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JYRKI K. VIRTANEN

Homocysteine, Folate and Cardiovascular Diseases

Doctoral dissertation

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Author´s address:
Research Institute of Public Health
University of Kuopio
P.O. Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 163547
E-mail: jyrki.virtanen@uku.fi

Supervisors:
Professor Jukka T. Salonen, M.D., Ph.D., M.Sc.P.H.
Research Institute of Public Health
University of Kuopio

Sari Voutilainen, Ph.D., R.D.
Research Institute of Public Health
University of Kuopio

Jari Kaikkonen, Ph.D.
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Opponent:
Professor Pekka Jousilahti, Ph.D.
Department of Epidemiology and Health Promotion
National Public Health Institute, Helsinki

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ABSTRACT

Homocysteine (Hcy) has been extensively studied after the first observations of vascular changes in patients with very high total Hcy (tHcy) levels in the 1960's. To date several meta-analyses have suggested that also less markedly elevated tHcy levels may be associated with increased risk of cardiovascular diseases (CVD). The purpose of this work was to study serum/plasma tHcy and two compounds closely linked to its metabolism, folate and methionine, and their role in the development of CVD in middle-aged men from Eastern Finland from the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) study.

The first study concerned with the effects of high dietary methionine intake on the risk of acute coronary events in 1981 men free of prior heart disease on the KIHD study baseline. The adjusted risk of an acute coronary event in the highest methionine intake quarter was about two times higher compared with the lowest quarter. High dietary methionine intake was not associated with increases in the plasma tHcy.

We then studied the effects of serum tHcy and folate on the risk of acute coronary events in 810 healthy men. We found that high serum tHcy concentration was not associated with the risk. However, men in the highest quarter of serum folate concentration had significantly decreased adjusted risk compared with the men in the lowest quarter.

In the next study we studied the effect of serum tHcy and folate on the risk of stroke in 1015 men free of prior stroke. The adjusted risk of any stroke and ischemic stroke in the highest serum tHcy third compared with the lowest third was significantly increased. On the other hand, high serum folate concentration was associated with significantly decreased risk.

The purpose of the fourth study was to explore the joint effect of increased serum tHcy concentration and other risk factors on the risk of CVD mortality in 802 men without a history of heart disease or stroke. The results of this study suggested that high serum tHcy concentration may increase the risk of CVD mortality and especially in men with other prevalent CVD risk factors.

Together these results suggest that high serum tHcy concentration may slightly increase the risk of CVD mortality and stroke in healthy middle-aged men, mortality especially when also other risk factors are present. On the other hand, high serum folate concentration would seem to protect against acute coronary events and stroke. And lastly, high dietary intake of methionine may increase the risk of acute coronary events by other means than by increasing blood tHcy concentrations.

National Library of Medical Classification: QU 60, QU 87, QU 145, QU 188, WA 105, WA 950, WG 120, WL 355

Medical Subject Headings: amino acids; cardiovascular diseases/epidemiology; cardiovascular diseases/metabolism; cerebrovascular accident/epidemiology; cerebrovascular accident/ metabolism; epidemiologic methods; epidemiologic studies; Finland; folic acid; homocysteine; male, methionine; middle aged; myocardial infarction; nutrition; risk factors

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YHTEENVETO

Homokysteiiniä on tutkittu laajasti 1960-luvulta lähtien. Silloin havaittiin, että potilailla, joilla oli erittäin suuri veren homokysteiinipitoisuus, oli myös muutoksia verisuonissa. Myöhemmissä meta-analyyseissä on ehdotettu, että myös lievemmin suurentuneet homokysteiinipitoisuudet voivat olla yhteydessä suurentuneeseen sydän- ja verisuonitautien (SVT) riskiin. Tämän väitöskirjatyön tarkoituksena oli tutkia seerumin/plasman homokysteiinipitoisuuden sekä kahden sen aineenvaihduntaan keskeisesti liittyvän yhdisteen, folaatin ja metioniinin, merkitystä SVT-riskiin keski-ikäisillä itäsuomalaisilla miehillä Sepelvaltimotaudin vaaratekijätutkimus-aineistossa (SVVT).

