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META-ANALYSIS OF TOTAL PLASMA HOMOCYSTEINE AND NUTRITIONAL STATUS OF B-VITAMINS OF HEALTHY AND DISEASED ADULTS

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By

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ABSTRACT

META-ANALYSIS OF TOTAL PLASMA HOMOCYSTEINE AND NUTRITIONAL STATUS OF B-VITAMINS OF HEALTHY AND DISEASED ADULTS

By

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Meta-analysis was performed to determine the relationship between plasma concentration ([Hcy]) and nutritional status of vitamins B_6, B_{12} and folate, to identify the reference value for plasma [Hcy] of healthy adults, and to determine the differences in the plasma [Hcy] between the healthy controls and diseased cases (CVD and CBVD) by age and gender. Data of 48 independent research studies (representing >30,000 subjects) were used to calculate weighted means, within-group variances and a fixed and random model. The mean [Hcy] of cases included in this meta-analysis, 14 μmol/l, was lower than the critical value cited by others (16 μmol/l). Significant differences in mean (±SD) plasma [Hcy] were observed between the cases (13.9±3.9 μmol/l) and controls (10.9±1.5 μmol/l); between healthy men and women (11.2±1.6 vs 9.5± 3.9 μmol/l); and between CVD or CBVD cases (12.6±2.1 vs 16.5± 3.3 μmol/l). Plasma [Hcy] was inversely associated with blood folate (β=-0.07, P<0.01), plasma vitamin B_6 (β=-0.19, P<0.01) and serum vitamin B_{12} (β=-0.17, P<0.01), dietary folate (β=-0.31, P<0.01), dietary B_{12}((β=-9.51, P<0.01), but not with dietary vitamin B_6 (excluded from the model). Findings on the predictability of plasma Hcy values can help public health officials and health educators set up recommendations for the public to reduce the risk of CVD.

To my mother, father and brother whom I love very much.

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INTRODUCTION

Introduction

Elevated plasma concentration of homocysteine (Hcy), a sulfur-containing amino acid, was initially reported in 1962 as a disease of genetic origin (Carlson & Neill, 1962). Inborn errors of the enzyme cystathionine b-synthetase (CBS) and/or methyltetrahydrofolate reductase (MTHFR), which are involved in Hcy metabolism, result in elevated Hcy concentration in plasma (hyperhomocysteinemia) and/or excretion in urine (homocysteinuria). Homozygous and heterozygous enzyme deficiency in humans results in high plasma concentration of Hcy, causing neurological abnormalities, mental retardation, fatal thrombosis and cardiovascular diseases (CVD) (Carlson & Neill, 1962).

Thirty five years after the first reported incidence, hyperhomocysteinemia is now recognized as an independent risk factor for CVD (Taylor et al, 1991; Stampfer et al, 1992; Verhoef et al, 1994, 1996; Perry et al, 1995; Arnesen et al, 1995). Hyperhomocysteinemia is no longer addressed as a disorder of solely genetic origin (Selhub & Miller, 1992;

Jacques et al, 1996; Dudman et al, 1996).

Hyperhomocysteinemia of nutritional origin, due to suboptimal blood concentrations of vitamins B_6, B_{12} and folate, has in recent years received public attentions (Ubbink et al, 1993; Guttormsen et al, 1996; Selhub et al, 1996). Suboptimal plasma concentrations of vitamins B_6, B_{12} and folate have been reported to cause a reduction in enzymatic activity of CBS or MTHFR, and an elevated Hcy concentration in plasma by Dudman et al in 1996. Inadequate nutritional status of vitamins B_6 B_{12} and folate with or without genetic defects have been reported to elevate plasma Hcy concentration (Jacques et al, 1996; Dudman et al, 1996; Guttormsen et al, 1996).

The prevalence of hyperhomocysteinemia reported in the U.S vary widely among studies. It ranges from 20-30% in the elderly of 67-96 years of age to 40% in those of over 80 years of age (Selhub et al, 1996). Prevalence of suboptimal plasma concentration of the vitamins B_6 (<30 nmol/l), B_{12} (<200 pmol/l) or folate (<5 nmol/l) was 67% in the elderly of 67-96 years of age (Selhub et al, 1993). Ubbink et al (1993) also reported that the prevalence of suboptimal plasma vitamins B_6 (<30 nmol/l), B_{12} (<200 pmol/l), and folate (<5 nmol/l) in men with hyperhomocysteinemia is 25%, 56% and 59%, respectively.

Most previous studies support the cause and effect relationship between suboptimal nutritional status of the three vitamins and elevated plasma Hcy concentrations (Selhub et al, 1993; Van den Berg et al, 1994; Pancharuniti et al, 1994; Verhoef et al, 1996). Many investigators (Pancharuniti et al, 1994; Van de Berg et al, 1994; Verhoef et al, 1996) reported significant inverse correlations between plasma Hcy concentration and plasma concentration of folate and vitamin B_{12} in both diseased with CVD (cases) and healthy individuals (controls). In contrast to these findings, Dalery et al (1995) reported no significant differences in blood vitamins B_6, B_{12} and folate in 150 CAD cases, when compared to 584 healthy controls (age<60 years).

Gender differences in plasma Hcy concentration of healthy people are also reported by different investigators. In 1992 Andersson et al reported lower plasma Hcy concentration in 83 healthy women (age: 20-69 years) compared to 74 healthy men of similar age (9.6±3.0 vs 10.7±2.6 μmol Hcy/l plasma). Cacan et al (1996) also reported lower plasma Hcy concentration in healthy women (age: 23-59 years) compared to healthy men (7.6±4.1 vs 9.7±4.9 μmol Hcy/l plasma) of similar age. In relation to gender differences in CVD cases, Robinson et al (1995) reported higher plasma Hcy concentration in 103 female cases

when compared to 201 male cases (15.3±5.7 vs 13.9±4.5 μmol Hcy/l plasma).

Investigators have classified hyperhomocysteinemia by different criteria. In a cross sectional study of the elderly in the Framingham study, Selhub et al (1993) defined hyperhomocysteinemia as (>14.0 μmol Hcy/l plasma), which is the cut-off point for plasma Hcy concentration of the 90th percentile in subjects of both genders (n=1160). The subjects had suboptimal plasma concentrations of three B-vitamins as defined by <70th percentiles: plasma pyridoxal-5-phosphate<77.7 nmol/l, vitamin B_{12}<389 pmol/l and folate<14.8 nmol/l. In 1995, Robinson et al reported (>13.5 μmol Hcy/l plasma) as hyperhomocysteinemia based on above the 80th percentile cutoff point in male patients (n=304) with established coronary artery disease. Hopkins et al (1995) suggested >9 μmol Hcy/l plasma as hyperhomocysteinemia that correspond to a progressive increase in CVD risk among men (n=162). In assessing the implications of this cause and effect relationship between nutrition and health in the public health arena, consistent definitions and reference values of the parameters are important.

Health professionals do not know if the current Recommended Dietary Allowances (RDA) for vitamins B_6, B_{12} and folate are adequate to minimize the risk of

hyperhomocysteinemia. Information currently available on the relationships between hyperhomocysteinemia and dietary intakes of the three vitamins is scarce (Verhoef et al, 1995; Selhub et al, 1996).

Individual studies (Andersson et al, 1992; Selhub et al, 1993; Nygard et al, 1995; Cacan et al, 1996; Riggs et al, 1996) identify age, disease, gender and nutritional status as possible risk factors for hyperhomocysteinemia. Findings of these studies are, however, inconsistent party due to sample variations. Individual studies identify different vitamins as the single most important nutrient in maintaining normal plasma Hcy concentration. Reference values used in determining hyperhomocysteinemia vary among the studies. No investigation has been made if hyperhomocysteinemia poses the same risk for CVD and cerebrovascular disease (CBVD). Findings of such an investigation will help to increase the public awareness for better health, and increase our knowledge to reduce the risk for CVD.

Meta-analysis offers the strength to combine systematically and quantitatively the previously reported research data on Hcy to draw a conclusion on nutritional status as a risk for hyperhomocysteinemia (Hedges & Olkin, 1985). Since many prior individual studies on Hcy included sample sizes too small to draw firm conclusions to apply in

the public health arena, the meta-analysis approach is proposed (Cooper & Hedges, 1994; Petitti, 1994; Hedges & Olkin, 1985). There has been only a single reported study of meta-analysis on homocysteine (Boushey et al, 1995). This meta-analysis determined the risk of hyperhomocysteinemia for arteriosclerotic vascular disease, estimated the potential reduction of CVD by increasing dietary or plasma folic acid.

The present study is designed to address the nutritional effect of vitamins B_6, B_{12}, and folate on hyperhomocysteinemia. Identification of modifiable factors for hyperhomocysteinemia, a risk factor for CVD, is important in public health arena.

Objectives of this study were:

1. To confirm that individuals with vascular diseases (cases) have a higher mean plasma Hcy concentration than healthy individuals (controls).
2. To determine if plasma Hcy concentration of men is higher than women for both vascular disease cases and healthy controls.
3. To establish reference values for plasma Hcy concentration of healthy controls and vascular disease cases.
4. To determine if plasma Hcy concentration differs between

cerebrovascular and cardiovascular disease cases.

5. To estimate the predictability of plasma Hcy concentration by plasma vitamin B_6, serum vitamin B_{12} and/or blood folate concentration.
6. To estimate the predictability of plasma Hcy concentration by dietary intake of vitamin B_6, vitamin B_{12} and/or folate.

Hypotheses of the study were that:

1. Vascular disease cases have a higher mean plasma Hcy concentration than healthy controls.
2. Men have a higher mean plasma Hcy concentration than women in both groups of vascular disease cases and healthy controls.
3. Reference values are lower than those reported.
4. Mean plasma Hcy concentration of healthy controls and vascular disease cases differ.
5. Plasma Hcy concentration can be predicted by plasma concentration of vitamin B_6, vitamin B_{12} and/or folate.
6. Plasma Hcy concentration can be predicted by dietary intake of vitamin B_6, vitamin B_{12} and/or folate.

Significance

Cardiovascular diseases are currently at epidemic levels worldwide in all developed and developing countries (McCully, 1997). The emerging information reveals the necessity of maintaining nutritional adequacy for disease prevention. However, the amount of dietary vitamins B_6, B_{12} and folate that are needed to prevent hyperhomocysteinemia of nutritional origin in healthy adults is not known.

Plasma Hcy concentration reference values for healthy adults and CVD patients vary among studies. Plasma levels of selected B-vitamins sufficient to maintain normal plasma Hcy levels have not been adequately addressed. Information on dietary and plasma levels of the B-vitamins in relation to Hcy is limited due to small numbers of studies and small sample sizes, which lead to limited generalizability of the findings to the whole population. Identification of unmodifiable risk factors (i.e. gender and age) and modifiable risk factors (i.e. nutritional status) for hyperhomocysteinemia is important. Findings on the predictability of plasma Hcy values based on plasma levels and dietary intake of vitamins B_6, B_{12} and folate can help public health officials and health educators set up recommendations for the public to reduce the risk of CVD.

Chapter Two

RELATED LITERATURE

Homocysteine metabolism and hyperhomocysteinemia

Homocysteine (Hcy) is an amino acid ($HSCH_2CH_2CH(NH_2)COOH$) produced by demethylation of methionine, as an intermediate in the biosynthesis of cysteine from methionine via cystathionine in humans (Figure 1) (Dudman et al, 1996). Hcy is present in blood in two forms: free or protein-bound. The sum of the two forms represents total Hcy. Under normal physiological conditions more than 50% of the total Hcy is bound to the protein. When plasma Hcy concentration is elevated percentage of free form in the blood is increased.

In the biosynthesis of Hcy, methionine, an essential amino acid and a precursor of Hcy is enzymatically methylated to s-adenosylmethionine through the action of methionine adenosyltransferase. S-adenosylmethionine, the first metabolite of methionine, is then demethylated by transferases to s-adenosylhomocysteine. Enzymatic hydrolysis of s-adenosylhomocysteine by adenosylhomocysteine hydrolase

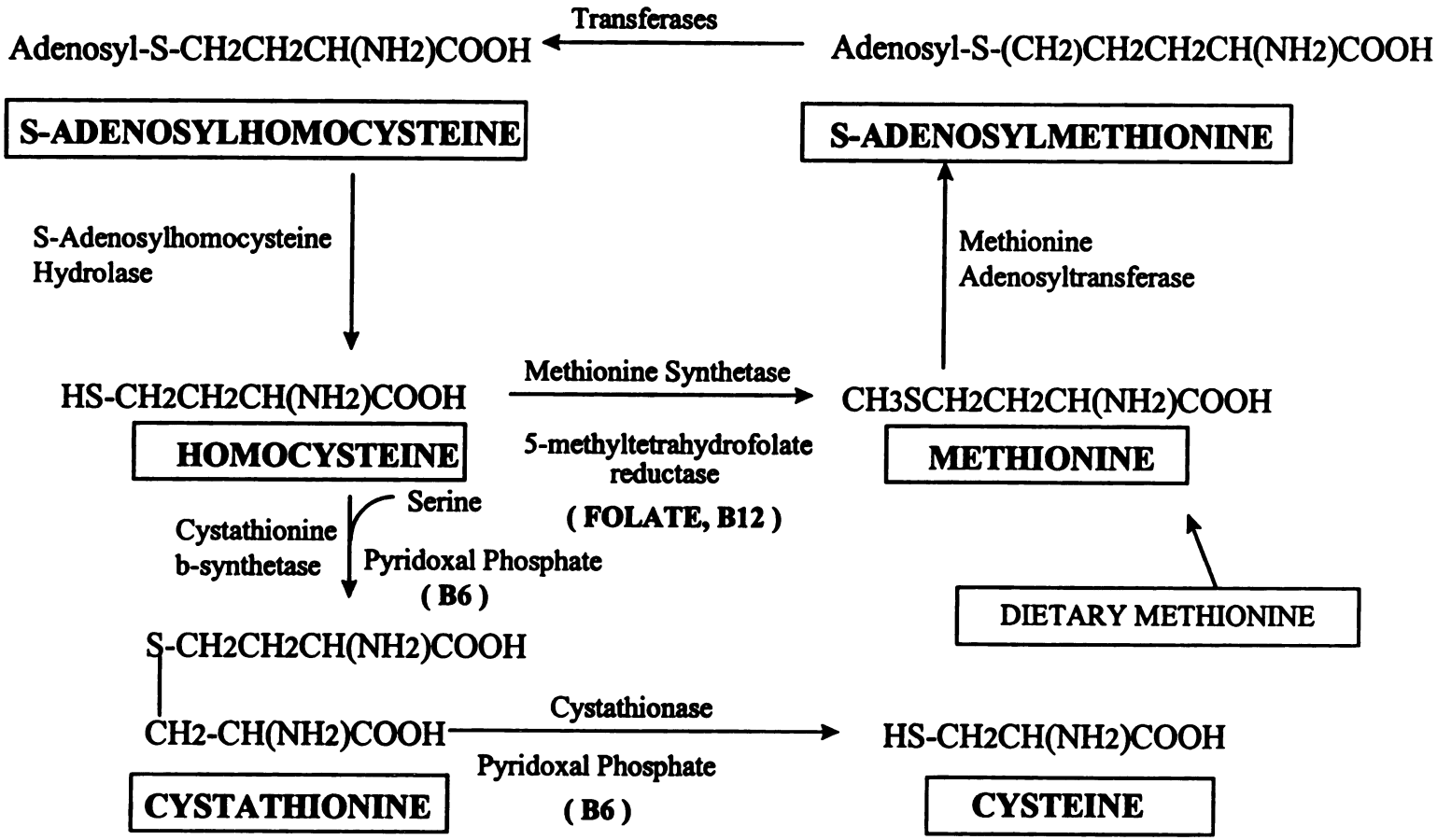

Figure 1. Pathways of homocysteine metabolism

yields the metabolite homocysteine, which can be metabolized via the irreversible trans-sulphuration pathway to cysteine. Major pathways of Hcy metabolism include first the remethylation of Hcy back to methionine. This remethylation reaction is catalyzed by methionine synthetase and 5-methyltetrahydrofolate reductase (MTHFR). MTHFR functions as the methyl group donor and requires folate as cofactor. The enzymatic conversion of Hcy to cystathionine is catalyzed by cystathionine b-synthetase, a vitamin B_6- dependent enzyme. Final conversion of cystathionine to cysteine is by the action of cystathionase, a vitamin B_6-dependent enzyme (Dudman et al, 1996).

