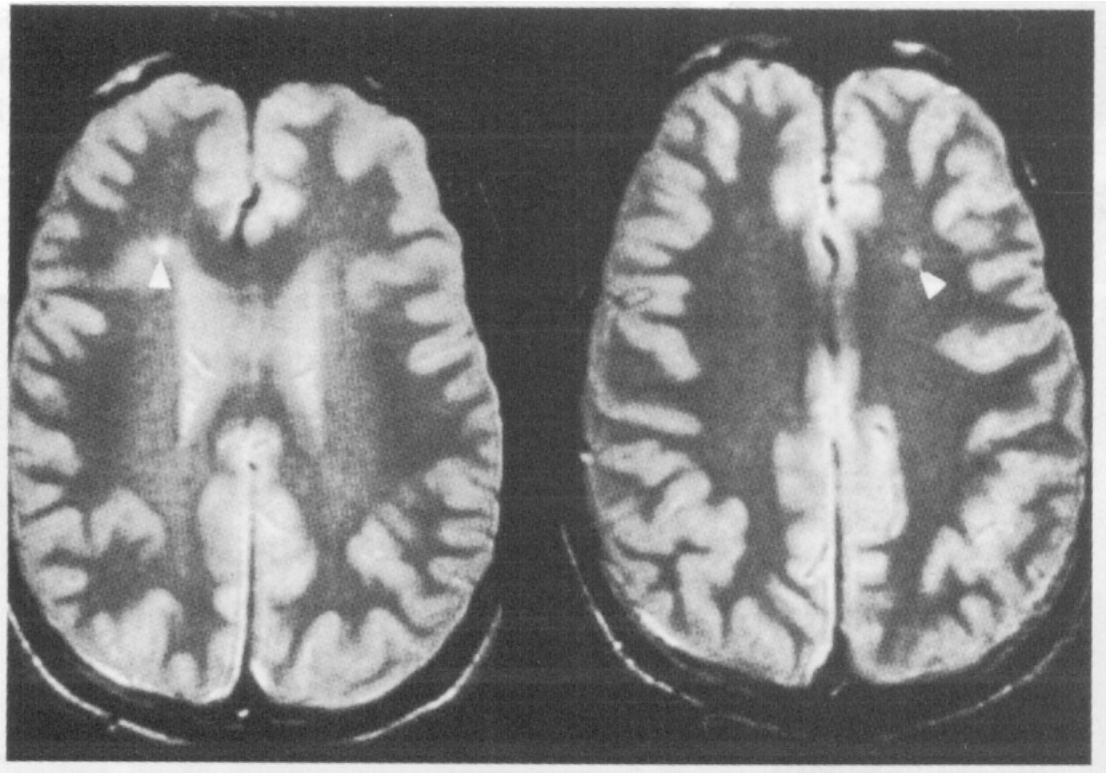


Figure I-2. Duplex image (including B-mode and Doppler) of the vertebral artery



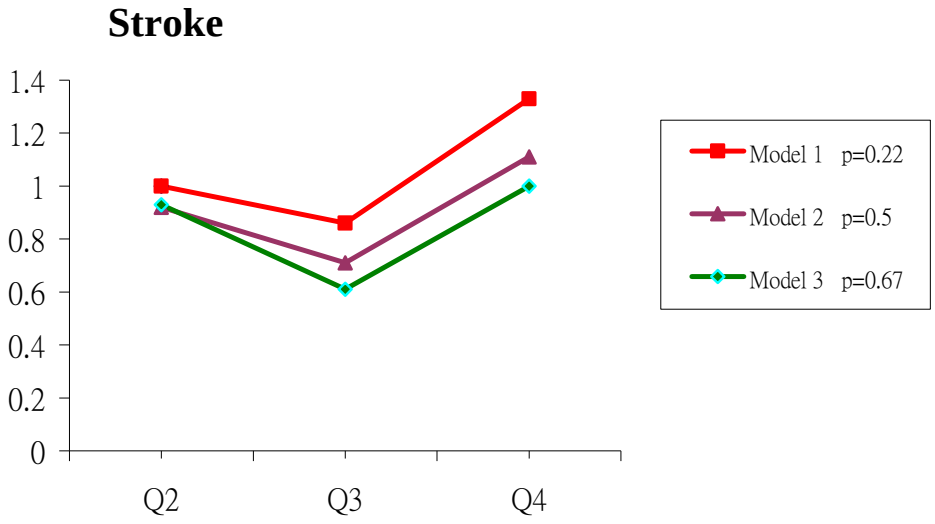
Figures for homocysteine and cerebral white matter lesions

Figure II-1. T2-weighted brain MR image with hyperintensity signals (pointed by white arrow) on the periventrcular (left) and subcortical area (right)



Figures for homocysteine and long-term vascular events

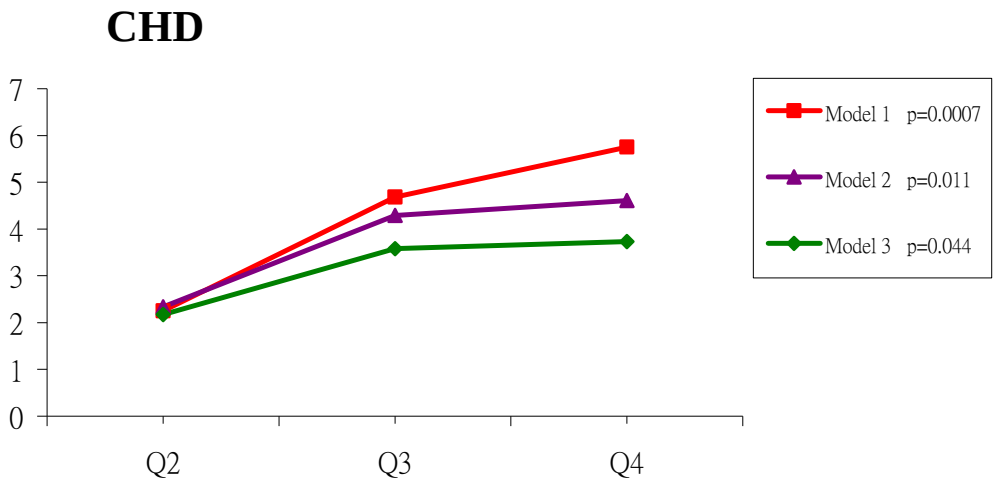
Figure Ⅲ-1. Relative risk for stroke during a median follow-up of 11.95 years, according to quartile of homocysteine concentration at baseline (1994-1995) in the Chin-Shan Community Cardiovascular Study