Ensimmäinen tutkimus käsitteli ravinnon suuren metioniinipitoisuuden yhteyttä sydäninfarktiriskiin 1981 miehellä, joilla ei ollut sydäntautia SVVT-tutkimuksen alussa. Vakioitu sydäninfarktiriski ylimmässä metioniininsaantineljänneksessä oli noin kaksi kertaa suurempi alimpaan neljännekseen verrattuna. Ravinnon runsas metioniinipitoisuus ei ollut yhteydessä suurentuneeseen plasman homokysteiinipitoisuuteen.

Seuraavassa tutkimuksessa tutkittiin seerumin homokysteiini- ja folaattipitoisuuksien yhteyksiä sydäninfarktiriskiin 810 terveellä miehellä. Tutkimuksessa havaittiin, ettei suurentunut seerumin homokysteiinipitoisuus ollut yhteydessä sydäninfarktiriskiin. Sen sijaan ylimpään folaatinsaantineljännekseen kuuluvilla miehillä oli merkitsevästi pienentynyt riski alimpaan neljännekseen kuuluviin miehiin verrattuna.

Kolmannessa tutkimuksessa tutkittiin suurentuneiden seerumin homokysteiini- ja folaattipitoisuuksien vaikutuksia ensimmäisen aivohalvauksen riskiin 1015 miehellä. Vakioitu aivohalvauksen ja iskeemisen aivohalvauksen riski oli merkitsevästi suurentunut ylimmässä seerumin homokysteiinipitoisuusneljänneksessä alimpaan neljännekseen verrattuna. Toisaalta suuri seerumin folaattipitoisuus oli yhteydessä merkitsevästi pienentyneeseen riskiin.

Neljännen tutkimuksen tarkoituksena oli tutkia suurentuneen seerumin homokysteiinipitoisuuden ja muiden riskitekijöiden yhteisvaikutusta SVT-kuolleisuuden riskiin 802:lla tutkimuksen alussa sydäntaudin ja aivohalvauksen suhteen terveellä miehellä. Tutkimuksen tulokset viittasivat siihen, että suurentunut seerumin homokysteiinipitoisuus voi lisätä SVT-kuolleisuuden riskiä ja varsinkin miehillä, joilla oli muita SVT-riskitekijöitä.

Yhdessä nämä tutkimukset viittaavat siihen, että suurentunut seerumin homokysteiinipitoisuus saattaa hiukan lisätä SVT-kuolleisuuden sekä aivohalvauksen riskiä terveillä keski-ikäisillä miehillä, sekä kuolleisuutta etenkin yhdessä muiden riskitekijöiden kanssa. Toisaalta suuri seerumin folaattipitoisuus näyttäisi suojaavan sydäninfarktilta ja aivohalvaukselta. Lisäksi runsas metioniinin saanti ravinnosta saattaa lisätä sydäninfarktiriskiä jollain muulla mekanismilla kuin suurentamalla veren homokysteiinipitoisuuksia.

Yleinen Suomalainen Asiasanasto: aivohalvaus; aminohapot; epidemiologia; ravitsemus; riskitekijät; sydäninfarkti; sydän- ja verisuonitaudit; vitamiinit

The only true wisdom
is in knowing you know nothing

- Socrates

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Jyrki Virtanen

ABBREVIATIONS

ACE	Angiotensin converting enzyme
ADMA	Asymmetric dimethylarginine
ANOVA	Analysis of variance
Apo-B	Apolipoprotein B
ARIC	Atherosclerosis Risk in Communities study
ASAP	Antioxidant Supplementation in Atherosclerosis Prevention study
ATP	Adenosine triphosphate
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
CBS	Cystathionine β-synthase
CHAOS-2	Cambridge Heart Antioxidant Study 2
CHD	Coronary heart disease
CHOL	Serum total cholesterol
CI	Confidence interval
CL	Cystathionine γ-lyase
COMT	Catechol-o-methyltransferase
CV	Coefficient of variation
CVD	Cardiovascular disease
DFE	Dietary folate equivalent
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EC	Enzyme Commission
eNOS	Endothelial nitric oxide synthase
FA	Folic acid
FAD	Flavin adenine dinucleotide
Fib	Plasma fibrinogen
FINMONICA	Finnish part of the MONICA project
GFR	Glomerular filtration rate
Hcy	Homocysteine
HDL	Serum high-density lipoprotein
HOPE-2	Heart Outcomes Prevention Evaluation-2 study
HPLC	High performance liquid chromatography
ICD	International Classification of Diseases
IHD	Ischemic heart disease
KIHD	Kuopio Ischaemic Heart Disease Risk Factor study
LDL	Serum low-density lipoprotein
MI	Myocardial infarction
MONICA	Monitoring of Trends and Determinants of Cardiovascular Disease
mRNA	Messenger ribonucleic acid
MRS	Methionyl-tRNA synthase
MS	Methionine synthase
MTHF	5-methyltetrahydrofolate
MTHFR	5,10-methylene-tetrahydrofolate reductase