Genetic defects causing an enzymatic deficiency of cystathionine b-synthetase or MTHFR reductase result in an accumulation of Hcy in the blood, leading to intermediate or severe hyperhomocysteinemia as defined by >16 µmol/l (Dudman et al, 1996). Current research also support that elevated plasma Hcy concentration may also be the result of nutritional inadequacies of vitamins B_6, B_{12} and/or folate (Ubbink et al, 1993) (Figure 2). Remethylation of Hcy to methionine is catalyzed by MTHFR, a folate and vitamin B_{12} dependent enzyme, (Dudman et al, 1996). Conversion of Hcy to cystathionine, and then to cysteine, is also a vitamin B_6-dependent reactions. Cystathionine b-synthetase and

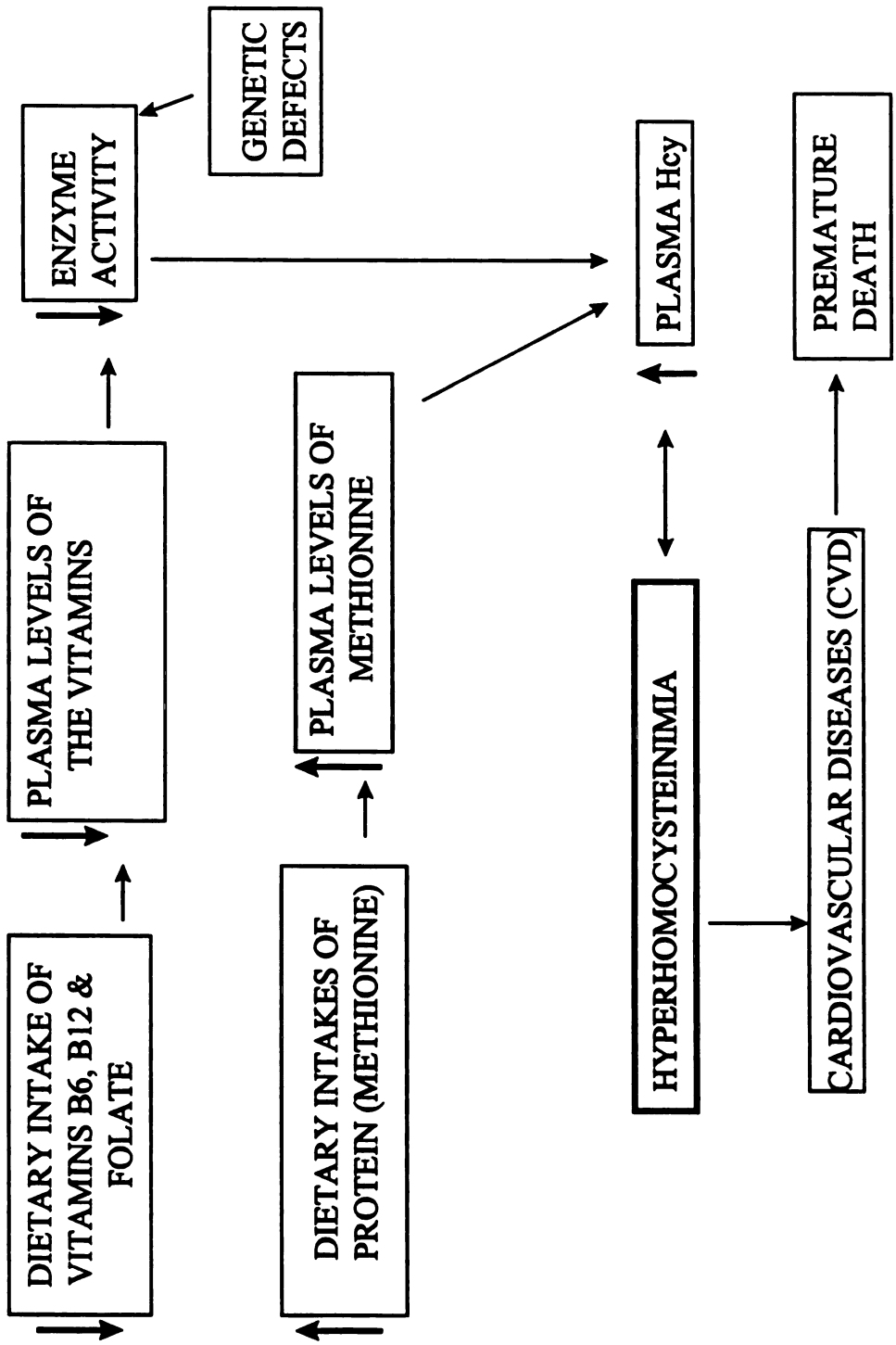

Figure 2. Diet and genetics in the development of cardiovascular diseases

cystathionase are both enzymes which require vitamin B_6 as a coenzyme.

Hyperhomocysteinemia has been, for epidemiological purposes, defined by Kang et al (1996), as plasma Hcy concentration >16 μmol/l whereas plasma Hcy concentration of healthy subjects as ≤16 μmol/l . When plasma Hcy concentration rises above 100 μmol/l, Hcy is also found in the urine (homocysteinuria) (Carlson and Neil, 1962).

Hyperhomocysteinemia is further classified in the literature as moderate, intermediate or severe, depending on plasma Hcy concentration (Table 1).

Table 1. Classification and clinical manifestations of hyperhomocysteinemia

Classification	Plasma Hcy (μmol/l)	Clinical manifestations	Etiology
Severe	>100	* neurological abnormalities * mental retardation * carotid thickening	homozygous and heterozygous enzyme deficiencies
Intermediate	31-100	* premature cerebrovascular peripheral and coronary artery disease * carotid thickening * thromboembolism	nutritional inadequacy with or without genetic defect
Moderate	16-30	* premature cerebrovascular peripheral and coronary artery disease	nutritional inadequacy with or without genetic defect
Normal	<16		

References: Kang S., 1996.

Hyperhomocysteinemia as a risk factor for cardiovascular diseases

Hyperhomocysteinemia was identified initially as a risk factor for cardiovascular diseases (CVD) through case series, cross-sectional and case control studies (Swift et al, 1986; Olszewski et al, 1991; Brattstrom et al, 1990; Clarke et al, 1991; Ubbink et al, 1993; Pancharuniti et al, 1994; Fermo et al, 1995). These studies consistently suggest a strong positive relationship (Odds Ratio (OR), 95% CI = 1.0-6.7) between elevated plasma Hcy concentration and the risk for CVD as measured by the degree of stenosis of the coronary artery (Swift et al, 1986; Olszewski et al, 1991; Brattstrom et al, 1990; Clarke et al, 1991; Miller et al, 1992; Ubbink et al, 1993; Pancharuniti et al, 1994; Selhub et al, 1995; Fermo et al, 1995). To date, a limited number of prospective studies on plasma Hcy and CVD have been reported (Taylor et al, 1991; Stampfer et al, 1992; Verhoef et al, 1994; Perry et al, 1995; Verhoef et al, 1995; Arnesen et al, 1995). These studies involved large cohorts to verify hyperhomocysteinemia as independent risk factor for CVD (Table 2)(Taylor et al, 1991; Stampfer et al, 1992; Verhoef et al, 1994; Perry et al, 1995; Verhoef et al, 1995; Arnesen et al, 1995).

Table 2. Risk factors for cardiovascular diseases

• Dietary cholesterol [1, 2, 3]
• Plasma cholesterol [2]
• Dietary protein [1]
• Dietary Fat [1]
• Plasma Homocysteine [1, 2, 3]
• Age [1]
• Hypertension [1]
• Smoking [1]
• Oral contraceptives [1]
• Sex [1]
• Alcoholism [1]
• Stress [1]
• Exercise [1]
• Diabetes [1]
• Body Mass Index [1]

References: [(1)] Gruberg et al, 1981, [(2)] Ubbink et al, 1996, [(3)] Pancharunti et al, 1994.

Prevalence of hyperhomocysteinemia among healthy individuals

In 1992, Anderson et al reported that 12 of 169 apparently healthy individuals in the city of Malmo, Sweden aged 20-69 had hyperhomocysteinemia (>16 μmol/l). Selhub et al (1994) also reported from the 20th biannual examination of the Framingham Heart Study (1989-90) that hyperhomocysteinemia (>90th percentile; plasma Hcy concentration >14.0 μmol/l) acounted for 30% of the entire cohort and over 40% of individuals aged 80 years and older (n=418 men and 623 women).

Hyperhomocysteinemia has been reported in people of all ages. Genetic defects and/or nutritional inadequacy of B-vitamins have been used in explaining hyperhomocysteinemia.

Prevalence of hyperhomocysteinemia in individuals with cardiovascular diseases

In 1991, Clarke et al reported that hyperhomocysteinemia (>16 μmol/l) was detected in 42% of 38 cerebrovascular disease patients, 28% of 25 peripheral vascular disease patients. After adjustments for the effects of conventional risk factors (i.e. hyperlipidemia, hypertension and cigarette smoking), patients with hyperhomocysteinemia had 3.2 OR for CVD. In 1992, Brattstrom et al reported that 40% of 142 stroke survivors and 6% of 66 control subjects had hyperhomocysteinemia. In 1995, Fermo et al also reported that moderate hyperhomocysteinemia (19.5-99.9 μmol Hcy/l plasma for male; 15.0-99.9 μmol Hcy/l plasma for female based on >95th percentile) was seen in 13.1% and 19.2% of patients with venous and arterial occlusive respectively. Dalery et al (1995) also reported a higher mean plasma Hcy concentration in coronary artery disease (CAD) patients (males and females) than healthy controls (men with CAD: 11.7±5.8 vs. controls: 9.7±4.9 nmol/ml; & women with CAD: 12.0±6.3 vs. controls: 7.6±4.1 nmol/ml, $P<0.01$ between cases and controls). The proportion of patients with CAD having Hcy levels >90th percentile of

controls (>14.5 μmol/l) was 18.1% for men and 44.4% for women (both P<0.01 compared to the controls).

Reference range of plasma Hcy concentration for both healthy and CVD or CAD patients vary among studies. It ranges from 7.6 to 16.3 μmol/l for healthy people, and from 13.5 μmol/l to 33.3 μmol/l for CVD or CAD patients. Many studies classified hyperhomocysteinemia as the >90th percentile of plasma Hcy concentration of healthy controls. With pooled data of numerous studies including a large number of population, reference values are hoped to be established in the proposed study.

Gender differences on plasma homocysteine concentration

In 1994, Jacobsen et al reported that 36 blood donors of apparently healthy men (X±SD: 34.4 ± 9.4 yrs) had a significantly higher range of plasma Hcy concentration (9.26-12.30 μmol/l) than 35 female blood donors (33.8 ± 6.2 yrs; 7.85-10.34 μmol/l). Brattstrom et al (1994) also reported that plasma Hcy concentration of 131 men was higher than that of 113 women with age ranging 35 to 95 years of age (mean±SD: 13.9±4.1 and 12.3±4.1 μmol/l respectively; P<0.001) and increased markedly with age (r= 0.488; P<0.001).

Gender differences in plasma Hcy concentration are not well understood biologically although most literature

supports the apparent gender differences. Gender needs to be more carefully examined in relation to the difference in plasma Hcy concentration in healthy and diseased stage.

Vitamin supplementation on plasma homocysteine concentration

Several studies reported effectiveness of megadose vitamin supplements in reducing plasma Hcy concentration. The doses used were several times higher than the RDA of vitamin B_6 (30 mg), vitamin B_{12} (25 µg) and folate (800 µg). In 1990, Brattstrom et al reported that daily supplementation of 240 mg pyridoxine hydrochloride and 10 mg folate for four weeks in 20 patients (heterozygotes for cystathionine b-synthatase and with moderate hyperhomocysteinemia) resulted in a reduction of 53% in the mean plasma Hcy concentration (baseline: 23.1±19.2 µmol/l; after supplementation: 10.9±7.2 µmol/l). Naurath et al (1995) also reported a prospective double-blind controlled study in which intramuscular vitamin injections were given daily to the elderly eight times over a three-week period. The vitamin injections contained 1 mg vitamin B_{12}, 1 mg folate, and 5 mg vitamin B_6. The elderly subjects in the study live at home (n=175; 65-96 years of age). The elderly who received the treatment (n=110), experienced 8% reduction in plasma Hcy concentration while those who received the placebo had no effect (Naurath et al, 1995). Ubbink et al (1995) also reported a reduction in plasma Hcy concentration

in white (n=18) and black (n=18) ranging from 19 to 24 years) when they were given 6 weeks daily oral vitamin supplementation of 1.0 mg folic acid, 400 µg vitamin B_{12} and 10 mg vitamin B_6. The participants were from the annual recruitment of new police at Pretoria's Police College. After the daily vitamin supplementation, fasting plasma Hcy concentration was reduced significantly from 9.6 ± 3.5 to 7.2 ± 1.6 µmol/l in whites ($P<0.05$) and from 8.4 ± 2.4 to 5.6 ± 1.4 µmol/l in blacks ($P<0.01$). The study of Ubbink et al (1995) also acknowledged the differences in plasma Hcy concentration between blacks and whites.

Supplementation of vitamin B_6, B_{12} and folate caused a significant reduction in mean plasma Hcy concentration in apparently healthy individuals. A greater reduction (53%) was achieved in individuals with hyperhomocysteinemia (Brattstorm et al, 1990). Thus nutritional adequacy of the B-vitamins may play a significant role in maintaining normal levels of plasma Hcy concentration.

Prevalence of suboptimal blood concentration of vitamins B_6, B_{12} and folate in healthy individuals

In 1992, Pennypacker et al reported that of 152 geriatric outpatients (65-99 yrs old) attending the Veteran's Administration Medical Center Geriatrics Clinic in Denver, Colorado 14.5% had vitamin B_{12} deficiency as diagnosed by ≤ 300 pg/ml serum. Yao et al (1992) reported

that 16% of the elderly (N=169) receiving primary care had vitamin B_{12} deficiency with < 200 pg/ml, and 21% had 201-299 pg/ml. In 1993, Joosten et al also reported that the free living elderly (>65 yrs old; n=145) and hospitalized elderly (>65 yrs old; n=135) had low serum concentration of the vitamin B_{12} (6% and 5%, respectively), of folate (5% and 19%, respectively) and of vitamin B_6 (9% and 51%, respectively).

Suboptimal plasma concentration of vitamins B_6, B_{12} and folate are very common in the elderly. If increasing prevalence of hyperhomocysteinemia with age is related to the high prevalance of vitamin deficiencies in the subgroup should be further investigated.

Prevalence of suboptimal blood concentration of vitamins B_6, B_{12} and folate in individuals with hyperhomocysteinemia

Ubbink et al (1993) reported that the prevalence of suboptimal concentration of plasma vitamin B_6 (<14 nmol/l), serum B_{12} (<150 pmol/l) and plasma folate (<5 nmol/l) status in people with moderate hyperhomocysteinemia (>16.3 μmol/l, n=44) was 25%, 56% and 59.1% respectively. In 1996, Selhub et al also reported these suboptimal plasma concentrations of one or more B-vitamins (B_6<12 nmol/l; B_{12}<140 pmol/l; folate<4 nmol/l) were detected in 67% of hyperhomocysteinemic subjects (≥16.3 µmol/l plasma Hcy) included in the Framingham Heart Study.

The prevalence of suboptimal blood concentration of the B-vitamins in hyperhomocysteinemic patients is very high (25%-67%). Among individuals with hyperhomocysteinemia, deficiency of folate and B_{12} is higher than that of vitamin B_6 .

Folate, vitamin B_6 and vitamin B_{12} : sources, deficiency, toxicity and reported intakes

Folate is a water-soluble vitamin found primarily in liver, yeast, leafy vegetables, legumes and some fruits (RDA, 1989; Combs, 1992). Folate was discovered in early 1930's in India, where it was found as a cure for macrocytic anemia. A few years later, the vitamin was named folic acid, a term derived from the Latin word *folium* meaning leaf. Folate is unstable in heat during cooking (RDA, 1989; Combs, 1992).

Serum folate concentration decreases when dietary intake of folate is inadequate. A serum folate concentration of <3 ng/ml indicates folate deficiency. Serum folate concentration, however, does not reflect depletion of body stores (Combs, 1992; Friedrich, 1988). Tissue depletion of folate is reflected by ≤140 ng/ml erythrocyte folate concentration. Tissue depletion of folate is also diagnosed by neutrophil hypersegmentation, a change in the morphology of the peripheral white blood cells. Hypersegmentation is caused by impaired DNA synthesis in white blood cells. The

end stage of folate deficiency is characterized by megaloblastic anemia, which is similar to vitamin B_{12} deficiency (Combs, 1992; Friedrich, 1988) (Table 3).

Table 3. Symptoms or signs of deficiency and toxicity of vitamins B_6, B_{12} and folate.

Vitamin B_6	Vitamin B_{12}	Folate
	Deficiency:	
* peripheral neuropathies * dermatitis * anemia	* macrocytic megaloblastic anemia * peripheral neuropathies * memory loss	* megaloblastic anemia * general weakness * depression * polyneuropathy * neural tube defects * pernicious anemia
	Toxicity:	
* convulsions * sensory neuropathy * ataxia * loss of small motor control	* innocuous (mice)	* epileptic responses (rats) * renal hypertrophy (rats)

Combs, F.G. The vitamins: Fundamental aspects in Nutrition. Academic Press; 1992.

In a folate and vitamin B_{12} deficiency, the red blood cells are large in size (macrocytic) while concentration of hemoglobin remains normal (normochromic). In contrast, iron deficiency leads to small sized red blood cells (microcytic) with low concentrations of hemoglobin (hypochromic) (Combs, 1992; Friedrich, 1988).

The RDA for folate is 200 µg for adult men, 180 µg for women, and 400 µg for pregnant women. For infants from birth to one year, the RDA for folate is set at 3.6 µg/kg body weight (Table 4).

Table 4. Recommended Dietary Allowances for vitamins B_6, B_{12} and folate

	Vitamin B_6 (mg)	Vitamin B_{12} (μg)	Folate (μg)
Males (15+ yrs)	2.0	2.0	200
Females (15+ yrs)	1.6	2.0	180
Pregnancy	2.2	2.2	400
Lactation	2.1	2.6	280

Source: Food and Nutrition Board. Recommended Dietary Allowances, 10th Ed. Washington, DC: National Academy Press;1989.

Vitamin B_6 is also a water soluble vitamin, found in meats, whole-grain products, vegetables, and nuts. It exists in various chemical forms in food (pyridoxine, pyridoxal, pyridoxamine). The various forms of the vitamin are converted in liver, in erythrocytes and in other tissues into pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP), which are the active forms of the vitamin (Combs, 1992; Friedrich, 1988; Bailey, 1990). PLP, serves as a coenzyme of transaminases and decarboxylases which are involved in the metabolism of amino acids; as a coenzyme for phosphorylases; in the biosynthesis of the neurotransmitters serotonin, epinephrine, and norepinephrine; coenzyme of glycogen phosphorylase (Combs, 1992; Friedrich, 1988; Bailey, 1990), and as a modulator of steroid hormone receptors. Severe deficiency of vitamin B_6 results in dermatologic and neurologic abnormalities (peripheral

neuropathies), weakness, cheilosis, glositis, and impaired cell-mediated immunity. The toxicity of vitamin B_6 occurs rarely in humans. Massive doses (10XRDA) of the vitamin have produced convulsions in rats (Combs, 1992; Friedrich, 1988; Bailey, 1990).

The requirement for vitamin B_6 varies depending on type of diet, health and some other factors. High intake of protein increases the dietary requirement for vitamin B_6. (Combs, 1992; Friedrich, 1988; Bailey, 1990). Human RDA of vitamin B_6 is established based on the ratio 0.016 mg pyridoxine per gram protein intake(RDA,1989; Combs, 1992). The ratio is to prevent or eliminate the appearance of biochemical indicators of deficiency when daily protein intakes range from 54 to 165 g (RDA, 1989; Combs, 1992). The RDA for vitamin B_6 was then established based on twice the RDA for protein (126 g/day for men, 100 g/day for women): 2.0 mg/day for men and 1.6 mg/day for women (RDA, 1989; Combs, 1992) (Table 4).

Vitamin B_{12} is synthesized exclusively by bacteria, and is found only in foods that have been bacterially fermented or derived from tissues of animals (Glusker, 1995; Combs, 1992; Friedrich, 1988; Bailey, 1990). Animal tissues that accumulate vitamin B_{12} (e.g. liver) are, therefore excellent food sources of the vitamin for humans.