Model 1, adjusted for age and sex; model 2, model 1 plus body mass index, education level, (<9 years, at least 9 years), occupation (no job, farmer/labor, professional/business), smoking (yes/no), alcohol drinking (regular/no), regular exercise habit (yes/no); model 3, model 2 plus baseline hypertension, diabetes mellitus (yes/no) and family history of stroke and coronary heart disease, fasting glucose, cholesterol, triglyceride, HDL-C, and LDL-C concentrations.

Study 3: Homocysteine and long-term vascular events

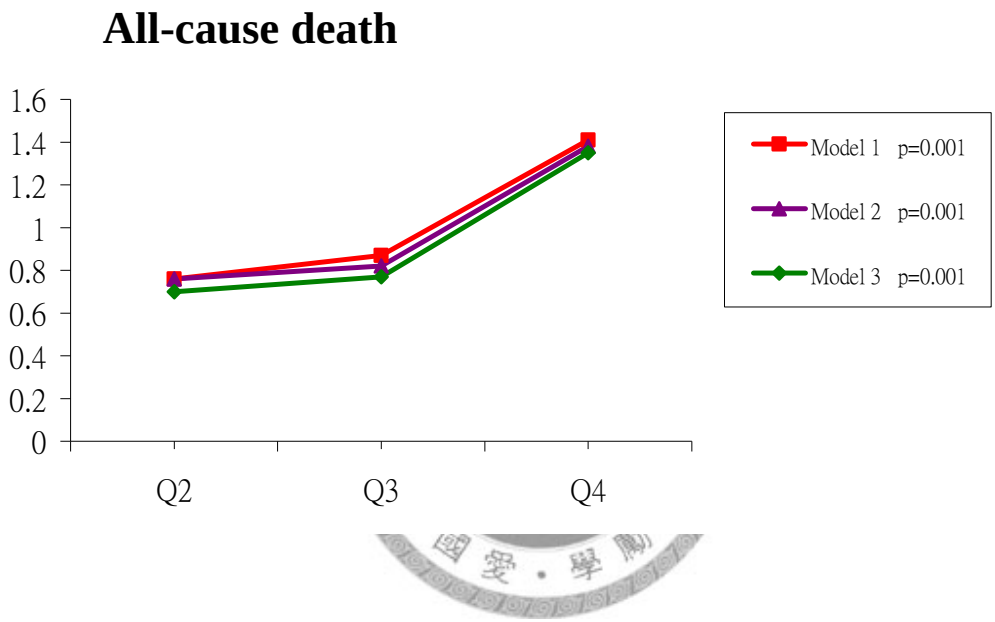
Figure Ⅲ-2. Relative risk for CHD during a median follow-up of 11.95 years, according to quartile of homocysteine concentration at baseline (1994-1995) in the Chin-Shan Community Cardiovascular Study



Model 1 to model 3 defined as figure III-1.

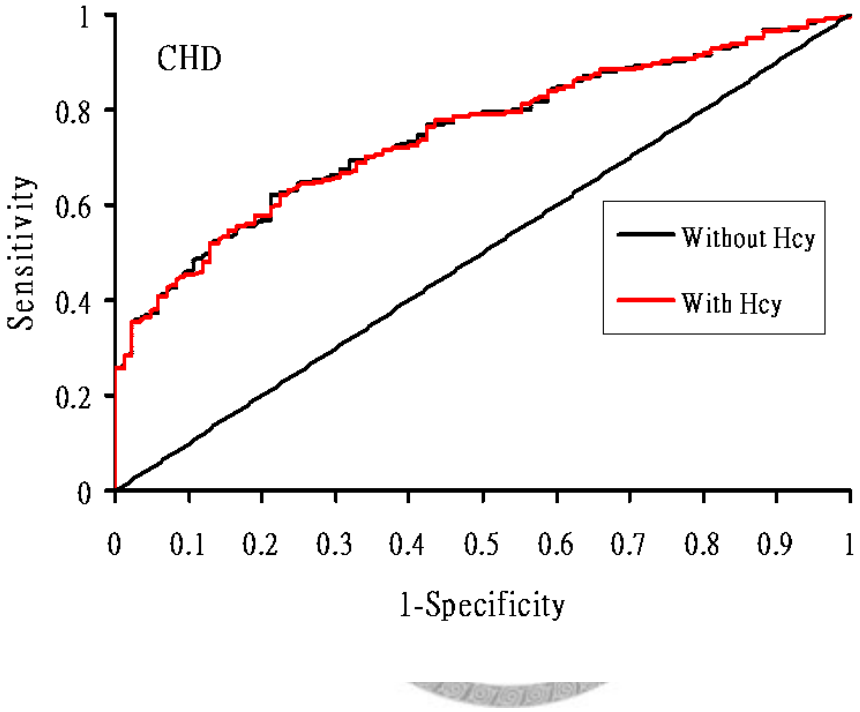
Study 3: Homocysteine and long-term vascular events

Figure Ⅲ-3. Relative risk for all-cause death during a median follow-up of 11.95 years, according to quartile of homocysteine concentration at baseline (1994-1995) in the Chin-Shan Community Cardiovascular Study