Vitamin B_{12} is involved in the maintenance of nervous tissue, normal blood formation, and general growth. Biochemical functions of the vitamin include conversion of methyl malonyl-CoA to succinyl CoA, intracellular synthesis of polyglutamate forms of folate, methylation of homocysteine to methionine and in nucleic acid metabolism (Glusker, 1995; Combs, 1992; Friedrich, 1988; Bailey, 1990). Vitamin B_{12} deficiency in humans causes delay of normal cell division, megaloblastic anemia, neurological abnormalities (neurological lesions), memory loss, and progressive nerve demyelination as part of a progressive neuropathy (Glusker, 1995; Combs, 1992; Bailey, 1990) (Table 3). The RDA for vitamin B_{12} is set at 2μg/day for adults, a value that is expected to prevent any signs of deficiency (Glusker, 1995; RDA, 1989; Combs, 1992) (Table 4).

During the last 35 years vitamins B_6, B_{12} and folate have been recognized to play a significant role in Hcy metabolism. Nutritional deficiency of any one of the vitamins may lead to an elevation of the amino acid, in plasma, which is risk factor for thrombotic disorders and CVD. Nutritional adequacy of the three vitamins may be important in maintaining plasma Hcy concentration and to reduce the risk for CVD.

Mechanisms by which Hcy may promote cardiovascular diseases

Hcy may promote cardiovascular diseases by any or combination of the following proposed mechanisms:

a. Protein C anticoagulant pathway

The blood clotting factors IX, X and prothrombin are synthesized in the liver in a process that requires vitamin K. Factor XI is activated via a process that requires calcium. Factor IX is then activated by the presence of the activated factor XI (XIa) and calcium (Dittman and Majerus, 1990; Rosendal et al, 1995). Activated factor IX (IXa), and calcium are required for the activation of factor X. During the final steps of the blood clotting cascade in humans, the coagulation factor prothrombin is converted to thrombin on the phospholipid membrane, in the presence of activated factor Xa, Va, VIIIa and calcium. Thrombin catalyzes the conversion of fibrinogen to soft clot fibrin. The soft clot fibrin is fragile and rapidly converted to a more stable hard clot in a reaction catalyzed by XIIIa (Dittman and Majerus, 1990; Rosendaal et al, 1995).

Individuals deficient in various clotting factors have a pronounced tendency to bleed. A blood clot consists of arrays of cross-linked fibrin that forms an insoluble fibrous network.

Protein C is a vitamin K-dependent plasma protein which inhibits blood coagulation by enzymatic cleavage of the factors Va and VIIIa, and thus interferes with the regulation of intravascular clot formation (Dittman and Majerus, 1990; Rosendaal et al, 1995) (Figure 3). Thrombin is a proteinase that converts fibrinogen into fibrin, causing blood coagulation, by hydrolyzing peptides. Thrombin is bound to a platelet and endothelial cell membrane receptor called thrombomodulin. Thrombomodulin is an anticoagulant glycoprotein that serves as a cofactor for the activation of protein C. Binding of thrombin to thrombomodulin activates protein C. Activated protein C cleaves and thus inactivates factors Va and VIIIa (Figure 3)(Dittman and Majerus, 1990; Rosendaal et al, 1995). By doing so, protein C causes an inhibition of the coagulant activity of the factors and helps maintaining blood fluidity (Svensson and Dahlback, 1994). Protein C deficient individuals often die in infancy of massive thrombotic complications.

Homocysteine interferes with protein C by two possible mechanisms. First, homocysteine prevents thrombomodulin transport to the cell surface (Figure 3) (Lentz and Sadler, 1991). This prevents the formation of thrombin-thrombomodulin complex and causes a reduction in protein C activation, less inhibition of factors Va and VIIIa and

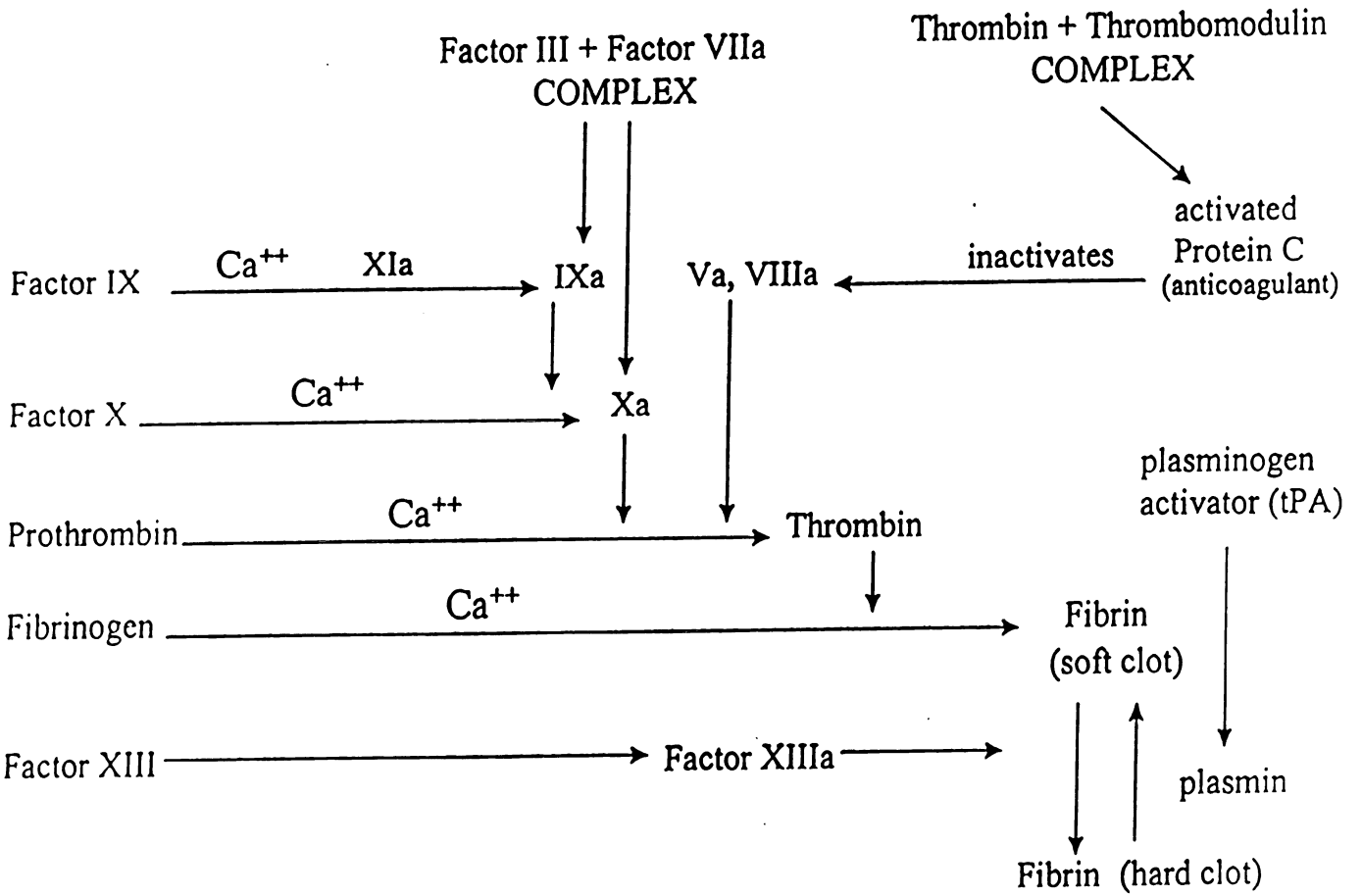

Figure 3. Blood clotting cascade in humans

promotion of blood coagulation. In the second mechanism homocysteine interferes the blood coagulation pathway by reducing the disulfide bonds between thrombomodulin and protein C. This decreases protein C activation and increases blood coagulation with thrombogenic events (Svensson et al, 1993; Rosendaal et al, 1995).

b. Heparin antithrombin activity

There are numerous physiological mechanisms that limit clot formation. Antithrombin reduces all active clotting factors by binding to them. The presence of heparin enhances the activity of antithrombin. Heparin is released by injury, activates antithrombin and thereby prevents clot formation (Rosenberg and Rosenberg, 1984; Nishinaga et al, 1993). Heparin is the most frequently used anticoagulant administered before and after surgery to retard clot formation.

Hcy causes a reduction in the binding affinity of endothelial cells for antithrombin. By doing so, less antithrombin binds to the endothelial cells and this decreases the anticoagulant properties of the factors (Rosenberg and Rosenberg, 1984; Nishinaga et al, 1993).

c. Plasminogen activator function

Blood clots (fibrin) are normally eliminated as wound repairs progress. Fibrin is dissolved by plasmin, an enzyme which cleaves fibrin (Figure 3). Plasmin is formed from precursor plasminogen activator. Plasminogen activators have received considerable medical attention since they rapidly dissolve the blood clots responsible for heart attacks and strokes.

Homocysteine causes a reduction of the cellular binding sites for the plasminogen activator (Hajjar et al, 1987; Hajjar et al, 1993). This causes accumulation of fibrin, reduces blood fluidity and increases the risk for thrombosis (Hajjar et al, 1987; Hajjar et al, 1993).

d. Fibrin binding activity of lipoprotein(a)

Lipoprotein(a) is an atherogenic lipoprotein which is composed of low density lipoprotein (LDL) particles. Lipoprotein(a) which has a very similar structure to plasminogen, competes for the plasminogen activator. This causes a reduction in plasmin formation and promotes thrombotic events (Harpel et al, 1992). Homocysteine enhance the binding of Lipoprotein(a) to fibrin and promotes the thrombogenic events.

e. Tissue coagulation factor stimulation

Tissue coagulation factor (Factor III) is a transmembrane glycoprotein that plays a major role in the initiation of blood coagulation. Cell surface factor III forms a complex with factor VIIa (Figure 3). This complex increases the activation of factors X, IX, which in the presence of VIIIa and Va convert prothrombin to thrombin. Plasma Hcy increases the factor III activity of endothelial cells resulting in increase of all other coagulation factors (Nemerson et al, 1992; Fryer et al, 1993).

f. Endothelial ADPase activity

The endothelial cell membrane catabolizes ADP to AMP. The energy released is utilized to modulate platelet reactivity. Hcy reduces the ADPase activity (Broekman et al, 1994) and thus platelet reactivity, which lead to platelet aggregation and blood clotting.

Summary

Coagulation factors and protein C have a significant role in the maintenance of blood fluidity. The proposed mechanisms by which Hcy promotes atherogenic events are not fully understood. A number of other possible mechanisms have also been proposed. In summary, elevated plasma concentration of Hcy leads to inactivation of the anticoagulant factors and/or activation of the procoagulant

factors . Activation or inactivation of pro or anti-coagulant factors causes disruption of the blood fluidity which leads to thrombotic events.

Meta analysis: purposes

The scientific literature provides a large number of replicated studies on plasma Hcy concentration in relation to age, gender, disease stage and nutritional status of B-vitamins. Such replication is essential to enhance the validity and accuracy of previous experiments, and to further our knowledge of the same topic.

Meta-analysis is a research method that combines previously reported research to arrive systematically and quantitatively at conclusions about the entire body of research on a selected topic. Meta-analysis attempts to integrate empirical research findings to derive generalizations (Cooper and Hedges, 1994). Meta-analysis, as a rigorous research synthesis method, attempts to make a sense of the rapidly expanding research literature (Glass, 1976).

To date, reported meta-analytic research on plasma Hcy is limited to only one (Boushey et al, 1995). The meta-analytic assessment examined plasma Hcy as a risk factor for vascular disease and the potential reduction of CAD by increasing folate intake.

Meta-analysis was chosen for the current study because of inconsistency in the results of independent studies. Meta-analysis accumulates results across studies and help us to gain accurate knowledge on the relationship between age, gender, disease and B-vitamins status and plasma Hcy concentration in humans. Meta-analysis is the best approach to establish reference values for healthy and CVD patients in an effort to establish prognostic tools that can be used to reduce CVD.

Meta-analysis: procedures

Meta-analysis follows five rigorous and systematic steps to arrive at conclusions: a) Problem formulation, b)Data collection, c) Data evaluation, d) Data analysis and interpretation and e) Presentation.

a. Problem formulation

Problem formulation is the stage to construct definitions that distinguish relevant studies from irrelevant studies, and to identify evidences to be included in the review. Formulation of a research problem should considers: a) the population to which generalizations are made, and b) the source of hypothesis. For the current meta-analysis, we assumed that the generalized population can be addressed by either fixed effects model (conditional), or

the random effects model (unconditional) (Cooper & Hedges, 1994).

In the conditional model, the population to which generalization is applied derived from the examined population. The only source of error is therefore presumed to be the sampling of subjects in the studies. In the unconditional model, the current study sample is presumed to be a sample from a hypothetical collection of studies. The population to which generalizations are applied in the current study is based on the studies from which the study sample is drawn. The source of hypotheses in problem formulation is theory and previously reported primary research studies and meta-analyses. The strength of synthesis compared to primary research lies in its ability to examine information accrued over multiple replications. The results of meta-analysis can also help direct future research.

b) Data collection

This process is aimed at determining a) sources of relevant studies and 2) procedures to identify the relevant evidence from the potential sources. The data collection procedure is designed to yield studies that are representative of the intended population of studies. First, critical terms descriptive of the topic under investigation

are identified. Correct definition of terms can accurately describe the topic at the appropriate level of specificity. The right terms are then linked to selected library reference databases (i.e. Medline, Agricola etc.) to identify potential relevant studies. If the research criteria are consistent with the population definition, potential studies should be representative samples from the population of studies (Cooper & Hedges, 1994). Potential studies are then examined based on inclusion criteria, and relevant studies are accumulated.

c) Data evaluation

Data evaluation is the stage in which features of interest, subject characteristics, and correlations, and any other measures of association reported in the included reported study are coded (Cooper & Hedges, 1994: pg 10-11). This coding process allows the researcher to assess the quality and amount of information present in each reported study for the present meta-analytic study.

d) Data analysis and interpretation

At this step we apply statistical procedures to draw inferences about the research questions, and use statistical procedures to make inferences about the literature as a whole. The meta-analyst generally draws inferences using one

of the following types of information from research reports: a) data that can be used to calculate effect-size estimates (e.g., means, S.D, test statistic values), b) statistical significance of hypothesis tests, and c) direction of the outcomes. The statistical procedure is chosen based on the statistical format and available data of the research studies (Cooper & Hedges, 1994). Meta-analytic findings should be then carefully interpreted based on discussion of research reports included in the meta-analysis.

e) Presentation

At this stage the meta-analyst apply editorial criteria to identify and organize important information to be included in the scientific report (Cooper & Hedges, 1994). Information need to be displayed in simple ways that can enhance the clarity of the conclusion. The purpose of this stage is to present meta-analytic findings accurately in order to help the readers grasp the big picture quickly (Cooper & Hedges, 1994).

Strengths and limitations of meta-analysis

Scientific literatures are cluttered with repeated studies of the same phenomena. Repetitive studies are sometimes because of inherent sampling error, investigator's lack of knowledge or because they are skeptical about the

results of past studies. Improved analytical methods are necessary. Meta-analysis increases reliability and allows quantitative and qualitative data analysis by combining the results of previously reported individual studies .

The revolution of computer technology has greatly expanded the ability and speed of literature searches, making meta-analysis possible based on thousands of journals and volumes of research data (Cooper & Hedges, 1994). Meta-analysis increases the precision of estimates by combining related studies. The approach is valuable in formulating hypotheses that could not have been tested in primary research studies. Meta-analysis can uncover results that raise interesting questions about relationships among variables (e.g. CBVD vs. CVD). By accumulating results across studies, one can gain an accurate representation of relationship that exists in the population.

Meta-analyses are sometimes subjected to criticisms for a lack of control or objectivity because the process involves comparisons or summaries of different studies. This criticism can be avoided if important distinctions are observed by various techniques, such as proper coding, to ensure that the researcher differentiates among studies. Lack of objectivity may be overcome by including variety of data including unpublished data such as theses or dissertations can reduce the bias.

Chapter Three

METHODOLOGY

Data Collection

Medline (1966-97) was searched for all relevant literature, including reviews, by using combinations of keywords: [plasma or blood] and [homocysteine], [plasma or blood or dietary] and [folate], [plasma or blood or dietary] and [B6 and/or pyridoxine], [plasma or blood or dietary] and [B12 and/or cobalamin]. Repeated instances of research reports were excluded by combining previous searches with the term [or]. The medline search identified 182 articles as potentially relevant to the meta-analysis (Table 5).

Table 5. Results of Medline search (1966-97)

	Keywords	Articles (n)
1	[blood or plasma] and [homocysteine]	828
2.	[plasma or blood or dietary] and [folate]	2263
3.	[plasma or blood or dietary] and [B_6 and/or pyridoxine]	1170
4.	[plasma or blood or dietary] and [B_{12} and/or cobalamin]	2471
5.	#1 and #2	140
6.	#1 and #3	37
7.	#1 and #4	86
8.	#5 or #6 or #7	182

Seventeen more articles were identified from the references cited in these reports, for a total of 199 research reports. Articles were systematically reviewed and some were excluded specifically: 1) review articles, 2) articles focusing on the mechanisms by which homocysteine may promote CVD, 3) animal studies, 4) studies with Blacks, 5) studies with homozygous or heterozygous homocysteinemic patients, 6) studies on analytical methods for plasma Hcy, 7) studies with subjects deficient in vitamin B6 and/or vitamin B12 and/or folate, and 8) studies with supplementation of vitamin B_6, vitamin B_{12} and folate. In all, 48 research reports met inclusion criteria while 151 were excluded from the meta-analysis (Table 6).

Coding of Research Reports

A standardized coding sheet (APPENDIX A) was developed to summarize data from each research report meeting the inclusion criteria. The coding sheet included four parts: a) characteristics of study and subjects, b) blood measurements, c) correlation matrix and d) relevant findings (Appendix A). The first part a) characteristics of study and subjects, included research-report reference characteristics (ID No, author, year, source and country), statistical

Table 6. Included and excluded number of articles from the meta-analysis.

	Articles (n)
Articles for meta-analytic inclusion	
1. Medline search	182
2. Reference cited in the articles	17
Total	199
Exclusion Criteria	
1. Review articles	19
2. Mechanisms of Hcy	25
3. Animal studies	22
4. African American subjects	4
5. Genetic Hyperhomocysteinemia	21
6. Analytical methods for Hcy	4
7. Suboptimal plasma B-vitamins	32
8. B-vitamin supplementation	24
Total	151
Total included in the meta-analysis	48

methods, number of subjects of both genders of cases and controls, and disease type of the cases. The second part b) blood measurements included analytical methods and means and standard deviations for concentrations of plasma homocysteine and blood vitamins. The third part c) correlation matrix included reported correlations of plasma homocysteine and vitamins B_6, B_{12} and folate, as well as correlations within the three vitamins for both cases and controls of both genders . The last part, d) relevant

findings, summarized relevant data or other information related to the examined variables. An example of a coded research report can be found in Appendix A.

Subject Variables

The subject variables were gender of the sample (coded as male, female or combined) and age (mean or range). The total population for the meta-analysis consisted of four groups: 1) cases of case-control studies, 2) controls of case-control studies, 3) healthy individuals included in cross-sectional studies and 4) diseased individuals included in cross-sectional studies. Cases were subjects diagnosed with pathological conditions of either a) cardiovascular disease (CVD), b) cerebrovascular disease (CBVD), or c) all other diseases (AOD). The CVD group included subjects with coronary artery disease, coronary heart disease, ischemic stroke, myocardial infarction, peripheral arterial occlusive disease, risk factors for CVD, transient ischemic attacks and subgroups with venous thrombosis. The CBVD group included subjects with acute stroke, stroke, cerebral bleeding, cerebral infarction and cerebral thrombosis. The third subgroup (AOD) included those subjects with asthma, dementia, end-stage renal disease, geriatric problems and alcoholism. Subjects falling within the AOD subgroup were excluded from the meta-analysis. Controls were subjects who

were free from any life-threatening diseases and matched for age and gender within each study with the cases.

Study Variables

Seven analytical methods of homocysteine measurement were reported in the 48 selected research reports. These included high performance liquid chromatography with fluorometric detection (HPLC-FD), electrochemical detection (HPLC-ED), radioenzymatic assay (HPLC-REA), gas chromatography-mass spectometry (HPLC-GCMS), amino acid analyzer (AAA), ion exchange chromatography (IEC) and non-specified high performance liquid chromatography (HPLC). Plasma Hcy concentrations were expressed in the literature in terms of the arithmetic mean (AM), geometric mean (GM) or median (MD).

Statistical Analysis

The Statistical Package for the Social Science (Windows version 7.5, 1997, SPSS Inc, Chicago, IL) was used for the statistical analyses of the present study. Procedures for the meta-analysis followed in this study are outlined in the Handbook of Research Synthesis (Cooper and Hedges, 1994).

Objective One: To determine whether vascular disease cases have a higher mean plasma Hcy concentration than healthy controls.

All 48 studies reported mean plasma Hcy concentration of cases and controls in the same measurement unit (μmol/L). Cross-sectional study subjects were not included in this analysis. Meta analysis for objective one followed steps by determining

1) A within-study mean difference in plasma Hcy concentration between the case and control groups (T_i: individual study effect size) was estimated by Formula 1.
2) The pooled variance within individual studies ($S_i^2{}_{pool}$) by Formula 2.
3) The variance of each individual study effect size (v_i) by Formula 3.
4) The individual study weight (w_i) by Formula 4.
5) The mean effect size ($T_{average}$) by Formula 5.

The individual study effect size is:

(Formula 1) $T_i = T_{1i} - T_{2i}$,

where T_i is the mean plasma [Hcy] difference of study i (effect size for study i),
T_{1i} is the mean plasma [Hcy] of the cases for study i, and
T_{2i} is the mean plasma [Hcy] of the controls for study i;

The pooled variance within individual studies is:

$$\text{(Formula 2)}\quad S_i^2{}_{pool} = \frac{(n_{1i}-1)S_{1i}^2+(n_{2i}-1)S_{2i}^2}{(n_{1i}+n_{2i}-2)},$$

where $S_i^2{}_{pool}$ is the pooled variance in study i,
n_{1i} is the sample size of the cases for study i,
n_{2i} is the sample size of the controls for study i,
S^2_{1i} is the variance of the cases for study i, and
S^2_{2i} is the variance of the controls for study i;

The variance of individual study effect size is:

$$\text{(Formula 3)}\quad v_i = S^2{}_{pool}[(1/n_{1i}+1/n_{2i})],$$

where v_i is the variance of T_i for study i,
$S_i^2{}_{pool}$ is the pooled variance for study i,
n_{1i} is the sample size of cases for study i, and
n_{2i} is the sample size of controls for study i;

The individual study weight is:

$$\text{(Formula 4)}\quad w_i = 1/v_i,$$

where w_i is the weight for study i, and
v_i is the variance of T_i for study i.

The mean effect size is:

$$\text{(Formula 5)}\quad T_{average} = \Sigma(w_i * T_i)/\Sigma w_i,$$

where $T_{average}$ is the mean effect size,
w_i is the weight for study i,
T_i is the effect size for study i.

A positive $T_{average}$ indicates that mean plasma Hcy concentration of the cases is higher than that of the controls in the population.

A homogeneity test is then performed to test the null hypothesis that differences in mean plasma Hcy concentration between the cases and the controls groups in all of the studies derived from the same population [$var(\Theta_i)=0$, where Θ_i is the population mean difference for study i]. The statistical test performed for homogeneity is given by Formula 6.

The homogeneity test is:

(Formula 6) $Q= \Sigma[(T_i-T_{average})^2*(w_i)]$,

where Q is a chi-square with k-1 df,
K is the number of studies,
T_i is the effect size for study i,
$T_{average}$ is the mean effect size, and
w_i is the weight for study i.

When all Θ_i are equal, the statistic Q is distributed as a chi-square with k-1 degrees of freedom (df), with k as the number of studies. If the critical value for a chi-square with k-1 df exceeds Q, the null hypothesis that all studies share a common mean difference is accepted. A fixed-effects model is then examined.

If Q exceeds the critical value for a chi-square with k-1 df, the null hypothesis that all studies share a common mean difference is rejected. A random-effects model is then examined.

Fixed effects model:

$T_i = \Theta_i + e_i$

where T_i is the effect size for study i,
Θ_i is the population mean for study i, and
e_i is the sampling error for study i.

Under the fixed effects model the data to be combined arise from a series of independent studies, in which the ith study reports one effect size T_i, with population effect size Θ_i. The fixed effects model is based in the assumption that $\Theta_1=...\Theta_k...=\Theta$, that all studies share a common effect size.

Random effects model:

$T_i = \Theta_i + e_i + u_i$,

where T_i is the effect size for study i,
Θ_i is the population mean for study i,
e_i is the sampling error for study i, and
u_i is the random effect for study i.

Under this model, Θ_i is not fixed, that is all studies do not share a common effect. Θ_i is random and has its own distribution.

A random effects variance of u_i is estimated using Formula 7 below. The studies are re-weighted by Formula 8, and a new $T_{average}$ is calculated using Formula 5 with the new weights.

The random effects variance of u_i is:

(Formula 7) $\sigma^2_e = K\{[Q_e/(K-p-1)]\}/\Sigma_{wi}$,

where σ^2_e is the random effects variance,
k is the number of studies,
Q_e is the weighted residual sum of square of the regression,
p is the number of parameters in the model, and
Σ_{wi} is the sum of individual studies weights;

The new individual study weight is:

(Formula 8) $w_i = 1/(\sigma^2_e + v_i)$,

where w_i is the new weight for study i,
σ^2_e is the random effects variance,
v_i is the variance of T_i for study i.

Onces fixed or random effects model is chosen based on the homogeneity test, confidence interval are calculated by Formulas 9 and 10.

The variance of μ is:

(Formula 9) $\sigma^2 = 1/\Sigma w_i$,

where σ^2 is the variance of μ, and
Σw_i is the sum of individual study weights;

The confidence interval for μ is:

(Formula 10) $95\%CI = T_{average} \pm 1.96\ (\sigma)$,

where $T_{average}$ is the mean effect size, and
σ is the standard deviation of the mean $T_{average}$.

If the 95% confidence interval does not contain zero, the null hypothesis that the mean population effect size between the vascular disease cases and healthy controls across studies is zero is rejected.

Objective Two: To determine if plasma Hcy concentration of men is higher than that of women for both vascular disease cases and healthy controls.

Data on mean plasma Hcy concentration of healthy subjects (males and females) from cross-sectional and case control studies were pooled together for gender differences analysis. Data were also pooled for mean plasma Hcy concentration differences among male and female subjects, diagnosed with CVD or CBVD (cases). Gender differences in mean plasma Hcy concentration within either the case or control group, individual study weights (w_i), mean effect size (T_i) and confidence intervals (CI) were calculated following formula 1-7 described in Objective One.

Selection of a fixed or random effects model was based on a homogeneity test, similar to that in Objective One, which was to examine gender differences in mean plasma Hcy concentration in either the case or the control group.

Objective Three: To establish reference values for plasma Hcy concentration for vascular disease cases and healthy control groups.

Reference values (mean±SD plasma Hcy concentration) were calculated for vascular disease cases and healthy controls (male or female) separately.

Objective Four: To determine if plasma Hcy concentration differs between CBVD and CVD case groups.

Data on mean plasma Hcy concentration of CBVD and CVD subjects (males and females) from cross-sectional and case control studies were pooled together for difference in mean plasma Hcy concentration. The within study comparison of the two disease groups was performed first with both male and female disease subjects in the same group. Subjects were then separated based on gender, and within study gender effect sizes were calculated as shown in Objectives One and Two.

A homogeneity test was performed to determine whether a random effects model was appropriate, for determining if plasma Hcy concentration differs among case groups. If Q exceeded a chi-square test with k-1 degrees of freedom then a random effects model is examined. A random effects variance is estimated using Formula 9 and the studies are re-weighted by Formula 10 as in Objective One. Based on the

new weights, a new $T_{average}$ and a new confidence interval were calculated, to test the null hypothesis that CBVD and CVD cases do not differ in mean plasma Hcy concentration. Gender difference within CBVD and CVD groups was also investigated.

Objective Five: To estimate the predictability of plasma Hcy concentration by blood vitamin B_6, vitamin B_{12} and/or folate concentration.

A weighted multiple regression was to determine whether blood B-vitamin status of participants was related to their mean Hcy concentration. A homogeneity test was performed for fixed or random effects model as described before (Objective 3). The random effects model examined is following:

Random effects model:

$$Hcy = B_0 + e_i + U_i + \beta_1(B6_i) + \beta_2(B12_i) + \beta_3(folate_i) + \beta_4 (B6_i \times B12_i) + \beta_5 (B12_i \times folate_i) + \beta_6 (B6_i \times folate_i) + \beta_7 (B6_i \times folate_i \times B12_i)$$

where Hcy is the study sample mean plasma Hcy concentration,
B_0 is the population mean plasma Hcy concentration,
e_i is the sampling error,
U_i is the random effects for study i,
β_{1-7} is the slope of the variable,
$B6_i$ is the mean plasma vitamin B_6 concentration in study i,
$B12_i$ is the mean serum vitamin B_{12} concentration in study i, and
$folate_i$ is the mean blood folate concentration in study i.
X is the interaction terms between the B-vitamins.

Objective Six: To estimate the predictability of plasma Hcy concentration by dietary intake of vitamin B_6, vitamin B_{12} and folate.

A weighted multiple regression was performed at the study level to determine whether dietary B-vitamins of controls and healthy cross-sectional subjects were related to their mean plasma Hcy concentrations. A homogeneity test was performed for fixed or random effects model as described before (Objective 3). The random effects model examined is the following:

Random effects model:

$$Hcy = B_0 + e_i + U_i + \beta_1(B6_i) + \beta_2(B12_i) + \beta_3(folate_i) + \beta_4 (B6_i \times B12_i) + \beta_5 (B12_i \times folate_i) + \beta_6 (B6_i \times folate_i) + \beta_7 (B6_i \times folate_i \times B12_i)$$

where Hcy is the study sample mean plasma Hcy concentration,
B_0 is the population mean plasma Hcy concentration,
e_i is the sampling error,
U_i is the random effects for study i,
β_{1-7} is the slope of the variable,
$B6_i$ is the dietary vitamin B_6 intake in study i,
$B12_i$ is the dietary vitamin B_{12} intake in study i,
$folate_i$ is the dietary folate intake in study i.
x is the interaction terms between the dietary B-vitamins.

Chapter Four

RESULTS

Research Reports and Subjects

Data included in this meta-analysis were from 48 primary research articles describing case-control studies (n =32) and cross-sectional studies (n=16), (Tables 7 and 8). Several articles reported data from multiple sub-studies with independent sub-group subjects.

The 48 studies included in this meta-analysis (Tables 7-9) represent a total of 26,132 healthy subjects (controls), and 4,549 vascular diseased cases (CBVD or CVD). Mean ages of healthy controls and vascular disease cases were 56.3 ± 11.0 yrs (range: 48.6 ± 8.0 yrs from 25 studies for healthy controls) and 61.6 ± 9.3 yrs (age range: 48.6 ± 7.8 yrs from 27 studies), respectively for vascular disease cases.

Differences in mean plasma Hcy concentration between vascular disease cases and healthy controls and between genders.

(Objective One and Two)

Table 7. Study designs of the research reports included in the meta-analysis.

	Authors	Design*	Cases	Controls
1a.	Alfthan et al (1994)	cc	Acute MI cases selected from a population-based random sample.	A five year age stratum was calculated after which equal numbers of controls were randomly selected.
1b.		cc	Acute stroke cases selected from a population-based random sample.	
2	Anderson et al (1992)	cs	N/A	City residents in good health, no history of actual or previuos renal hepatic or vascular disease, took no medication or vit. supplements.
3a.	Araki et al (1989)	cc	Inpatients who had cerebral infarction (CI) and survived acute stroke.	Normotensive inpatients admitted to the hospital with no suffering from any acute vascular disease nor history of MI or stroke.
3b.		cc	Inpatients who had cerebral bleeding (CB) and survived acute stroke.	
4	Arnesen et al (1995)	cc	Identified from a population based cohort as patients with CHD or died suddenly after onset of chest pain.	Subjects from same population based cohort who were without disease at the time of diagnosis and matched for age and sex.
5a.	Brattstrom et al (1988)	cs	N/A	Subjects with normal blood biochemical variables, free of actual or previous renal, hepatic or vascular disease were divided randomly into three groups.
5b.		cs	N/A	
5c.		cs	N/A	
6a.	Brattstrom et al (1990)	cc	Patients with intermittent claudication or Leriche's disease by aorto-iliac disease, and underwent a by-pass surgery.	Healthy individuals, randomly selected from participants in a health screening program, no medication and no symptoms of any disease.
6b.		cc	Patients with a history of transient ischemic attacks, stroke, evidence of carotid artery stenosis or occlusion.	
6c.		cc	Patients with cerebral thrombosis, in whom cerebral infarction was verified by computerized tomography.	
7a.	Brattstrom et al (1992)	cc	Patients with history of acute stroke falling within 38-72 age range.	Healthy individuals, randomly selected from participants in a health screening program.
7b.		cc	Patients with history of acute stroke falling with 73-90 age range.	

* cc = case control
cs = cross sectional
N/A = not applicable
a.,b.,c. = denote subpopulation studies reported in an article

Table 7 (cont'd)

	Authors	Design	Cases	Controls
8a.	Brattstrom et al (1994)	cs	N/A	Randomly selected from the local population records with age ranging from 35-69 years.
8b.		cs	N/A	Randomly selected from the local population records with age ranging from 70-95 years.
9	Cacan et al (1996)	cs	N/A	White-collar workers employed by a major utility company with no metabolic disorders and no clinical or ECG manifestations of CVD.
10	Chadefaux et al (1994)	cs	N/A	Healthy individuals randomly selected from laboratory personnel.
11a.	Coull et al (1989)	cc	Acute stroke patients recruited via a Medical Center.	Healthy volunteers recruited through university faculty and staff.
11b.		cc	Patients with transient ischemic attacks.	
11c.		cc	Patients with history of hypertension, diabetes or CVD.	
12	Chu et al (1988)	cs	N/A	Serum samples for routine hospital assays from young subjects.
13	Cravo et al (1996)	cc	Chronic alcoholics and active drinkers.	Healthy volunteers from medical staff, matched for age and sex with cases.
14	Dalery et al (1995)	cc	CAD patients from a cardiology clinic or referred from the cardiology services in Montreal.	Healthy subjects free of major CAD risk factors, white-collar workers employed by a major utility company.
15	Den Heijer et al (1995)	cc	Patients who had first episode of deep-vein thrombosis were selected from anticoagulation clinics files.	Healthy neighbors or friends of cases matched for sex and age.
16	Den Heijer et al (1996)	cc	Patients who had had two or more episodes of venous thrombosis.	Invited people through a general practice for participation in a health survey.
17	Fermo et al (1995)	cc	Unrelated patients with a history of venous or arterial occlusive disease before the age of 45.	Healthy individuals, members of the hospital staff taking no medication and having no vascular disease.
18	Gary et al (1984)	cs	N/A	Subjects were healthy elderly volunteers who were seen as outpatients in a clinical research center in New Mexico Hospital.
19	Genest et al (1990)	cc	Patients with premature CAD after diagnostic cardiac catheterization and angiography.	Framingham Heart Study subjects not on a b-adrenergic blocker and clinically free of vascular disease.
20	Herzlich et al (1996)	cc	Patients undergoing coronary angiography at Maimonides Medical center.	Healthy subjects

Table 7 (cont'd)

	Authors	Design	Cases	Controls
21	Hopkins et al (1995)	cc	Unrelated subjects with early familial CAD who had survived a MI angioplasty or coronary artery bypass.	Selected from a random population sampling or were spouses of hypertensive siblings who participated in previous studies.
22	Israelsson et al (1988)	cc	Patients with their first MI before the age of 55	Selected on the basis of as few first degree relatives as possible with no history of MI or stoke.
23a.	Israelsson et al (1993)	cc	Patients who survived an ischaemic stroke.	Healthy people from the same data base matched with cases.
23b.		cc	Patients who survived an acute MI.	
24a.	Jacobsen et al (1994)	cs	N/A	Healthy laboratory personnel.
24b.		cs	N/A	
25a	Joosten et al (1993)	cc	Elderly hospitalized patients with common geriatric disease	Healthy young people belonging to the medical and nursing staffs who did not suffer from any concomitant disease and no medication
25b.		cc		Healthy elderly subjects selected by a practitioner, living at home and able to carry out all normal daily activities.
26	Landgren et al (1995)	cc	MI patients.	Free of previous vascular events and no regular intake of vitamins.
27	Lewis et al (1992)	cc	Angiographically demonstrated CAD.	Healthy people matched for sex and age with controls.
28a.	Lindenbaum et al (1994)	cs	N/A	Healthy young medical personnel employed by Columbia University.
28b.		cs	N/A	Surviving members of the Framingham Heart Study.
29a.	Malinow et al (1989)	cc	Patients with clinical evidence of arterial disease recruited from a medical center.	Healthy individuals from the staff of a research center less or equal to sixty years of age.
29b.		cc		Healthy individuals from the staff of a research center greater than sixty years of age.
30	Molgaard et al (1992)	cc	Recruited from an epidemiological study as intermittent claudication patients (IC).	Randomly selected healthy subjects from same population-based sample, sex and age-matched.
31a.	Nilsson et al (1994)	cc	Non-intitutionalized dementia patients.	Healthy subjects with no history of any vascular, hepatic or renal
31b.		cc		disease non-dementia subjects with psychiatric disorders.
32a.	Nilson et al (1996)	cc	Elderly patients with dementia	Elderly population with no history of any major vascular, hepatic or renal disease.
32b.		cc		Non-dementia subjects with psychiatric disorders.