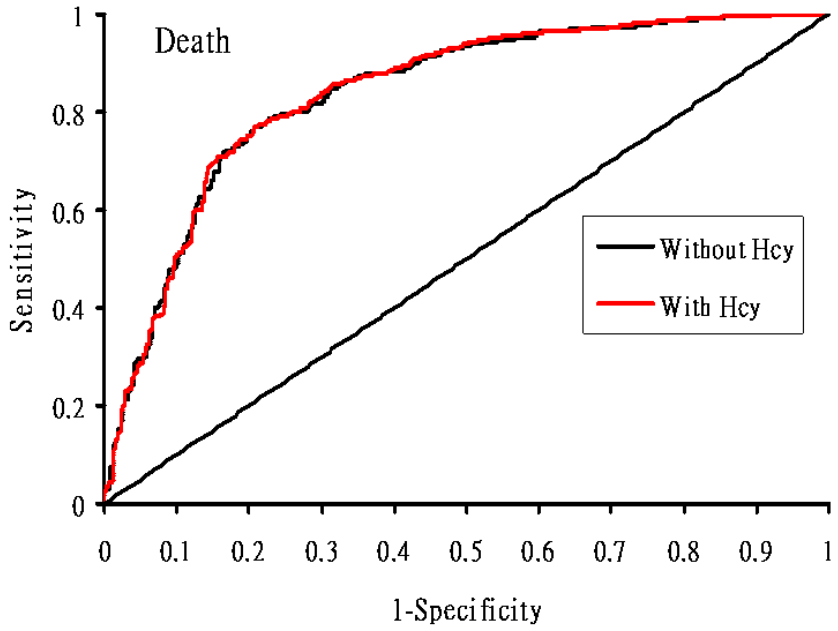
Model 1 to model 3 defined as figure III-1.

Figure Ⅲ-4. Receiver-Operating Characteristic Curves for coronary heart disease (CHD) events during a median follow-up of 11.95 years



For each end point, curves are based on prediction models adjusted for conventional risk factors with or without homocysteine (Hcy). The conventional risk factors in these models included age, sex, body mass index, educational level, occupation, alcohol intake, smoking, regular exercise, hypertension, diabetes and family history of stroke and coronary heart disease.

Figure Ⅲ-5. Receiver-Operating Characteristic Curves for death events during a median follow-up of 11.95 years



For each end point, curves are based on prediction models adjusted for conventional risk factors with or without homocysteine (Hcy). The conventional risk factors in these models included age, sex, body mass index, educational level, occupation, alcohol intake, smoking, regular exercise, hypertension, diabetes and family history of stroke and coronary heart disease.

Figures for homocysteine-lowering therapy

Figure Ⅳ-1. Patients enrollment and completion throughout the study period

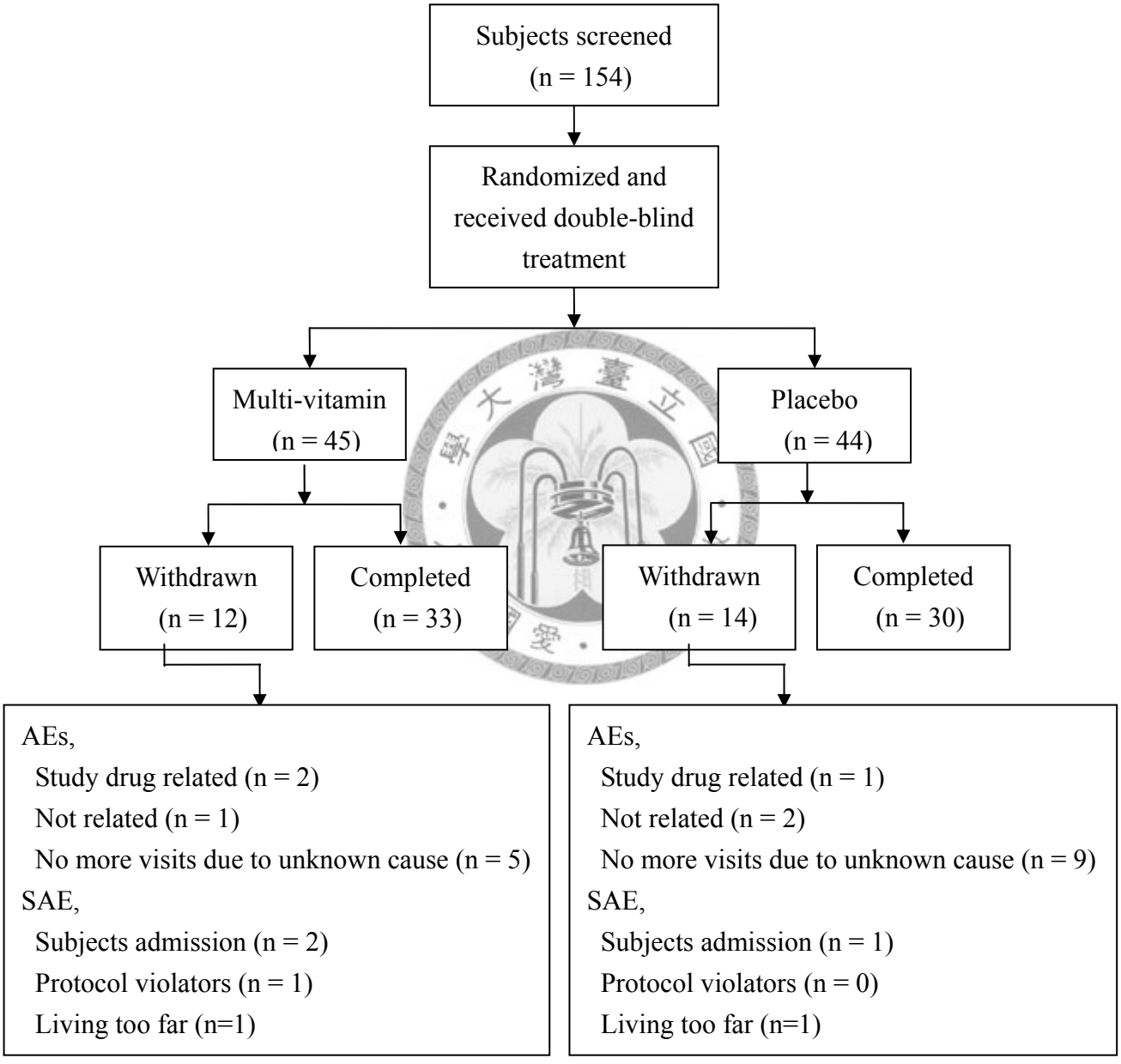


Figure Ⅳ-2. Odds ratios for the effects on cognition and daily living scores of 26 weeks of combined multivitamins or placebo in patients with mild or moderate Alzheimer's dementia

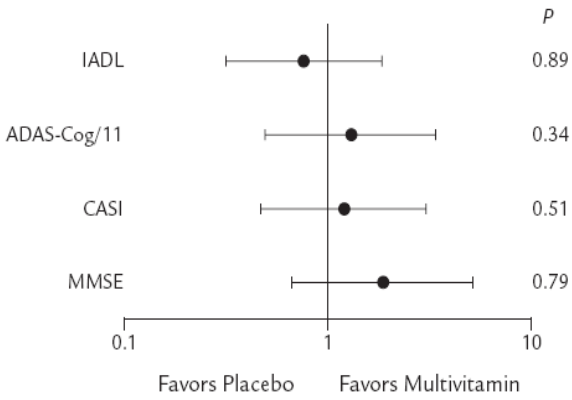


Figure 2. Odds ratios (95% CI) for the effects on cognition and daily living scores of 26 weeks of combined multivitamins or placebo in patients with mild to moderate Alzheimer's dementia. Outcomes were defined as dichotomous variables with improvement or not on the cognition as comparing the scores between baseline and 26 weeks. IADL = Instrumental Activities of Daily Living Scale[33]; ADAS-Cog/11 = Alzheimer's Disease Assessment Scale[26–28] 11-item Cognition subscale; CASI = Cognitive Abilities Screening Instrument[29–31]; MMSE = Mini-Mental State Examination.[21,22]

Study4: Homocysteine lowering therapy

Figure Ⅳ-3. Mean changes form baseline in cognition scale in the homocysteine-increase and homocysteine-decrease groups with 26 weeks of combined multivitamins or placebo in patients with mild or moderate Alzheimer's dementia

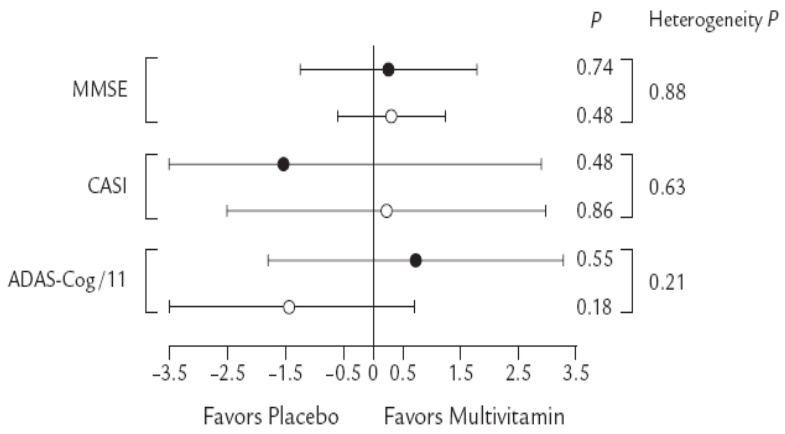


Figure 3. Mean (95% CI) changes from baseline in cognition scale scores in the homocysteine-increase (○) and homocysteine-decrease (●) groups with 26 weeks of combined multivitamins or placebo in patients with mild to moderate Alzheimer's dementia. MMSE = Mini-Mental State Examination[21,22]; CASI = Cognitive Abilities Screening Instrument[29–31]; ADAS-Cog/11 = Alzheimer's Disease Assessment Scale[26–28] 11-item Cognition subscale.

Tables for homocysteine and cerebral hemodynamic status

Table Ⅰ-1. Characteristics of the study population according to homocysteine quartiles

	Quartiles of Homocysteine*				
	Q1 (n=135)	Q2 (n=133)	Q3 (n=134)	Q4 (n=133)	*P*
Age, y	50	53	56	61	<0.0001
Men, %	56	56	56	56	0.99
Hypertension, %	21	13	25	25	0.13
Diabetes mellitus, %	1.3	5.2	4.8	7.3	0.30
Current smoking, %	27	38	48	55	0.008
Systolic blood pressure, mmHg	120	124	128	132	0.0002
Diastolic blood pressure, mmHg	74	74	75	78	0.11
Body mass index	29	25	25	25	0.021
Laboratory					
Fasting glucose, mg/dl	98	98	100	99	0.90
High density lipoprotein, mg/dl	53	53	50	49	0.07
Serum creatinine, mg/dl	0.87	0.94	0.99	1.04	0.0002
Serum uric acid, mg/dl	6	5.9	6.3	6.6	0.005

*Homocysteine (μmol/L) quartiles : first, 1.91-7.36; second, 7.37-9.07; third, 9.08-11.32; and fourth, 11.33-58.7

Table Ⅰ-2. Hemodynamic parameter values of the carotid artery according to homocysteine quartiles

	Quartiles of Homocysteine*				
	Q1	Q2	Q3	Q4	*P*
Common carotid artery					
Systolic velocity	82.52	79.84	82.93	78.63	0.25
Diastolic velocity	26.48	25.62	25.02	24.55	0.02
Resistance index	0.67	0.67	0.69	0.68	0.05
Pulsatility index	1.34	1.37	1.47	1.42	0.02
Internal carotid artery					
Systolic velocity	66.76	63.05	64.53	60.78	0.01
Diastolic velocity	31.00	29.33	29.53	27.91	0.006
Resistance index	0.53	0.53	0.54	0.53	0.77
Pulsatility index	0.81	0.82	0.86	0.83	0.77
External carotid artery					
Systolic velocity	70.09	68.35	71.81	71.92	0.26
Diastolic velocity	14.12	14.76	15.03	14.68	0.35
Resistance index	0.79	0.78	0.79	0.79	0.99
Pulsatility index	1.95	1.89	1.98	1.99	0.24

*Footnote as table I-1.