Table 7 (cont'd)

	Authors	Design	Cases	Controls
33a.	Nygard et al (1995)	cs	N/A	Selected from the National Population Registry with no previous diagnosis of vascular or any other disease aging 40-42 years.
33b.	Nygard et al (1995)	cs	N/A	Selected from the National Population Registry with no previous diagnosis of vascular or any other disease aging 43-64 years.
33c.	Nygard et al (1995)	cs	N/A	Selected from the National Population Registry with no previous diagnosis of vascular or any other disease aging 65-67 years.
34	Pancharuniti et al (199	cc	Patients who underwent cardiac catheterization for presumptive CAD at the clinic.	Randomly selected from a commercial listing of telephone numbers of men living in the same counties area.
35	Perry et al (1995)	cc	Stroke patients.	Randomly selected from same population-based cohort without history of stroke or MI and matched by town and age group.
36	Riggs et al (1996)	cs	N/A	First to complete the full battery of tests which are being administered
37	Robinson et al (1996)	cc	Patients on hemodialysis or peritoneal dialysis with end-stage renal disease (ESRD).	Subjects attending an executive health screening program with no ECG evidence of vascular disease or renal failure.
38	Robinson et al (1995)	cc	Patients with established CAD.	Consecutive subjects attending an executive health screening program with no clinical or ECG evidence of CAD .
39a.	Selhub et al (1993)	cs	N/A	Surviving members of the Framingham cohort study 67-74 years of age.
39b.		cs	N/A	Surviving members of the Framingham cohort study 75-79 years of age.
39c.		cs	N/A	Surviving members of the Fram. cohort study 80 years of age or older.
40	Selhub et al (1995)	cs	N/A	Surviving members of the Framingham Heart Study Cohort who participated in the biennial examination.
41	Stampfer et al (1992)	cc	MI patients from the Physicians Health Study	Randomly selected from the same population as cases and paired matched.
42	Swift et al (1986)	cc	Volunteers were recruited and rated as high risk for coronary heart disease.	Volunteers recruited through the Center for Health Promotion and rated as low risk for coronary heart disease.
43	Ubbink et al (1993)	cs	N/A	Subjects employed by five different major employers in the vicinity of the author's laboratory.
44	Ubbink et al (1995)	cs	N/A	Healthy subjects recruited from the annual recruitment of new police.
45	Ubbink et al (1996)	cc	Asthma patients.	Healthy volunteers without any other life-threating conditions.

Table 7 (cont'd)

	Authors	Design	Cases	Controls
46	Verhoef et al (1996)	cc	Boston area patients hospitalized with a first MI and no history of previous MI	Randomly selected from the residents' list of the same town with no previous MI or angina, and matched for age and gender.
47	Verhoef et al (1994)	cc	Physician's Health Study sample with diagnosed ischemic stroke.	Participants were randomly selected and free from any type of cerebrovascular disease.
48	Wu et al (1994)	cc	Familial CAD probands who were ascertained from computerized discharge records from area hospitals.	Random population sample of parents listed on family history forms or spouses of hypertensive siblings who participated in other studies

Table 8. Characteristics and analytical methods of studies included in the meta-analysis.

	Authors	Analytical Methods		Cases	Percentile/ Hcy (umol/L)	No of Cases, Sex	No of Controls, Sex	Age, y
		Hcy	Vitamins					
1a.	Alfthan et al (1994)	HPLC-FD		MI	95 / 15.2	92M,99F	141M,128F	40-60
1b.				S	95 / 15.2	42M,32F	141M,128F	40-60
2	Anderson et al (1992)	IEC	TD, RTA				74M,83F	20-69
3a.	Araki et al (1989)	HPLC-FD		CI		30M,15F	30M,15F	39-79
3b.				CB		13M,7F	30M,15F	39-80
4	Arnesen et al (1995)	HPLC-FD		CHD		110M,12F	430M,48F	34-61
5a.	Brattstrom et al (1988)	AAA					14MF	43-56
5b.							13MF	43-56
5c.							15MF	43-56
6a.	Brattstrom et al (1990)	AAA	TD, RTA	IC		21M,16F	22M,24F	24-59
6b.				TIA		12M,6F	22M,24F	24-59
6c.				CT		8M,9F	22M,24F	24-59
7a.	Brattstrom et al (1992)	HPLC-ED	RTA	S		54M,16F	34M,32F	38-72
7b.						49M,23F	34M,32F	73-90
8a.	Brattstrom et al (1994)	HPLC	RTA				69M,52F	35-69
8b.							62M,61F	70-95
9	Cacan et al (1996)	HPLC-FD	TD, RA, MA		95 / 17.85		380M, 204F	23-59
10	Chadefaux et al (1994)	HPLC-PC	RDA				26MF	<50
11a.	Coull et al (1989)	HPLC-ED		AS		33M,8F	16M,15F	40-90
11b.				TIA		24M,3F	16M,16F	40-90
11c.				RCV		28M,3F	16M,17F	40-90
12	Chu et al (1988)	HPLC-REA					18MF	Young
13	Cravo et al (1996)	HPLC-FD	TD, RA, MA	AL		24M,8F	19M, 12F	29-60
14	Dalery et al (1995)	HPLC-FD	TD, RA, MA	CAD	95 / 15.5	123M,27F	380M,204F	20-59
15	Den Heijer et al (1995)	HPLC-FD		VT	95 / 18.5	269MF	269MF	<70
16	Den Heijer et al (1996)	HPLC-FD		VT	95 / 22.2	185MF	220MF	20-97
17	Fermo et al (1995)	HPLC	IA	PAOD	95 / 15.5	73M,84F	30M,30F	<45
18	Gary et al (1984)		RA, RA				130M,150F	60-93

Table 8 (cont'd)

	Authors	Analytical Methods		Cases	Percentile/ Hcy (umol/L)	No of Cases, Sex	No of Controls, Sex	Age, y
		Hcy	Vitamins					
19	Genest et al (1990)	HPLC-ED		CAD		170M	255M	40-60
20	Herzlich et al (1996)	HPLC-ED	RA, RA	CAD		367MF		73(6.7)
21	Hopkins et al (1995)	HPLC-ED	RA, CIBA System	CAD		120M,42F	85M,70F	38-68
22	Israelsson et al (1988)	AAA		MI		21M	36M	48-58
23a.	Israelsson et al (1993)	HPLC-FD		IS		13M	21M	44-70
23b.		HPLC-FD		MI		16M	29M	44-70
24a.	Jacobsen et al (1994)	HPLC-FD	TD, RA,RA				36M,35F	22-66
24b.							36M,35F	22-66
25a.	Joosten et al (1993)	HPLC-GCMS	TD, RA, RA	GP		115M,171F	53M,46F	19-88
25b.						115M,171F	20M,44F	19-88
26	Landgren et al (1995)	HPLC		MI	95 / 17.3	54M,14F	50M,30F	28-81
27	Lewis et al (1992)	HPLC-ED	MA	CAD		101M	108M	<50
28a.	Lindenbaum et al (1994)	HPLC-GCMS	RA, RA				58M,59F	22-63
28b.							200M,348F	22-63
29a.	Malinow et al (1989)	HPLC-ED		PAOD		26M,21F	35M,39F	<60
29b.						26M,21F	18M,11F	>60
30	Moolgard et al (1992)	HPLC-ED	RA	IC		78M	98M	45-69
31a.	Nilsson et al (1994)	IEC	RA, RA	DM	95 / 19.9	58M,106F	18M,27F	74(9)
31b.							41M,91F	77(12)
32a.	Nilsson et al (1996)	AAA	RA, RA	DM		121M,174F	84M,79F	75(7)
32b.							65M,150F	75(7)
33a.	Nygard et al (1995)	HPLC					5918M,6348F	40-42
33b.							287M,305F	43-64
33c.							1386M,1932F	65-67
34	Pancharuniti et al (1994)	HPLC-ED	RA, MA	CAD		101M	108M	30-50
35	Perry et al (1995)	HPLC-FD		S		107M	118M	40-59
36	Riggs et al (1996)	HPLC-FD	RA, RA				70M	54-81
37	Robinson et al (1996)	HPLC-FD	RLA	ESRD	95 / 16.3	97M,79F	185M,46F	56(15)

Table 8 (cont'd)

	Authors	Analytical Methods		Cases	Percentile/ Hcy (umol/L)	No of Cases, Sex	No of Controls, Sex	Age, y
		Hcy	Vitamins					
38	**Robinson et al (1995)**	**HPLC-FD**	**TD, RLA**	**CAD**	**80 / 13.5**	**201M,103F**	**185M,46F**	**62(11)**
39a.	**Selhub et al (1993)**	**HPLC-FD**	**TD, RA, MA**		**90 / 14.0**		**239M,310F**	**67-74**
39b.	**Selhub et al (1993)**						**110M,204F**	**75-79**
39c.	Selhub et al (1993)						108M,189F	80+
40	Selhub et al (1995)	HPLC-FD	TD, RA, MA				418M,623F	67-96
41	Stampfer et al (1992)	HPLC-ED	MA	MI	95 / 15.8	271M	271M	40-84
42	Swift et al (1986)	HPLC	TD, RA	RCV		5M	9M	48-66
43	Ubbink et al (1993)	HPLC-FD	RA, RA			44M	274M	19-71
44	Ubbink et al (1995)	HPLC-FD					74M	19-24
45	Ubbink et al (1996)	HPLC-FD	RA	A		8M,14F	8M,16F	>21
46	Verhoef et al (1996)	HPLC-ED	TD	MI		97M,33F	81M,37F	<76
47	Verhoef et al (1994)	HPLC-ED		IS		109M	427M	40-84
48	Wu et al (1994)	HPLC-ED	RE	CAD		170M,63F	87M,81F	23-75

Analytical methods

AAA: Amino Acid Analyzer
ED: Electrochemical detection
FD: Fluorometric Detection
GCMS: Gas Chromatography-Mass Spectometry
HPLC: High Performane Liquid Cromatography
IA: Immunoassay
IEC: Ion Exchange Chromatography
MA: Microbial Assay
PC: Paper Cromatography
RA: Radio Assay
RDA: Radiodilution Assay
REA: Radioenzymatic Assay
RLA: Radioligand
RTA: Routine Analysis
TD: Tyrosine Decarboxylase

Diseases

AL: Alcoholics
AS: Acute Stroke
CAD: Coronary Heart Disease
CB: Cerebral Bleeding
CHD: Coronary Heart Disease
CI: Cerebral Infraction
CT: Cerebral Thrombosis
DM: Dementia
ESRD: End Stage renal Disease
GP: Geriatric Problems
IC: Intermittent Claidication
IS: Ischemic Stroke
MI: Myocardial Infraction
PAOD: Peripheral Arterial Occlusive Disease
RCV: Risk Factors for Cardiovascular Disease
S: Stroke
TIA: Transient Ischemic Attacks
VT: Venous Thrombosis

Table 9. Number of studies and subjects used for this meta- analysis

		No of articles (N_1)	No of studies (N_2)	No of subjects (n)
Objective One				
	controls			4549
	cases			3080
total		41	42	
Objective Two				
control	males			1520
	females			1327
total		9	10	
case	males			426
	females			143
total		5	6	
Objective Three				
	controls	41	42	23587
	cases	41	42	3080
Objective Four				
cases	CVD			460
	CBVD			191
total		4	5	
CVD	males			50
	females			63
total		2	2	
CBVD	males			145
	females			71
total		2	3	
Objective Five				
cases &	B_6			2778
controls	B_{12}			3081
	folate			3623
total		27	29	
Objective Six				
controls	B_6			2319
	B_{12}			2319
	folate			2319
total		9	9	

Differences in mean plasma Hcy concentration between vascular disease cases and healthy controls and between genders.

(Objective One and Two)

Forty two research reports presented data on mean plasma Hcy concentration for vascular disease cases subjects [(n=3,080; males(n)=1,812 (58.8%); females(n)=735 (23.9%); both(n)=533 (17.3%) and controls (n=4549; males(n)=2596 (57%); females(n)=735 (16.3%) and both(n)=1,218 (26.7%)]. Data from case-control studies were only used for meta-analysis of this objective. Cross-sectional data were excluded.

Mean plasma Hcy concentration of cases (13.9±3.9 μ mol/l; range:9.4-33.8 μmol/l) was significantly ($P<0.05$) higher than healthy controls (10.9±1.5 μmol/l; range:7.3-14.0 μmol/l, $T_{average}=2.6$, 95%CI=2.42, 2.78). The difference in mean plasma Hcy of cases and healthy controls was about 3 μmol/l (Table 10).

An overall test of homogeneity (H_T) was conducted to test the null hypothesis that differences in mean plasma Hcy concentrations between vascular disease cases and healthy among the studies represent that they all were derived from the same population. The overall H_T value, 328.75, was greater than the critical value for a chi-square test with 41 degrees of freedom ($X^2=55.7$) (Table 11).

Table 10. Mean plasma Hcy concentration of sub-population groups.

Groups		n	Hcy (SD) (μmol/l)	Range (μmol/l)	Significance
cases			13.9 (3.9)	9.4-33.8	*
controls			10.9 (1.5)	7.3-14.0	
cases	men		14.2 (2.6)	11.7-20.0	ns
	women		13.7 (3.1)	12.0-21.7	
healthy	men		11.2 (1.6)	9.3-14.0	*
	women		9.5 (2.1)	7.6-13.2	
cases	CBVD		16.5 (3.3)	13.7-21.7	*
	CVD		12.6 (2.1)	12.7-18.5	

(± SD)
ns: not significant at P<0.05*
n = subject number

Table 11. Fixed effect sizes and homogeneity tests of mean plasma Hcy concentration.

effect size	$T_{average}$	95%CI	K	df	H_T	X^2	Significance	
cases-controls	2.6	2.4, 2.8	42	41	328.8		55.7	*
cases: men vs. women	0.5	1.6, -0.6	6	5	4.7		11.0	ns
controls: men vs. women	1.6	1.3, 1.9	10	9	18.8		16.9	*
cases : CBVD vs. CVD	0.6	0.4, 0.8	4	3	3.5		7.8	ns

P <0.05

(P<.05); therefore, the null hypothesis of homogeneity among the 42-study mean differences between cases and controls was rejected. A random effects model was then applied by estimating the random effects variance and the new weights. The mean plasma Hcy difference calculated from the random effects model was $T_{average}$=2.6 (95%CI=2.2, 3.0).

Graphic representation of mean plasma Hcy concentration of cases and controls when the effect of age was controlled (Figure 4), showed that for all age groups, the cases had a higher mean plasma Hcy concentration than the healthy controls.

Difference in plasma Hcy concentration between men and women (Objective Two)

A homogeneity test (H_T) was conducted, to test the null hypothesis that gender differences in plasma Hcy concentration within healthy and diseased groups denote that all samples were derived from the same population. The overall value H_T for gender difference in plasma Hcy concentration within the healthy group, (H_T =18.8) was larger than the chi-square critical value of 16.9 of 9 degrees of freedom (P<0.05). A random effects model was then applied.

The H_T value for plasma concentration between the diseased men and women (H_T= 4.7), was smaller than the chi-square critical value of 11.0 with 5 degrees of freedom

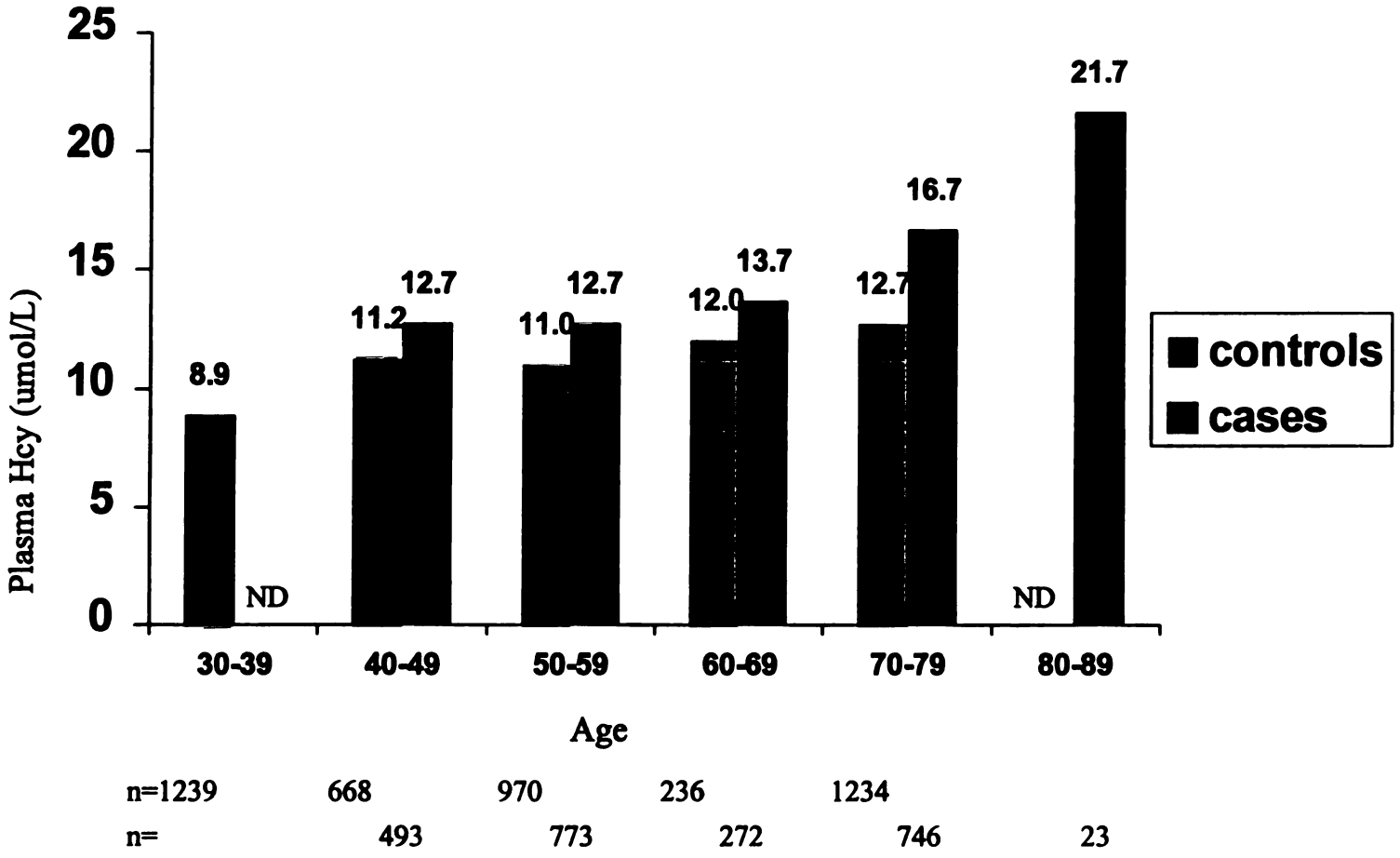

Figure 4. Weighted mean plasma Hcy concentration of cases and controls by age group.

controls: healthy people without any life-threatening diseases.
cases: people with cerebrovascular or cardiovascular diseases.
ND: no data available

($p<0.05$) (Table 11). A fixed effect model was subsequently used.