Table Ⅰ-3. Hemodynamic parameter values of the vertebral artery according to homocysteine quartiles

	Quartiles of Homocysteine*				
	Q1	Q2	Q3	Q4	*P*
Velocity					
Peak systolic velocity, cm/sec	44.4	40.5	38.8	35.9	0.0006
End diastolic velocity, cm/sec	17.6	16.4	16.2	15.3	0.19
Time average flow velocity, cm/sec	27.5	25.0	24.1	22.3	<0.0001
Resistance					
Resistance index	0.57	0.52	0.51	0.47	0.004
Pulsatility index	0.94	0.87	0.85	0.79	0.012
Diameter and flow volume					
Diameter, cm	0.26	0.25	0.25	0.23	0.08
Flow volume, cm3/sec	100.9	98.4	96.4	88.7	0.13

*Footnote as table I-1.

Table Ⅰ-4. Adjusted mean values of flow parameters of carotid artery across homocysteine quartiles

	Quartiles of Homocysteine*				
	Q1	Q2	Q3	Q4	*P* for Trend
Common carotid artery					
Systolic velocity					
Model 1†	81.74	79.41	82.00	81.20	0.92
Model 2‡	79.32	77.58	80.02	79.85	0.03
Diastolic velocity					
Model 1†	25.43	25.30	25.21	25.90	0.51
Model 2‡	25.55	25.10	25.36	26.06	0.48
Resistance index					
Model 1†	0.68	0.68	0.69	0.68	0.70
Model 2‡	0.67	0.67	0.68	0.67	0.74
Pulsatility index					
Model 1†	1.39	1.38	1.43	1.39	0.93
Model 2‡	1.33	1.36	1.39	1.34	0.96
Internal carotid artery					
Systolic velocity					
Model 1†	65.11	62.60	65.18	62.44	0.39
Model 2‡	64.15	60.09	65.58	60.56	0.39
Diastolic velocity					
Model 1†	29.82	28.99	29.87	29.18	0.71
Model 2‡	29.99	28.37	30.50	29.19	0.83
Resistance index					
Model 1†	0.54	0.53	0.54	0.53	0.19
Model 2‡	0.53	0.53	0.53	0.52	0.24
Pulsatility index					
Model 1†	0.83	0.83	0.86	0.81	0.42
Model 2‡	0.80	0.80	0.83	0.78	0.57

*Footnote as table I-1.

† Model 1: adjusted for age, sex.

‡ Model 2: Model 1 plus current smoking, systolic blood pressure, serum creatinine, body mass index, uric acid, and high-density lipoprotein

Table Ⅰ-5. Adjusted mean values of flow parameters of vertebral artery across homocysteine quartiles

	Quartiles of Homocysteine*				
	Q1	Q2	Q3	Q4	*P* for Trend
Diameter, cm					
Model 1†	0.24	0.24	0.25	0.25	0.43
Model 2‡	0.23	0.24	0.24	0.24	0.35
Peak systolic velocity, cm/sec					
Model 1†	41.1	38.7	39.5	40.2	0.72
Model 2‡	37.4	35.3	37.5	38.8	0.35
End diastolic velocity, cm/sec					
Model 1†	16.1	15.6	16.5	17.3	0.17
Model 2‡	14.9	14.5	16.2	17.4	0.038
Time average flow velocity, cm/sec					
Model 1†	25.5	23.9	24.6	24.9	0.77
Model 2‡	23.5	22.2	23.5	24.5	0.33
Resistance index					
Model 1†	0.53	0.50	0.52	0.51	0.48
Model 2‡	0.49	0.47	0.48	0.49	0.82
Pulsatility index					
Model 1†	0.89	0.84	0.86	0.86	0.53
Model 2‡	0.80	0.77	0.80	0.80	0.81
Flow volume, cm3/sec					
Model 1†	92.8	94.1	98.2	99.4	0.12
Model 2‡	87.7	90.9	94.6	97.0	0.13

*Footnote as table I-1.

† Model 1: adjusted for age, sex.

‡ Model 2: Model 1 plus current smoking, systolic blood pressure, serum creatinine, body mass index, uric acid, and high-density lipoprotein

Table Ⅰ-6. Relations of plasma homocysteine to the flow parameter of carotid artery

	Model 1 †			Model 2 ‡		
	Regression Coefficients* (95% CI)		*P*	Regression Coefficients (95% CI)		*P*
Common carotid artery						
Systolic velocity	-0.12	(-1.52 to 1.28)	0.87	-0.92	(-2.90 to 1.04)	0.36
Diastolic velocity	0.04	(-0.45 to 0.52)	0.89	0.02	(-0.68 to 0.71)	0.96
Resistance index	-0.0003	(-0.006 to 0.005)	0.91	-0.003	(-0.01 to 0.004)	0.4
Pulsatility index	0.006	(-0.02 to 0.03)	0.66	-0.009	(-0.05 to 0.68)	0.61
Internal carotid artery						
Systolic velocity	-1.15	(-2.66 to 0.37)	0.14	-1.42	(-3.52 to 0.67)	0.18
Diastolic velocity	-0.42	(-1.12 to 0.29)	0.24	-0.35	(-1.38 to 0.68)	0.5
Resistance index	-0.002	(-0.008 to 0.003)	0.46	-0.005	(-0.013 to 0.002)	0.17
Pulsatility index	-0.001	(-0.017 to 0.015)	0.9	-0.007	(-0.029 to 0.015)	0.52

*Linear regression coefficients represent difference of hemodynamic parameters of the carotid artery for one-SD increment of plasma homocysteine

† Model 1: adjusted for age, sex.

‡ Model 2: Model 1 plus current smoking, systolic blood pressure, serum creatinine, body mass index, uric acid, and high-density lipoproteine

Table Ⅰ-7. Relations of plasma homocysteine to the flow parameter of vertebral artery

	Model 1 †			Model 2 ‡		
	Regression Coefficients* (95% CI)		*P*	Regression Coefficients (95% CI)		*P*
Velocity						
Peak systolic velocity, cm/sec	0.24	(-1.0 to 1.5)	0.71	0.89	(-0.88 to 2.67)	0.32
End diastolic velocity, cm/sec	0.35	(-0.31 to 1.02)	0.29	0.82	(-0.18 to 1.82)	0.11
Time average velocity, cm/sec	0.12	(-0.67 to 0.91)	0.77	0.69	(-0.44 to1.82)	0.23
Resistance						
Resistance index	0.0005	(-0.02 to 0.02)	0.95	0.002	(-0.02 to 0.02)	0.89
Pulsatility index	0.003	(-0.03 to 0.03)	0.83	0.0005	(-0.04 to 0.04)	0.98
Diameter and flow volume						
Diameter, cm	0.004	(-0.004 to 0.01)	0.32	0.005	(-0.006 to 0.02)	0.37
Flow volume, cm^3/sec	2.3	(-1.06 to 5.69)	0.18	3.8	(-0.95 to 8.62)	0.12

*Linear regression coefficients represent difference of hemodynamic parameters of the vertebral artery for one-SD increment of plasma homocysteine

† Model 1: adjusted for age, sex.