Mean plasma Hcy concentration of healthy men (11.2±1.6 μmol/l; range:9.3-14.0 μmol/l) was significantly higher ($P<0.05$) than that of healthy women (9.5±2.1 μmol/l; range:7.6-13.2 μmol/l) with an average difference of 1.7 μmol/l , (95%CI=1.3, 1.9).

The gender difference persisted even when the effect of age was controlled (Figure 5). In all age groups, healthy men had a higher mean plasma Hcy concentration than healthy women. No data are available for comparison of healthy women to men for the age range of 50-59 years.

Mean plasma Hcy concentration of the diseased men (14.2±2.6 μmol/l; range:11.7-20.0 μmol/l) was not statistically higher than that of diseased women (13.7±3.1 μmol/l; range:12.0-21.7 μmol/l), with an average difference of 0.5μmol/l, (95% CI=-0.6, 1.6) (Table 9). Male cases had a similar mean plasma Hcy concentration to female cases for the age groups of 50-59, 60-69, and 70-79 years (Figure 6). No data are available for women cases of 40-49 year range, and males of 80-89 year age range.

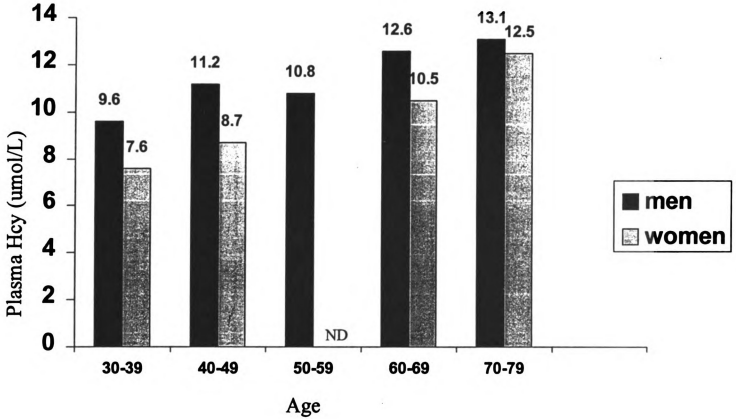

Figure 5. Weighted mean plasma Hcy concentration of healthy controls by gender and age.

controls: healthy people without any life-threatening disease.
ND: no data available

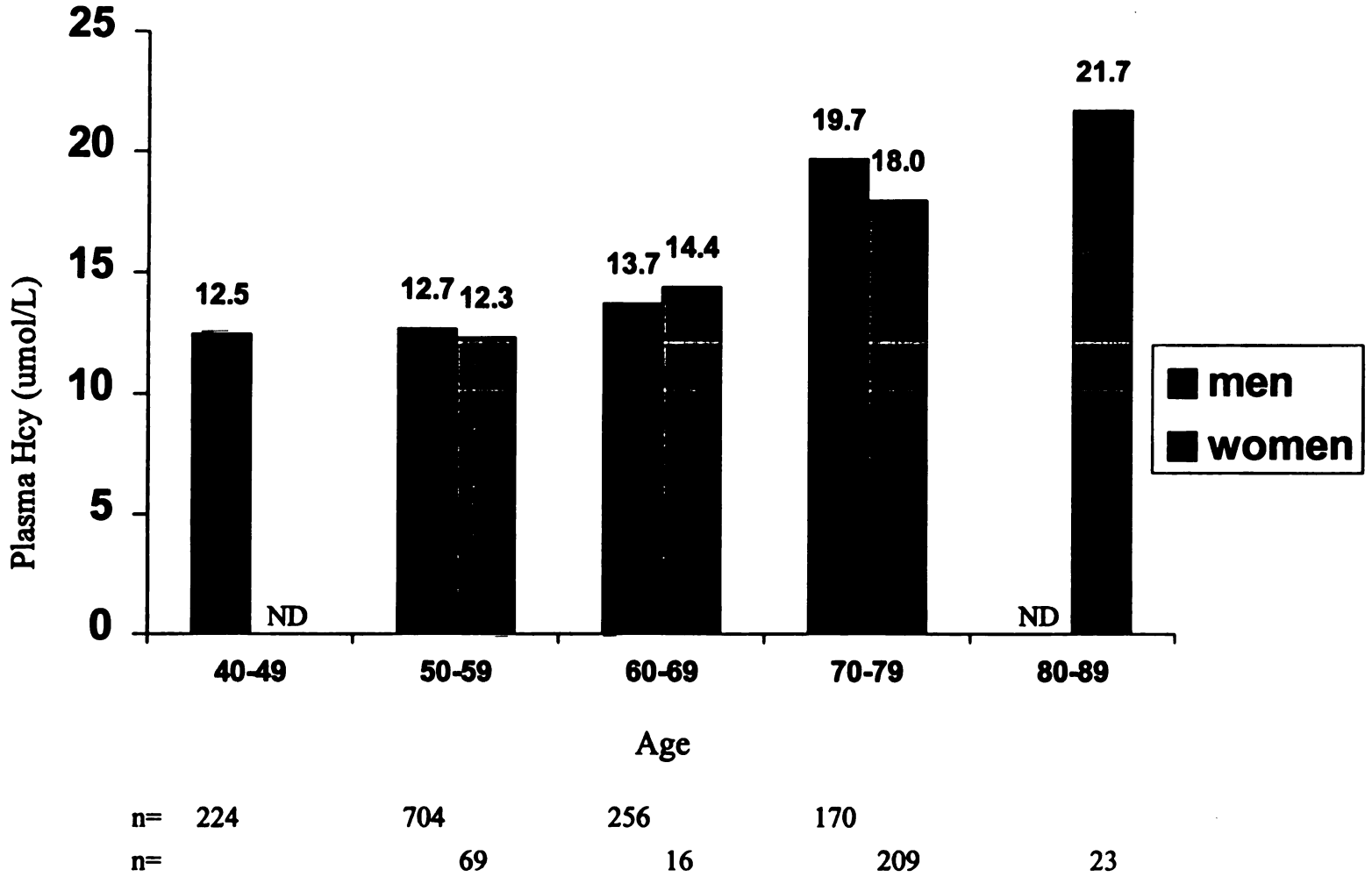

Figure 6. Weighted mean plasma Hcy concentration of cases by gender and age.

cases: people with cerebrovascular or cardiovascular diseases.
ND: no data available

Normal range of plasma Hcy concentration of healthy adults and a cut-off point for hyperhomocysteinemia.

(Objective Three)

We calculate the normal plasma Hcy concentration value (mean ± SD) which can be used as a reference value of healthy adults. Normal range of plasma Hcy concentration of healthy adults were estimated based on data used for Objectives One, Two (Table 12).

Table 12. Mean plasma Hcy concentration of healthy adults and those with vascular diseases.

Group	Gender	Hcy (SD) (umol/l)	Range (umol/l)
vascular disease cases	both	13.9 (3.9)	9.4-33.8
	men	14.2 (2.6)	11.7-20.0
	women	13.7 (3.1)	12.0-21.7
healthy controls	both	10.9 (1.5)	7.3-14.0
	men	11.2 (1.6)	9.3-14.0
	women	9.5 (2.1)	7.6-13.2

The large number of healthy men and women included in this meta-analysis had a mean(SD) plasma Hcy concentrations of 11.2(1.6) μmol/l and 9.5(2.1) μmol/l, respectively. Mean(SD) plasma Hcy concentration of vascular disease men did not

differ from that of vascular disease women (Objective Four), but 13.9(3.9) μmol/l been to indicate a critical value for hyperhomocysteinemia and increase risk for vascular diseases.

Difference in mean plasma Hcy concentration of CBVD and CVD cases

(Objective Four)

Mean plasma Hcy concentration of CBVD cases (16.5±3.3 μmol/l; range:13.7-21.7 μmol/l) was significantly ($P<0.05$) higher than that of CVD cases (10.9±1.5 μmol/l; range:7.3-14.0 μmol/l, $T_{average}=2.6$, 95%CI=2.42, 2.78).

The weighted mean plasma Hcy concentration of CBVD patients was significantly higher than that of CVD patients even after age (Figure 7) and gender (Figure 8) were controlled. Insufficient studies and samples were available for CBVD in the age range of 40-49 and the CVD group for the 80-89 age range.

A separate analysis of differences in mean plasma Hcy concentration among men and women within CBVD or CVD case groups, showed no significant differences within CBVD (CI=-0.4, 0.6) and CVD (CI=-0.2, 0.4) groups among men and women.

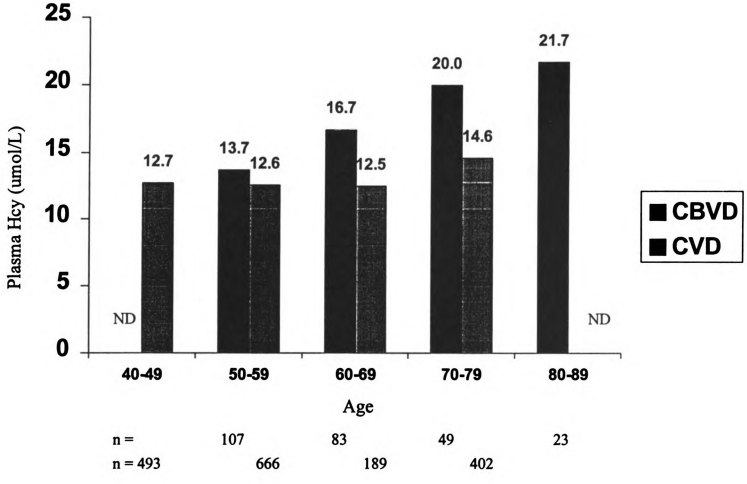

Figure 7. Weighted mean plasma Hcy concentration of CBVD & CVD groups by age.

CBVD: Cerebrovascular diseases (e.g. stroke, cerebral bleeding, cerebral infarction, cerebral thrombosis)
CVD: Cardiovascular diseases (e.g. coronary artery disease, myocardial infraction, peripheral arterial disease)
ND: no data available

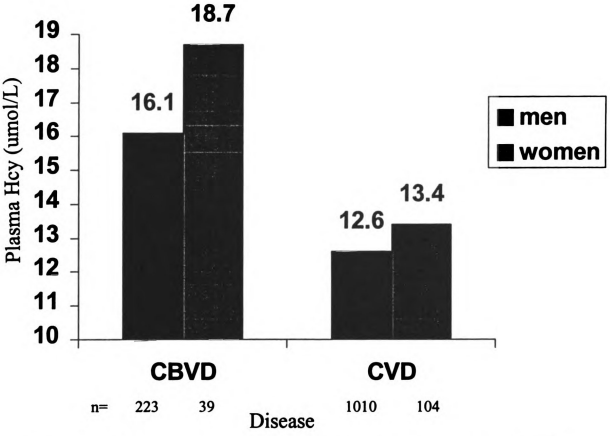

Figure 8. Weighted mean plasma Hcy concentration of cases by gender and disease.

CBVD: Cerebrovascular disease patients
CVD: Cardiovascular disease patients

The predictability of plasma Hcy concentration by blood B-vitamin concentration.

(Objective Five)

Pearson's correlations were calculated to assess blood B-vitamin interactions (Table 12). All correlations within the B-vitamins were highly significant (P<.01). Blood folate had a correlation of 0.66 with plasma B_6 and a correlation of 0.42 with serum B_{12}. Serum B_{12} was also highly correlated

Table 13. Pearson correlations coefficients among plasma concentrations and dietary intakes of B-vitamins.

	blood			dietary	
	serum B_{12}	blood folate		dietary B_{12}	dietary folate
plasma B_6	0.58**	0.66**	dietary B_6	0.72**	0.28**
serum B_{12}		0.42**	dietary B_{12}		0.19**

** P< 0.01

with plasma B_6 (r=0.58).

Simple linear regressions for predicting mean plasma Hcy concentration by individual mean blood B-vitamin concentration was performed (Table 14, Model 1-3). Data were pooled together from both vascular disease cases and healthy controls. All three blood B-vitamins had significant negative associations with plasma Hcy concentration (β_{pB6} = -0.016; β_{sB12} = -0.004; and β_{bfol} = -0.033) (Table 14). The

negative association between the blood B-vitamins and mean plasma Hcy concentration is presented in Figures 9-11.

Weighted multiple regression of plasma Hcy concentration of all three vitamins without interaction terms also regressed significance (β_{pB6} = -0.192; β_{sB12} = -0.172; and β_{bfol} = -0.068).

When the weighted multiple regression model included all three blood B-vitamins plus their interactions (Table 14, Model 5), backward elimination retain serum B_{12} (β_{sB12} = -

Table 14. Simple and multiple regression of blood B-vitamin concentration to plasma Hcy concentration.

Model		Coefficients B	Std. Error	t	Sig
1	(Constant)	13.598	0.117	116.228	
	Vitamin B_6	**-0.016**	0.002	-9.255	0.000
2	(Constant)	14.125	0.225	62.913	
	Vitamin B_{12}	**-0.004**	0.001	-5.941	0.000
3	(Constant)	29.665	1.550	19.142	
	folate	**-0.033**	0.005	-7.141	0.000
4	(Constant)	94.082	0.000	633.100	
	Vitamin B_6	**-0.192**	0.000	-114.000	0.000
	Vitamin B_{12}	**-0.172**	0.000	-413.000	0.000
	folate	**-0.068**	0.000	-5370.000	0.000
5	(Constant)	94.082	0.000	922.000	
	Vitamin B_{12}	**-0.152**	0.000	-619.000	0.000
	folate	**-0.067**	0.000	-750.000	0.000
	Vitamins B_6*B_{12}	**-0.001**	0.000	-235.000	0.000

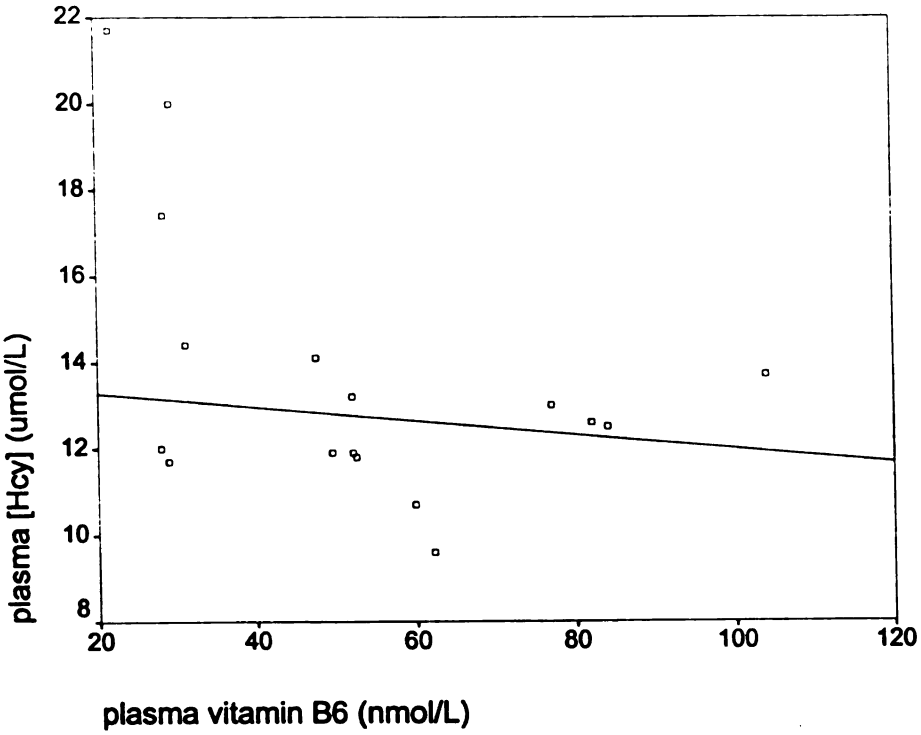

Figure 9. Scatterplot of plasma Hcy concentration and plasma vitamin B_6 concentration.

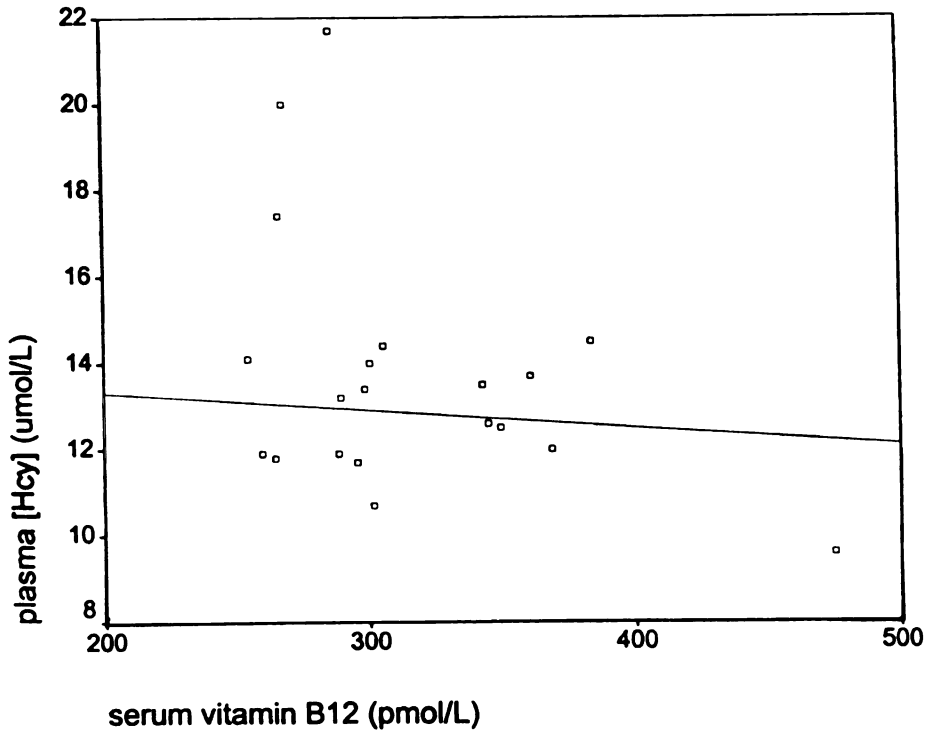

Figure 10. Scatterplot of plasma Hcy concentration and serum vitamin B_{12} concentration.

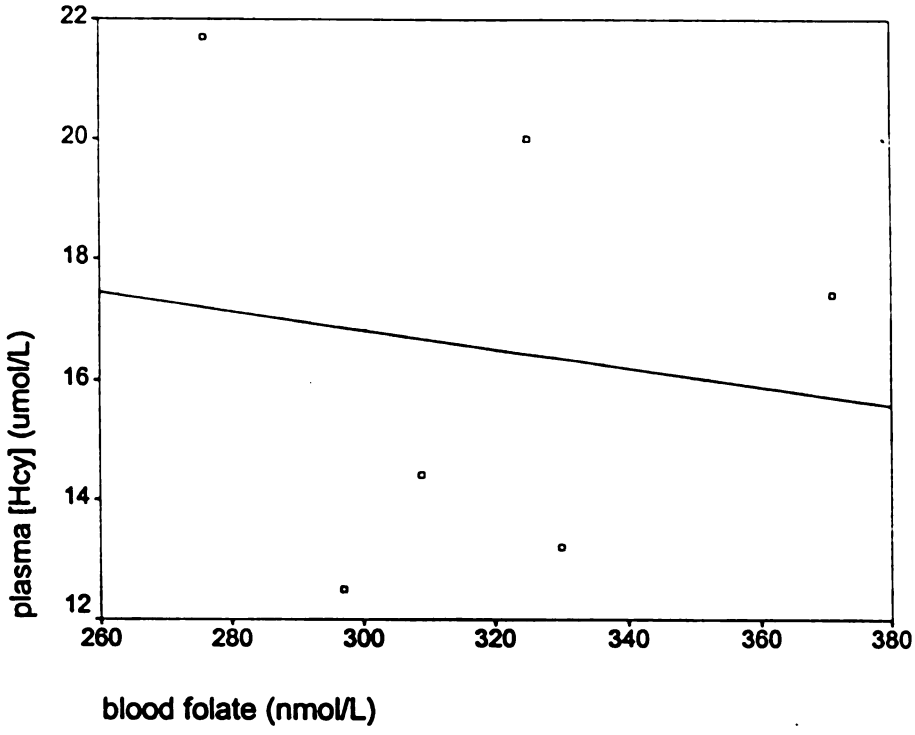

Figure 11. Scatterplot of plasma Hcy concentration and blood folate concentration

0.152), blood folate (β_{bfol} = -0.067) and the interaction between plasma vitamin B_6 and serum vitamin B_{12} (β_{B6*B12} = -0.001). All three predictors showed significantly (P<0.001) negative association with mean plasma Hcy concentration.

The predictability of plasma Hcy concentration by dietary intake of B-vitamins.

(Objective Six)

Pearson's correlations among intake of dietary vitamins B_6, B_{12} and folate of healthy control subjects (Table 12) were highly significant (P<0.01). Dietary folate correlated

with dietary vitamin B_6 (r=0.28) and dietary vitamin B_{12} (r=0.19). Dietary vitamin B_{12} was also highly correlated with vitamin B_6 (r=0.72, P<0.05).

A weighted multiple regression model for dietary intakes of B-vitamins and their interactions (Table 15)

Table 15. Multiple regression output of dietary B-vitamins predicting plasma Hcy

Model		Coefficients B	Std. Error	t	Sig
1	(Constant)	59.434	1.202	49.426	
	B_{12}	**-9.515**	0.353	-26.976	0.000
	folate	**-0.307**	0.005	-58.870	0.000
	B_6*B_{12}	-0.461	0.035	-13.370	0.000
	B_{12}*folate	0.064	0.002	41.466	0.000
	B_6*folate	0.009	0.001	11.632	0.000

resulted in significant negative regressions (β_{B12} = -9.515; β_{folate} = -0.307). Dietary vitamin B_6 was excluded from the model by backward elimination.

Chapter Five

DISCUSSION AND CONCLUSION

Discussion

Hyperhomocysteinemia has frequently been defined in literature by the 90-95th percentile of plasma Hcy concentration of healthy controls (Genest et al, 1990; Heijer et al, 1993; Selhub et al, 1993; Robinson et al, 1996). Reported critical values of plasma Hcy concentration of healthy adults range from 14.0 μmol/l (Selhub et al, 1993) to 19.0 μmol/l (Genest et al, 1990). This wide range of critical value for plasma Hcy concentration makes no distinction between genders, and does not reflect a reference range that can be generalized for public health. These critical values of individual studies, each of which was based on a small percent of the general population, have not been consistent across studies.

Genest et al (1990) reported 90th and 95th percentile values of 15.0 and 19.0 μmol/l for healthy controls and Heijer et al (1993) reported 95th percentile values of 18.5 μmol/l among their controls. Moreover, Selhub et al (1993)

defined hyperhomocysteinemia as 14 μmol/l, and Robinson et al (1996) reported 95th percentile values of 16.3 μmol/l in healthy controls. This approach led scientists to adopt a wide range of plasma Hcy concentration for healthy adults based on individual study findings. Previous research (Boushey et al, 1995), reported that an increase in plasma Hcy concentration by 1 umol/l is associated with 10% higher risk for CVD, and a 5 μmol/l increment in plasma Hcy may be associated with ≥50% increase in risk of vascular disease.

The last ten years a substantial amount of data on plasma Hcy have been accumulated (normal ranges by age for men and women). Utilizing the unique statistical power of incorporating findings of individual studies to make generalization, this meta-analysis identified healthy and diseased reference ranges of plasma Hcy concentration for men and women. However, hyperhomocysteinemia a new independent risk factor for CVD (Clarke et al, 1991; Stampfer et al, 1991), can be linearly associated with the risk for CVD only in certain ranges. The need for plasma Hcy reference values that can be adopted as a screening tool to help maintain plasma Hcy levels within the normal range for disease prevention becomes an important issue.

I confirmed, in this meta-analysis, a significantly higher mean plasma Hcy concentration of vascular disease cases than that of healthy controls (Malinow et al, 1989;

Stampfer et al, 1992; Pancharuniti et al, 1994; Robinson et al, 1995). The difference in mean plasma Hcy concentration of vascular disease cases and healthy controls in this meta-analysis was 3 μmol/l. Verhoef et al in 1996 reported that a 3 μmol/l increase in plasma Hcy was associated with 1.35 odds ratio for myocardial infarction. Among healthy subjects, we observed higher mean plasma Hcy concentration in men compared to women. The mean plasma Hcy difference of healthy men and women was about 2 μmol/l. No significant differences were observed in mean plasma Hcy concentration among vascular disease men and women. Similar insignificant findings have also been reported by Dalery and colleagues in 1995; (11.7 ± 5.8 μmol/l and 12.0 ± 6.3 μmol/l). These findings suggest healthy reference ranges for men of 9.3-14.0 μmol/l with a mean ± SD of 11.2 ± 1.6 μmol/l and 7.6-13.2 μmol/l with a mean ± SD of 9.5 ± 2.1 μmol/l for women. Mean plasma Hcy value of ≥14 μmol/l was associated with vascular disease cases. Robinson et al (1995) reported that a plasma Hcy concentration of 14 μmol/l conferred an odds ratio of coronary disease of 4.8.

Nygard et al (1998) reported different reference ranges for apparently healthy participants of the Hordland Homocysteine Study as 6.2-18.7 μmol/l and 5.1-16.5 μmol/l for men and women, respectively. The reported findings of

Nygard et al, have similar lower reference ranges for both men and women with findings of this meta-analysis. Differences in the upper reference value may be attributed to the fact that researchers excluded previously diagnosed subjects of vascular diseases but no vascular assessment for possible development of the disease at the time of the study was performed. The normal health ranges, of plasma Hcy concentration established in this meta-analysis based on data of 2319 healthy and 3628 vascular disease subjects may be useful in identifying population risk groups for vascular diseases.

Evaluation of mean plasma Hcy concentration of CBVD and CVD disease groups identify significant differences between the two groups. CBVD cases had higher mean plasma Hcy concentration than CVD cases. In my knowledge, this is the first time that separate evaluation for mean plasma Hcy concentration of CBVD and CVD disease groups is performed. There are no proposed mechanisms for the difference in mean plasma Hcy concentration among the disease groups. Further investigation is needed to establish reasoning.

Studies (Ubbink et al, 1993; Jacques et al, 1996; Dudman et al, 1996), report suboptimal blood concentration of vitamins B_6, B_{12} and folate as modifiable risk factors for hyperhomocysteinemia. In this meta-analysis, vascular disease cases compared to the controls had lower

concentration of plasma vitamin B_6 (47.4±29.0; 65.9±38.7 nmo/l), lower blood folate (298.9±29.0; 339.2±22.1 nmol/l) but had comparable serum vitamin B_{12} (308.9±77.9; 310.5±73.2 pmol/l). Blood folate (β=-0.68), serum vitamin B_{12} (β=-0.172), and plasma B_6 (β=-0.192) exhibited significant ($P<0.001$) inverse associations with mean plasma Hcy concentration for both cases and controls, when controlling for interactions of the blood B-vitamins. Blood folate exhibited strong positive correlations with plasma vitamin B_6 ($r=0.66$) and serum vitamin B_{12} ($r=0.42$). Plasma B_6 was also positively correlated with serum vitamin B_{12} ($r=0.58$). When plotting blood folate levels against plasma Hcy concentration we observed that suggested normal plasma Hcy concentration of 11.0 μmol/l is associated with blood folate levels of >360 nmol/l. In relation to serum vitamin B_{12}, optimal plasma Hcy concentration can be achieved for serum concentrations of vitamin B_{12} greater than 350 pmol/l, while greater than 60 nmol/l of plasma B_6 are needed for the same purpose. Based on these findings, I am suggesting the above critical values of blood vitamins B_6, B_{12} and folate to maintain plasma Hcy concentration below the disease level (11.0 μmol/l).

Mean dietary intakes of folate and vitamin B_{12} showed significant inverse association (β_{folate}=-0.307; β_{B12}=-9.515)

with mean plasma Hcy concentration for the healthy subjects, when dietary vitamin interactions where controlled. Dietary vitamin B_6 showed no association with mean plasma Hcy concentration, and was excluded from the model by backward elimination. Univariate analysis of dietary vitamin B_6 also showed no association, although plasma vitamin B_6 concentration exhibited a significant inverse association (β= -0.012) with mean plasma Hcy concentration. The conflicting findings on plasma as opposed to dietary vitamin B_6 I attribute to the limited number of studies which included information on dietary intakes of vitamin B_6. Most studies reported data on dietary folate and dietary vitamin B_{12}. Dietary data were only reported for the healthy control group, and no dietary B-vitamin comparison can be made with vascular disease cases. Dietary folate exhibited strong positive correlations with dietary vitamins B_6 (r=0.28) and B_{12} (r=0.19). Dietary vitamin B_6 also had a significant positive correlation with dietary vitamin B_{12} (r=0.72).

Elderly people have higher mean plasma Hcy concentration than younger people, are frequently deficient in blood B-vitamins (Garry et al,1984; Joosten et al, 1993), and are in a higher risk for CVD. Suboptimal blood B-vitamin concentration may partially explain the increase risk for CVD in this group. Blood B-vitamin deficiency in the elderly may be attributed to socioeconomic status, intestinal

vitamin malabsorption or decreased appetite. These findings address the significance of nutritional adequacy of the B-vitamins for vascular disease prevention.

Conclusion

In conclusion, this meta-analysis confirmed that vascular disease cases have higher mean plasma Hcy than healthy controls. Men have higher mean plasma Hcy concentration than women for the healthy group, but similar mean plasma Hcy concentration for the vascular disease group. CBVD cases had higher mean plasma Hcy than CVD cases. Furthermore, this meta-analysis addressed the view that plasma Hcy concentration is inversely associated with blood or dietary B-vitamins. Adequate intake of B-vitamins for maintenance of optimal levels in the blood may be an important step for maintaining plasma Hcy concentration to normal levels, to reduce the risk for vascular diseases.

Chapter Six

ASSUMPTIONS, LIMITATIONS, IMPLICATIONS, AND RECOMMENDATIONS

Assumptions

In conducting the present study, the following assumptions were made:

1. The errors of estimation e_i are statistically independent, each with a mean of zero and estimation variance v_i.
2. Each random effect U_i is independent with a mean of zero.
3. In the random effects model part of the variability of the true effect sizes is unexplained by the model.

Limitations

This study has some limitations that should be addressed:

1. The majority of the studies included in the meta-analysis represent case- control and cross-sectional study designs. No prospective studies were included in this meta-analysis.

2. The case-control studies included in this meta-analysis had relatively higher percentage of male subjects for both vascular disease and healthy groups when compared to female subjects.
3. The results of this meta-analysis are based on the group and not on the individual level. Certain limitations for generalization may apply.
4. Dietary data were limited to a very small number of studies.

Implications

Hyperhomocysteinemia has recently been identified as an independent risk factor for CVD. Reference values for healthy and vascular diseased people have not yet been established. This meta-analysis proposes reference values for healthy or diseased adult Caucasian men and women. These values are lower than the currently proposed in the literature. These reference values can be used as a screening tool for prevention, to help reduce the risk for CVD.

We have shown that dietary and consequently blood B-vitamins are significant factors for predicting mean plasma Hcy concentration. Adequate dietary intakes of the B-vitamins may be needed to normalize levels of plasma Hcy and thereby lower vascular diseases.

Recommendations

In this meta-analysis CBVD patients had a higher mean plasma Hcy concentration than CVD patients. No distinction was made by gender of the diseased due to limited data. More studies are accumulated and gender within diseased group should be further investigated.

As individual research on plasma Hcy concentration and dietary intakes of vitamins B_6, B_{12} and folate accumulates, a new meta-analysis is needed to establish dietary intakes of the B-vitamins, to help maintain normal plasma Hcy concentration. RDA are established for disease prevention. The new scientific findings on plasma Hcy concentration and the effect of the B-vitamins address the need to reconsider the RDA.

Hyperhomocysteinemia is an independent risk factor for CVD. Monitoring of plasma Hcy concentration should be part of the standard blood analysis. This will help identify hyperhomocysteinemic people, help implement nutritional intervention with B-vitamin supplementation, and help reduce the risk of CVD.

In our study, all three blood B-vitamins were significant determinants of mean plasma Hcy concentration. Blood folate concentration was the most significant factor. This relationship should be further examined by controlled supplementation studies of individuals and with combinations

of the B-vitamins. New blood B-vitamin reference values need to be established to help maintain normal plasma Hcy concentration in an effort to reduce the risk for CVD.

APPENDICES

APPENDIX A

CODING SHEET

APPENDIX A. CODING SHEET

ID No	30		
Author	Hopkins et al.	Dietary assesment by	
Year	1995	Homocysteine measured by	High Pressure Liquid Chromatography (HPLC)
Source	Art Thr Vasc B	Plasma vitamins measured by	Radioenzymatic Method (B6), CIBA (B12, folate)
Country	U.S	Statistical methods used	SAS, t-test, multiple logistic regression, ANCOVA

		number	age: x (SD), range	comments (ethnicity, patients)
control	males	120		
	females	42		
	total	162	, 38-68	Coronary Artery Disease (CAD) patients from families in which at least one other sibling had early CAD.
case	males	85		
	females	70		
	total	155	, 38-68	Selected from a random population sampling who were spouses of hypertensive siblings.
Total # subjects:		317		

APPENDIX A. (CONT'D)

		Hcy (umol/L) x±SD/SE	Plasma B6 (nmol/L) x±SD/SE	Plasma B12 (pmol/L) x±SD/SE	Plasma folate (nmol/L) x±SD/SE	Diet B6 (mg/day) x±SD/SE	Suppl B6 (mg/day) x±SD/SE	Diet+Suppl B6 (mg/day) x±SD/SE
cases	males	13.7±4.5	104±113	361±134	23.7±10.7			
	females	12.6±5.6	82±87	345±134	27.6±13.6			
	x							
controls	males	11.3±2.9	131±135	353±149	22.6±11.5			
	females	8.9±2.7	139±164	356±157	27.7±14.7			
	x							
p-value								
			Diet B12 (ug/day) x±SD/SE	Suppl B12 (mg/day) x±SD/SE	Diet+Sup B12 (ug/day) x±SD/SE	Diet folate (ug/day) x±SD/SE	Suppl folate (mg/day) x±SD/SE	Diet+Suppl fol (ug/day) x±SD/SE
cases	males							
	females							
	x							
controls	males							
	females							
	x							
p-value								

APPENDIX A. (CONT'D)

	Plasm B6	Plasm B12	Plasm fol	Diet B6	Sup B6	Diet+ Sup B6	Diet B12	Sup B12	Diet+Sup B12	Diet fol	Sup fol	Diet+Sup fol
Hcy	c= -0.21 o= -0.20	c= -0.27 o= -0.33	c= -0.46 o= -0.39									
Plasma B6		c= 0.30 o= 0.17	c= 0.26 o= 0.23									
Plasma B12			c= 0.33 o= 0.30									
Plasma fol												
Diet B6												
Suppl B6												
Diet+ Sup B6												
Diet B12												
Suppl B12												
Diet+Sup B12												
Dietary folate												
Suppl folate												

c = cases
o = controls

APPENDIX 1. (CONT'D)

Significant findings:
High plasma Hcy concentration is an independent contributor to risk for CAD.
Subjects with CAD had significantly lower levels of folate in their plasma.
There was a progressive increase in risk for CAD among both men and women as plasma Hcy rose above 9 umol/L.
A 10-umol/l increase in Hcy was associated with an 8.1-fold increase in risk after adjustment for other
risk factors (Age, Smoking, BMI, BP, Chol)
Notes:
The Author states that Hyperhomocysteinemia appears to agregate atherosclerosis by three mechanisms.
1) Endothelial cell toxicity. 2) Increased platelet adhesiveness. 3) modification by clotting factors.
Also Author uses the following plasma values for comparison:
plasma B12: normal levels: 148-664 pmol/L plasma folate: normal levels: 6.8 - 36 nmol/L
deficiency: <66-74 pmol/L