‡ Model 2: Model 1 plus current smoking, systolic blood pressure, serum creatinine, body mass index, uric acid, and high-density lipoprotein

Tables for homocysteine and cerebral white matter lesions

Table Ⅱ-1. Demographic, laboratory data and homocysteine levels in subjects with normal brain MRI and patient with white matter lesions (WML) on MRI

	Normal (n=193)		WML (n=95)		*p*
Categorical variables	n	%	n	%	
Women	79	40.93	34	52.31	0.11
Hypertension	18	18.6	15	39.47	0.01
Diabetes mellitus	4	4.2	4	10.53	0.16
Current smoker	29	29.9	8	21.05	0.3
Alcohol drinking	29	29.9	6	15.79	0.09
Continuous variables, mean±SD	Mean	SD	Mean	SD	
Homocysteine, μmol/L	8.9	5.5	9.1	4.7	0.83
Age, year	47.1	11.3	61.9	11.6	<0.0001
Systolic blood pressure, mmHg	119.6	19.4	131.5	21.6	0.0025
Body mass index, km/m^2	27.9	16.7	29.3	20.2	0.68
Glucose, mg/dL	97.2	23.5	103.1	32.6	0.19
Creatinine, mg/dL	1.0	0.4	1.0	0.5	0.67
Uric acid, mg/dL	6.1	1.6	6.3	2.0	0.47
High-density lipoprotein, mg/dL	50.4	15.0	52.2	14.8	0.41

Abbreviation: WML, white matter lesion

Study 2: Homocysteine and cerebral white matter lesions

Table Ⅱ-2. Effects of homocysteine on the risk of white matter lesions on the brain MRI adjusting for potential confounders

	Model 1			Model 2			Model 3		
	Estimate	95%CI	*P*	Estimate	95%CI	*P*	Estimate	95%CI	*P*
Homocysteine	1.14	(1.02-1.28)	0.02	1.12	(1.02-1.31)	0.02	1.15	(1.01-1.31)	0.03
Age	1.12	(1.07-1.17)	<0.0001	1.12	(1.07-1.18)	<0.0001	1.11	(1.05-1.17)	<0.0001
Sex	0.24	(0.08-0.69)	0.008	0.31	(0.10-1.00)	0.04	0.35	(0.10-1.29)	0.11
Hypertension	1.60	(0.57-4.45)	0.37	1.76	(0.61-5.10)	0.30	1.30	(0.40-4.24)	0.67
Diabetes mellitus				0.93	(0.16-5.29)	0.94	0.86	(0.05-14.11)	0.92
Current smoker				0.63	(0.16-2.53)	0.52	0.82	(0.19-3.65)	0.80
Alcohol drinking				0.64	(0.16-2.50)	0.52	0.74	(0.18-3.13)	0.68
BMI				1.01	(1.00-1.04)	0.38	1.02	(1.00-1.05)	0.16
Systolic blood pressure							1.02	(1.00-1.06)	0.15
Glucose							1.01	(1.96-1.05)	0.52
Creatinine							0.86	(0.34-2.14)	0.74
Uric acid							1.04	(0.75-1.45)	0.80
High-density lipoprotein							1.02	(0.98-1.06)	0.31

Tables for homocysteine and long-term vascular events

Table Ⅲ-1 .Characteristics of the study population according to homocysteine quartiles

	Quartiles of Homocysteine*				*P*
	Q1 (n=507)	Q2 (n=499)	Q3 (n=503)	Q4 (n=500)	
Categorical variables, %					
Women	88.9	62.7	45.7	28.5	<0.0001
Education level, %					0.23
＜ 9 year	91.7	94.2	94.3	92.1	
≧ 9 year	8.3	5.8	5.7	7.9	
Job status, %					<0.0001
No job	51.2	48.0	45.9	49.3	
farmer, labor	25.2	32.9	39.3	35.8	
professional, business	23.6	19.1	14.8	14.8	
Regular exercise (yes), %	12.5	14.3	16	20.2	0.005
Family history of stroke and coronary heart disease, %	26.8	29.1	28.9	28.1	0.84
Current smoker (yes), %	11.9	27.3	39.3	50.1	<0.0001
Alcohol drinking (yes), %	15.9	21.7	32.4	42.6	<0.0001
Hypertension	18.1	21.3	32.2	38	<0.0001
Diabetes mellitus	1.6	2.4	3.7	3.5	0.15
Continuous variables, mean ± SD	Mean SD	Mean SD	Mean SD	Mean SD	P
Age, years	51.1 9.1	55.8 10.3	60.0 10.7	64.2 11.4	<0.0001
Body mass index, km/ m^2	24.3 3.4	24.3 3.4	24.2 3.2	24.4 3.7	0.88
Systolic blood pressure, mmHg	121.4 17.6	123.3 18.1	127.3 18.9	131.1 20.4	<0.0001
Diastolic blood pressure, mmHg	75.2 12.6	75.5 11.3	76.8 11.7	76.9 12.4	0.05
Fasting glucose, mg/dl	110.8 30.5	114.4 34.5	113.2 29.4	112.7 29.3	0.32
Total cholesterol, mg/dl	205.7 43.7	207.2 40.4	205.8 43.1	207.3 50.2	0.90
Triglycerides	104.5 88.9	110.0 100.8	117.1 107.4	121.3 110.0	0.04
High density liporotein, mg/dl	43.0 11.8	40.6 10.8	40.1 11.6	38.5 10.8	<0.0001
Low density liporotein, mg/dl	126.1 38.6	132.0 36.6	130.6 38.1	130.1 41.4	0.09

*Homocysteine (μmol/L) quartiles: first: 3.2-7.4, second: 7.5-9.1, third: 9.2-11.4, fourth: 11.5-63.5

Study 3: Homocysteine and long-term vascular events

Table Ⅲ-2. Incidence cases, person-years, incidence rates, and relative risk (RR) for stroke, CHD and all-cause death outcomes during a median follow-up of 11.95 years, according to quartile of homocysteine concentration at baseline (1994-1995) in the Chin-Shan Community Cardiovascular Study.*

	Quartiles of Homocysteine				*P*, trend
	Q1	Q2	Q3	Q4	
Median homocysteine,	6.53	8.36	10.26	11.67	
Stroke					
Case, n	14	22	26	52	
person-years, n	5857	5717	5421	5144	
incidence rate	2.4	3.8	4.8	10.1	
RR, model 1**	1	1.59 (0.81-3.11)	1.99 (1.04-3.79)	4.40 (2.43-7.94)	<0.0001
RR, model 2	1	1.00 (0.51-1.99)	0.86 (0.43-1.70)	1.33 (0.67-2.64)	0.22
RR, model 3	1	0.92 (0.46-1.84)	0.71 (0.35-1.45)	1.11 (0.55-2.25)	0.5
RR, model 4	1	0.93 (0.46-1.87)	0.61 (0.30-1.26)	1.00 (0.49-2.05)	0.67
CHD					
Case, n	4	14	31	46	
person-years, n	5885	5773	5380	5224	
Incidence rate	0.7	2.4	5.8	8.8	
RR, model 1	1	3.25 (1.06-9.97)	8.72 (3.09-24.61)	13.90 (5.00-38.65)	<0.0001
RR, model 2	1	2.25 (0.72-6.99)	4.68 (1.60-13.69)	5.75 (1.92-17.20)	0.0007
RR, model 3	1	2.34 (0.75-7.29)	4.29 (1.45-12.70)	4.61 (1.52-13.97)	0.011
RR, model 4	1	2.17 (0.70-6.78)	3.58 (1.20-10.72)	3.73 (1.22-11.40)	0.044
All-cause death					
Case, n	41	53	88	198	
person-years, n	5895	5819	5506	5324	
Incidence rate	7.0	9.1	16.0	37.2	
RR, model 1	1	1.28 (0.85-1.93)	2.26 (1.56-3.27)	5.59 (3.99-7.84)	<0.0001
RR, model 2	1	0.76 (0.50-1.15)	0.87 (0.59-1.29)	1.41 (0.96-2.07)	0.001
RR, model 3	1	0.76 (0.49-1.17)	0.82 (0.55-1.24)	1.38 (0.92-2.07)	0.001
RR, model 4	1	0.70 (0.45-1.09)	0.77 (0.51-1.17)	1.35 (0.90-2.03)	0.001

*Incidence rates are presented per 1000 person-years, and relative risk are presented as RR (95% CI).
**Model 1, univariate; model 2, adjusted for age and sex; model 3, model 1 plus body mass index, education level, (<9 years, at least 9 years), occupation (no job, farmer/labor, professional/business), smoking (yes/no), alcohol drinking (regular/no), regular exercise habit (yes/no); model 4, model 3 plus baseline hypertension, diabetes mellitus (yes/no) and family history of stroke and coronary heart disease, fasting glucose, cholesterol, triglyceride, HDL-C, and LDL-C concentrations.

Table Ⅲ-3. Subgroup analyses of relative risk (RR) for ischemic stroke, hemorrhagic stroke, and ischemic versus hemorrhagic stroke; RR for cardiovascular (CV) death, non-cardiovascular (non-CV) death, and cardiac versus non-cardiac death in fully adjusted model*

	Quartiles of Homocysteine				*P* , trend
	Q1	Q2	Q3	Q4	
Stroke					
RR, ischemic stroke	1	0.93 (0.43-2.01)	0.51 (0.22-1.16)	0.89 (0.40-2.00)	0.77
RR, hemorrhagic stroke	1	0.90 (0.17-4.71)	1.20 (0.26-5.45)	1.67 (0.36-7.74)	0.44
RR, ischemia v.s. hemorrhage	1	0.92 (0.43-2.01)	0.51 (0.22-1.15)	0.87 (0.39-1.96)	0.92
Death					
RR, CV death	1	1.97 (0.20-19.46)	4.10 (0.51-32.89)	9.63 (1.23-75.56)	0.0002
RR, non-CV death	1	0.66 (0.42-1.04)	0.67 (0.44-1.04)	1.16 (0.76-1.79)	0.017
RR, CV v.s. Non-CV death	1	2.25 (0.23-21.95)	5.31 (0.66-42.49)	9.30 (1.18-73.52)	0.002

*Fully adjusted model as the model 4 in table III-2, adjusted for age, sex, body mass index, education level (<9 years, at lease 9 years), occupation (no job, farmer/labor, professional/business), smoking (yes/no), alcohol drinking (regular/no), regular exercise habit (yes/no), hypertension, diabetes mellitus (yes/no), family history of stroke and coronary heart disease, fasting glucose, cholesterol, triglyceride, HDL-C, and LDL-C concentrations.