APPENDIX B

CODED VARIABLES

APPENDIX B. CODED VARIABLES

sequence	variable	code	explanation
1.	id#	1-48	Coding number of journal article
2.	first author	1	Alfthan
		2	Andersson
		3	Araki
		4	Arnesen
		5	Brattstrom
		6	Cacan
		7	Chadefaux
		8	Coull
		9	Chu
		10	Cravo
		11	Dalery
		12	Den Heijer
		13	Fermo
		14	Gary
		15	Genest
		16	Herzlich
		17	Hopkins
		18	Israelsson
		19	Jacobsen
		20	Joosten
		21	Landgren
		22	Lewis
		23	Lindenbaum
		24	Malinow
		25	Moolgard
		26	Nilsson
		27	Nygard
		28	Pancharuniti
		29	Perry
		30	Riggs
		31	Robinson
		32	Selhub
		33	Stampfer
		34	Swift
		35	Ubbink
		36	Verhoef
		37	Wu
3.	study design	1	cases-control
		2	cross-sectional

APPENDIX B. (cont'd)

sequence	variable	code	explanation
4.	disease [cases]	1	cerebrovascular diseases
			acute stroke
			stroke
			cerebral bleeding
			cerebral infraction
			cerebral thrombosis
		2	cardiovascular diseases
			coronary artery disease
			coronary heart disease
			ischemic stroke
			myocardial infraction
			peripheral arterial occlusive disease
			risk factors for CVD
			transient ischemic attacks
			venous thrombosis
		3	all other diseases
			asthma
			alcoholics
			dementia
			end stage renal disease
			geriatric problems
			Intermittent
5.	hcymeth [analytical methods for Hcy]	1	HPLC-FD
		2	HPLC-ED
		3	HPLC-REA
		4	HPLC-GCMS
		5	AAA
		6	HPLC
		7	IEC
6.	gender	1	male
		2	female
		3	both
7.	agecas	1	age for cases
8.	sdagca	1	standard deviation of age for cases

APPENDIX B. (cont'd)

sequence	variable	code	explanation
9.	ageco	1	age for controls
10.	sdagco	1	standard deviation of age for controls
11.	cases	1	number of cases
12.	controls	1	number of controls
13.	typme	1 2 3	Arithmetic Mean Geometric Mean Median
14.	mehcy	1 2 3 4	plasma serum blood red cell
15.	mhca	1	mean plasma homocysteine for cases
16.	sdhcas	1	standard deviation
17.	mhcon	1	mean plasma homocysteine for controls
18.	sdhco	1	standard deviation
19.	meb6	1 2 3 4	plasma serum blood red cell
20.	mb6ca	1	mean vitamin B6 for cases
21.	sdb6cas	1	standard deviation
22.	mb6co	1	mean vitamin B6 for controls
23.	sdb6co	1	standard deviation
24.	mb12ca	1	mean vitamin B12 for cases
25.	sdb12ca	1	standard deviation

APPENDIX B. (cont'd)

sequence	variable	code	explanation
26.	mb12co	1	mean vitamin B12 for controls
27.	sdb12co	1	standard deviation
28.	mefol	1 2 3 4	plasma serum blood red cell
29.	mfolca	1	mean folate for cases
30.	sdmfolca	1	standard deviation
31.	mfolco	1	mean folate for controls
32.	sdfolco	1	standard deviation
33.	Kcalca	1	daily Calorie intake for cases
34.	sdKca	1	standard deviation
35.	Kcalco	1	daily calorie intake for controls
36.	sdKco	1	standard deviation
37.	dieb6ca	1	dietary b6 for cases
38.	sddb6ca	1	standard deviation
39.	dieb6co	1	dietary b6 for controls
40.	sddb6co	1	standard deviation
41.	dib12ca	1	dietary b12 for cases
42.	sddb12ca	1	standard deviation
43.	dib12co	1	dietary b12 for controls
44.	sddb12co	1	standard deviation

APPENDIX B. (cont'd)

sequence	variable	code	explanation
45.	difolca	1	dietary folate for cases
46.	sddfolca	1	standard deviation
47.	difolco	1	dietary folate for controls
48.	sddfoco	1	standard deviation

APPENDIX C

REPORTED CORRELATIONS OF HOMOCYSTEINE AND VITAMINS B_6, B_{12} AND FOLATE

APPENDIX C. Reported correlations of homocysteine and vitamins B6, B12 and folate

ID#	UTHOR	GENDER	DISCAT	NoCASES	NoCONTR	GROUP	ORLTYPE	MEAS1	HCY-B6	MEAS2	HCY-B12	MEAS3	HCY-FOL
2.00	2.00	1.00			74.00	2.00	1.00	1.00	0.56	2.00	0.22	3.00	0.34
2.00	2.00	2.00			83.00	2.00	1.00	1.00	0.15	2.00	0.31	3.00	0.25
7.00	5.00	3.00	1.00	70.00		1.00	1.00	1.00	0.36	2.00	0.14	3.00	0.36
7.00	5.00	3.00			66.00	2.00	1.00	1.00	0.30	2.00	0.19	3.00	0.23
8.00	5.00	1.00			69.00	2.00	1.00			2.00	0.35	3.00	0.42
8.00	5.00	2.00			52.00	2.00	1.00			2.00	0.30	3.00	0.34
14.00	11.00	1.00	2.00	123.00		1.00	1.00	1.00	0.02	1.00	0.06	1.00	0.20
14.00	11.00	2.00	2.00	27.00		1.00	1.00	1.00	0.13	1.00	0.13	1.00	0.30
14.00	11.00	1.00			380.00	2.00	1.00	1.00	0.08	1.00	0.19	1.00	0.17
14.00	11.00	2.00			204.00	2.00	1.00	1.00	0.11	1.00	0.17	1.00	0.18
20.00	16.00	3.00	2.00	367.00		1.00	1.00			2.00	0.35	2.00	0.33
21.00	17.00	3.00	2.00	162.00		1.00	2.00	1.00	0.21	1.00	0.27	1.00	0.46
21.00	17.00	3.00			155.00	2.00	2.00	1.00	0.20	1.00	0.33	1.00	0.39
24.00	19.00	1.00			36.00	2.00	2.00			1.00	0.50	1.00	0.36
24.00	19.00	2.00			35.00	2.00	2.00			1.00	0.40	1.00	0.36
25.00	20.00	3.00			99.00	2.00	1.00	1.00	0.19	1.00	0.27	1.00	0.38
26.00	21.00	3.00	2.00	68.00		1.00	1.00			1.00	0.30	1.00	0.37
28.00	23.00	3.00			117.00	2.00	1.00			2.00	0.50	2.00	0.42
30.00	25.00	1.00	3.00	78.00			1.00	1.00	0.19	1.00	0.27	1.00	0.38
31.00	26.00	3.00	3.00	164.00		1.00	2.00			2.00	0.20	3.00	0.24
31.00	26.00	3.00			45.00	2.00	2.00			2.00	0.36	3.00	0.33
32.00	26.00	3.00	3.00	295.00		1.00	1.00			2.00	0.23	3.00	0.41
32.00	26.00	3.00			163.00	2.00	1.00			2.00	0.31	3.00	0.43
34.00	28.00	1.00	2.00	101.00		1.00	2.00			1.00	0.23	1.00	0.40
34.00	28.00	1.00			108.00	2.00	2.00			1.00	0.42	1.00	0.48
36.00	30.00	3.00			70.00	2.00	1.00			1.00	0.36	1.00	0.58
37.00	31.00	3.00	2.00	304.00		1.00	1.00	1.00	0.19	1.00	0.39	1.00	0.29
37.00	31.00	3.00			231.00	2.00	1.00	1.00	0.28	1.00	0.38	1.00	0.41
38.00	31.00	3.00	3.00	176.00		1.00	2.00	1.00	0.41	1.00	0.25	1.00	0.48
38.00	31.00	3.00			231.00	2.00	2.00	1.00	0.29	1.00	0.37	1.00	0.43
41.00	33.00	1.00			271.00	2.00	1.00	1.00	0.28				
43.00	35.00	1.00	1.00	44.00		1.00	1.00	1.00	0.29				
46.00	36.00	3.00	2.00	130.00		1.00	2.00	1.00	0.22	1.00	0.35	1.00	0.38
46.00	36.00	3.00			118.00	2.00	2.00	1.00	0.23	1.00	0.19	1.00	0.49
48.00	37.00	3.00	2.00	170.00		1.00	2.00	1.00	0.18	1.00	0.34	1.00	0.25

APPENDIX C. (cont'd)

ID#	AUTHOR	GENDER	DISCAT	NoCASES	NoCONTR	GROUP	ORLTYPE	MEAS4	B6-B12	MEAS5	B6-FOL	MEAS6	B12-FOL
2.00	2.00	1.00			74.00	2.00	1.00						
2.00	2.00	2.00			83.00	2.00	1.00						
7.00	5.00	3.00	1.00	70.00		1.00	1.00						
7.00	5.00	3.00			66.00	2.00	1.00						
8.00	5.00	1.00			69.00	2.00	1.00						
8.00	5.00	2.00			52.00	2.00	1.00						
14.00	11.00	1.00	2.00	123.00		1.00	1.00						
14.00	11.00	2.00	2.00	27.00		1.00	1.00						
14.00	11.00	1.00			380.00	2.00	1.00						
14.00	11.00	2.00			204.00	2.00	1.00						
20.00	16.00	3.00	2.00	367.00		1.00	1.00						
21.00	17.00	3.00	2.00	162.00		1.00	2.00	1.00	0.30	1	0.26	1	0.33
21.00	17.00	3.00			155.00	2.00	2.00	1.00	0.17	1	0.23	1	0.30
24.00	19.00	1.00			36.00	2.00	2.00						
24.00	19.00	2.00			35.00	2.00	2.00						
25.00	20.00	3.00			99.00	2.00	1.00						
26.00	21.00	3.00	2.00	68.00		1.00	1.00						
28.00	23.00	3.00			117.00	2.00	1.00						
30.00	25.00	1.00	3.00	78.00			1.00						
31.00	26.00	3.00	3.00	164.00		1.00	2.00						
31.00	26.00	3.00			45.00	2.00	2.00						
32.00	26.00	3.00	3.00	295.00		1.00	1.00						
32.00	26.00	3.00			163.00	2.00	1.00						
34.00	28.00	1.00	2.00	101.00		1.00	2.00					1	0.13
34.00	28.00	1.00			108.00	2.00	2.00					1	0.39
36.00	30.00	3.00			70.00	2.00	1.00			1	0.21	1	0.42
37.00	31.00	3.00	2.00	304.00		1.00	1.00						
37.00	31.00	3.00			231.00	2.00	1.00						
38.00	31.00	3.00	3.00	176.00		1.00	2.00						
38.00	31.00	3.00			231.00	2.00	2.00						
41.00	33.00	1.00			271.00	2.00	1.00						
43.00	35.00	1.00	1.00	44.00		1.00	1.00						
46.00	36.00	3.00	2.00	130.00		1.00	2.00						
46.00	36.00	3.00			118.00	2.00	2.00						
48.00	37.00	3.00	2.00	170.00		1.00	2.00	1.00	0.39	1	0.33	1	0.26

APPENDIX D

REPORTED VALUES FOR CODED VARIABLES OF EIGHT STUDIES

APPENDIX D. Reported values for coded variables of eight studies

ID#	AUTHOR	DESI	DISE	HCYME	GEN	AGECAS	SDAGCA	AGECO	SDAGCO	GENDER	NoCASES	NoCONTR
1.00	1.00	1.00	2.00	1.00	1.00					1.00	92.00	141.00
1.00	1.00	1.00	2.00	1.00	2.00					2.00	99.00	128.00
1.00	1.00	1.00	1.00	1.00	1.00					1.00	42.00	
1.00	1.00	1.00	1.00	1.00	2.00					2.00	32.00	
2.00	2.00	2.00		7.00	1.00					1.00		74.00
2.00	2.00	2.00		7.00	2.00					2.00		83.00
2.00	2.00	2.00		7.00	3.00					3.00		
3.00	3.00	1.00	1.00	1.00	1.00					1.00	30.00	30.00
3.00	3.00	1.00	1.00	1.00	2.00					2.00	15.00	15.00
3.00	3.00	1.00	1.00	1.00	3.00	63.30	10.90	62.90	10.80	3.00		
3.00	3.00	1.00	1.00	1.00	1.00					1.00	13.00	
3.00	3.00	1.00	1.00	1.00	2.00					2.00	7.00	
3.00	3.00	1.00	1.00	1.00	3.00	65.40	9.70			3.00		
4.00	4.00	1.00	2.00	1.00	1.00					1.00	110.00	430.00
4.00	4.00	1.00	2.00	1.00	2.00					2.00	12.00	48.00
4.00	4.00	1.00	2.00	1.00	3.00	51.30	7.30	51.20	7.30	3.00		
5.00	5.00	2.00		5.00	3.00			51.00		3.00		14.00
5.00	5.00	2.00		5.00	3.00			51.00		3.00		13.00
5.00	5.00	2.00		5.00	3.00			51.00		3.00		15.00
6.00	5.00	1.00	3.00	5.00	1.00					1.00	21.00	22.00
6.00	5.00	1.00	3.00	5.00	2.00					2.00	16.00	24.00
6.00	5.00	1.00	3.00	5.00	3.00	52.20	6.00	52.20	7.00	3.00		
6.00	5.00	1.00	2.00	5.00	1.00					1.00	12.00	
6.00	5.00	1.00	2.00	5.00	2.00					2.00	6.00	
6.00	5.00	1.00	2.00	5.00	3.00	51.90	6.00			3.00		
6.00	5.00	1.00	1.00	5.00	1.00					1.00	8.00	
6.00	5.00	1.00	1.00	5.00	2.00					2.00	9.00	
6.00	5.00	1.00	1.00	5.00	3.00	41.20	8.00			3.00		
7.00	5.00	1.00	1.00	2.00	1.00	62.40	6.70	61.60	4.90	1.00	54.00	34.00
7.00	5.00	1.00	1.00	2.00	2.00	63.20	10.20	61.40	5.00	2.00	16.00	32.00
7.00	5.00	2.00	1.00	2.00	1.00	77.60	3.20			1.00	49.00	
7.00	5.00	2.00	1.00	2.00	2.00	80.60	5.50			2.00	23.00	
8.00	5.00	2.00		6.00	1.00					1.00		69.00
8.00	5.00	2.00		6.00	2.00					2.00		52.00
8.00	5.00	2.00		6.00	1.00					1.00		62.00
8.00	5.00	2.00		6.00	2.00					2.00		61.00

APPENDIX D. (co

ID#	AUTHOR	TYPME	MEHCY	MHCA	SDHCA	MHCON	SDHCO	MEB6	MB6CA	SDB6CA	MB6CO	SDB6CO
1.00	1.00	1.00	2.00	9.83	3.00	9.82	3.17					
1.00	1.00	1.00	2.00	9.39	2.42	9.28	2.59					
1.00	1.00	1.00	2.00	10.40	2.72							
1.00	1.00	1.00	2.00	10.10	3.23							
2.00	2.00	1.00	1.00			10.70	2.60					
2.00	2.00	1.00	1.00			9.60	3.00					
2.00		1.00	1.00					1.00			27.90	15.00
3.00	3.00	1.00	1.00									
3.00	3.00	1.00	1.00									
3.00	3.00	1.00	1.00	13.10	5.60	7.30	2.90					
3.00	3.00	1.00	1.00									
3.00	3.00	1.00	1.00									
3.00	3.00	1.00	1.00	9.60	3.90							
4.00	4.00	1.00	2.00									
4.00	4.00	1.00	2.00									
4.00	4.00	1.00	2.00	12.70	4.70	11.30	3.70					
5.00	5.00	1.00	1.00			10.70	.70					
5.00	5.00	1.00	1.00			19.90	4.40					
5.00	5.00	1.00	1.00			12.00	1.30					
6.00	5.00	1.00	1.00									
6.00	5.00	1.00	1.00									
6.00	5.00	1.00	1.00	18.70	14.90	11.00	3.40	1.00	17.90	8.60	39.70	24.90
6.00	5.00	1.00	1.00									
6.00	5.00	1.00	1.00									
6.00	5.00	1.00	1.00	13.20	4.20			1.00	20.50	8.10		
6.00	5.00	1.00	1.00									
6.00	5.00	1.00	1.00									
6.00	5.00	1.00	1.00	12.50	7.80			1.00	13.70	7.00		
7.00	5.00	1.00	1.00	17.40	7.40	12.70	2.50	1.00	28.50	15.20	24.40	12.20
7.00	5.00	1.00	1.00	14.40	4.50	11.10	3.60	1.00	31.20	22.90	26.20	14.80
7.00	5.00	1.00	1.00	20.00	7.80			1.00	29.50	14.70		
7.00	5.00	1.00	1.00	21.70	8.90			1.00	21.90	11.40		
8.00	5.00	1.00	1.00			12.10	2.40					
8.00	5.00	1.00	1.00			10.70	3.30					
8.00	5.00	1.00	1.00			15.90	4.70					
8.00	5.00	1.00	1.00			13.60	4.30					

APPENDIX D. (cont'd)

ID#	AUTHOR	MEB12	MB12CA	SDB12CA	MB12CO	SDB12CO	MEFOL	MFOLCA	SDFOLCA	MFOLCO	SDFOLCO
1.00	1.00										
1.00	1.00										
1.00	1.00										
1.00	1.00										
2.00	2.00										
2.00	2.00										
2.00	2.00	2.00			270.00	111.00	3.00	#NULL!	#NULL!	307.00	94.00
3.00	3.00										
3.00	3.00										
3.00	3.00										
3.00	3.00										
3.00	3.00										
3.00	3.00										
4.00	4.00										
4.00	4.00										
4.00	4.00										
5.00	5.00										
5.00	5.00										
5.00	5.00										
6.00	5.00										
6.00	5.00										
6.00	5.00	2.00	302.00	142.00	243.00	106.00	4.00	296.00	106.00	306.00	81.00
6.00	5.00										
6.00	5.00										
6.00	5.00	2.00	271.00	123.00				330.00	114.00		
6.00	5.00										
6.00	5.00										
6.00	5.00	2.00	289.00	103.00				297.00	147.00		
7.00	5.00	2.00	267.00	78.00	234.00	73.00	3.00	371.00	156.00	287.00	58.00
7.00	5.00	2.00	306.00	77.00	265.00	105.00	3.00	309.00	62.00	308.00	99.00
7.00	5.00	2.00	269.00	190.00			3.00	325.00	117.00		
7.00	5.00	2.00	287.00	125.00			3.00	276.00	105.00		
8.00	5.00	2.00			274.00	199.00	3.00			387.00	158.00
8.00	5.00	2.00			274.00	91.00	3.00			346.00	169.00
8.00	5.00	2.00			239.00	120.00	3.00			334.00	166.00
8.00	5.00	2.00			264.00	118.00	3.00			358.00	161.00

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