Table Ⅲ-4. Sensitivity, specificity and best Youden's index of the cutoff values, and the hazard ratio for the risk of stroke, cardiovascular events and all-cause of death under the cutoff value of homocysteine

	Hcy > 9.70 for stroke	Hcy >9.47 for coronary heart disease	Hcy >11.84 for all-cause of death
Sensitivity, %	65.8	81.1	49.7
Specificity, %	57.2	54.3	84.0
Youden's index	0.23	0.35	0.34
Hazard ratio	0.94	2.3	2.4
95% CI	0.57-1.54	1.24-4.18	1.76-3.32
P value	0.79	0.008	<0.0001

Tables for homocysteine lowering therapy

Table Ⅳ-1. Demographic, baseline Hcy, B12, folic acid and cognitive function of the study population.

variables	multivitamin (n=45)		placebo (n=44)		p value
	number, mean	%, SD	number, mean	%, SD	
sex, n (%)					
male	21	23.6%	24	27%	0.4
age					
median	75		77		
mean (SD)	74.9	-7.1	74.6	-7.5	0.9
Hcy, mean (SD)	11.6	-3.7	11.2	-3.5	0.7
B12, mean (SD)	488	-378	409.5	-307.4	0.3
folic acid, mean (SD)	9	-4.5	8.4	-6.6	0.6
MMSE, mean (SD)	18.7	-4.63	18.6	-5.3	0.9
ADAS-cog, mean (SD)	24	12.3	21.2	10.5	0.3
CASI, mean (SD)	54.8	-14.4	55.5	-16.1	0.8

Abbreviation: SD, standard deviation; Hcy, homocysteine; MMSE, mini-mental state examination; CASI, cognitive abilities screening instrument; ADAS-cog, Alzheimer's disease assessment scale-cognition.

Study4: Homocysteine lowering therapy

Table Ⅳ-2. Serum concentrations from baseline to 26 weeks with combined multivitamins or placebo in patients with mild to moderate Alzheimer's dementia.* Values are median interquartile range.

Component	Multivitamin (n=45)			Placebo (n=44)			p
	median	25% (Q1)	75% (Q3)	median	25% (Q1)	75% (Q3)	
Baseline plasma folic acid (ng/mL)	7.6	5.5	10.7	7.3	4.5	10.1	0.14
Change in folic acid (ng/mL)	9.9	5	23.2	0.2	-2.5	1.5	<0.0001
Baseline plasma vitamin B12 (pg/mL)	359	244	545	333	223.5	530.5	0.19
Change in vitamin B12 (pg/mL)	237	73	640	-38	-135	28	<0.0001
Baseline plasma homocysteine (μmol/L)	10.6	8.8	12.7	11.3	7.2	12.6	0.48
Change in homocysteine (μmol/L)	-1.4	-3.1	0	1.3	-0.7	2.7	0.0004

*Intent-to-treat analysis set using nonparametric Wilcoxon 2-sample test (Mann-whitney U test)

Study4: Homocysteine lowering therapy

Table Ⅳ-3. Changes from baseline to 26 weeks in cognition and daily living function with combined multivitamins or placebo in patients with mild to moderate Alzheimer's dementia (intent-to-treat population).

Scale	Multivitamin (n = 45)	Placebo (n =44)	*P*
ADAS-Cog/11			
Mean	+0.67	-0.9	0.34
95%CI	-2.33 to 3.69	-2.77 to 0.85	
MMSE			
Mean	+0.15	+0.41	0.79
95%CI	-1.06 to 1.35	-1.12 to 1.93	
CASI			
Mean	-1.7	+0.12	0.51
95%CI	-5.30 to 1.89	-4.32 to 4.55	
ADL			
Mean	-0.33	-0.19	0.70
95%CI	-1.03 to 0.36	-0.57 to 0.20	
IADL			
Mean	+0.04	-0.04	0.89
95%CI	-1.01 to 1.08	-0.41 to 0.33	

ADAS-Cog/11 = Alzheimer's Disease Assessment Scale 11-Item Cognition subscale; MMSE = Mini-Mental State Examination ; CASI = Cognitive Abilities Screening Instrument; ADL = Simplified Barthel Activities of Daily Living Index; IADL = Instrumental Activities of Daily Living Scale.

Study4: Homocysteine lowering therapy

Table Ⅳ- 4. Adverse events (AEs) experienced with 26 weeks of combined multivitamins or placebo in patients with mild to moderate Alzheimer's dementia (intent-to-treat population). Values are no. (%) of patients.

System/AE	Multivitamin (n = 45)	Placebo (n = 44)	System/AE	Multivitamin (n = 45)	Placebo (n = 44)
Cardiovascular			Psychiatric		
Need for adjustment of antihypertensive medication dosage	2 (4.4)	1 (2.3)	Insomnia	4 (8.9)	4 (9.1)
			Delirium	4 (8.9)	1 (2.3)
			Depression	1 (2.2)	0
			Others		
Chest pain	1 (2.2)	0	Dizziness	3 (6.7)	1 (2.3)
Respiratory			Need to adjust dosage of antiparkinsonian drug	2 (4.4)	1 (2.3)
Asthma	1 (2.2)	0			
Cough	1 (2.2)	0	Allergy	0	1 (2.3)
URTI	0	1 (2.3)	SAE		
Gastrointestinal			Parkinson's disease with rigidity worsening	1 (2.2)	0
Constipation	2 (4.4)	1 (2.3)			
Diarrhea	2 (4.4)	0	Stroke	1 (2.2)	0
Gastric upset	1 (2.2)	0	Ischemic hart disease	0	1 (2.3)
Nausea	1 (2.2)	0	Totals		
Neuromuscular			Patients who experienced ≧1 AE	21 (46.7)	14 (31.8)
Muscle pain	5 (11.1)	3 (6.8)			
Cramp	1 (2.2)	2 (4.5)	Patients who experienced ≧1 SAE	2 (4.4)	1 (2.3)
Neuralgia	1 (2.2)	0			

URTI = upper respiratory trat infection; SAE = serious adverse event.

Appendix

1. **Sun Y**, Lai MS, Lu CJ, Chen RC. How long can patients with mild or moderate Alzheimer's dementia maintain both the cognition and the therapy of cholinesterase inhibitors: a national population-based study. *Eur J Neurol.* 2008;15(3):278-283.

2. **Sun Y**, Lu CJ, Chien KL, Chen ST, Chen RC. Efficacy of multivitamin supplementation containing vitamins B6 and B12 and folic acid as adjunctive treatment with a cholinesterase inhibitor in Alzheimer's disease: a 26-week, randomized, double-blind, placebo-controlled study in Taiwanese patients. *Clin Ther.* 2007;29(10):2204-2214.

3. **Sun Y**, Lai MS, Lu CJ. Effectiveness of vitamin B12 on diabetic neuropathy: systematic review of clinical controlled trials. *Acta Neurol Taiwan.* 2005;14(2):48-54.