NO	Nitric oxide
NORVIT	Norwegian Study of Homocysteine Lowering with B-vitamins in Myocardial Infarction
NOS	Nitric oxide synthase
NTD	Neural tube defect
O_2^-	Superoxide anion
OR	Odds ratio
PACIFIC	Prevention with a Combined Inhibitor and Folate in Coronary Heart Disease study
PON	Paraoxonase
r	Pearson's correlation coefficient
RNA	Ribonucleic acid
RR	Rate ratio
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAMS	S-adenosylmethionine synthase
SEARCH	Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine study
tHcy	Total homocysteine
THF	Tetrahydrofolate
tRNA	Transfer ribonucleic acid
VISP	Vitamin Intervention for Stroke Prevention study
VITATOPS	Vitamins to Prevent Stroke Study
VITRO	Vitamins and Thrombosis trial
WACS	Women's Antioxidant Cardiovascular Study
WENBIT	Western Norway B Vitamin Trial

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications referred to in the text by their Roman numerals I-IV:

I Virtanen JK, Voutilainen S, Rissanen TH, Happonen P, Mursu J, Laukkanen JA, Poulsen H, Lakka TA, Salonen JT. High dietary methionine intake increases the risk of acute coronary events in middle-aged men. Nutrition, Metabolism & Cardiovascular Diseases 2005. In press.

II Voutilainen S, Virtanen JK, Rissanen TH, Alfthan G, Laukkanen J, Nyyssönen K, Mursu J, Valkonen V-P, Tuomainen T-P, Kaplan GA, Salonen JT. Serum folate and homocysteine and incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. American Journal of Clinical Nutrition 2004;80:317-23.

III Virtanen JK, Voutilainen S, Happonen P, Alfthan G, Kaikkonen J, Mursu J, Rissanen TH, Kaplan GA, Korhonen MJ, Sivenius J, Salonen JT. Serum homocysteine, folate and risk of stroke: Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. European Journal of Cardiovascular Prevention and Rehabilitation 2005. In press.

IV Virtanen JK, Voutilainen S, Alfthan G, Korhonen MJ, Rissanen TH, Mursu J, Kaplan GA, Salonen JT. Homocysteine as a risk factor for CVD mortality in men with other CVD risk factors: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. Journal of Internal Medicine 2005; 257:255-262.

CONTENTS

1. INTRODUCTION

Cardiovascular diseases (CVD) are a major public health problem in the Western countries. Despite the decline in CVD mortality rates during the recent decades, over 40% of the deaths are still caused by CVD in Finland (1). The major risk factors for CVD are known, such as smoking, high blood pressure, high serum cholesterol levels and diabetes. Still there are people who, despite not having these risk factors, suffer heart disease. The search for other risk factors continues and one that has raised much interest is homocysteine (Hcy).

Hcy, a normal metabolite of the essential amino acid methionine, has been extensively studied after the first observations of vascular changes in patients with homocystinuria [that is, total Hcy (tHcy) levels in blood as high as 20-fold compared to normative range] in 1960's (2, 3). This and the subsequent finding in the next decade that tHcy levels one magnitude lower than in homocystinuric patients were significantly higher in patients with coronary heart disease (CHD) than in controls (4), lead to the hypothesis that even moderately elevated tHcy levels may be of importance in the progression of CVD. Since then the number of studies on the effects of tHcy on the risk of CVD has increased exponentially and several meta-analyses has been conducted (5-11). Although case-control studies suggest that increased tHcy concentration is a strong and graded risk factor for CVD, it is still uncertain whether it is a risk factor itself or just a marker of some other causal risk factor (6).

Folate belongs to the vitamin B group. It is an important metabolite in the Hcy metabolism, since it acts as a methyl donor in the remethylation of Hcy back to methionine. This has raised interest in the role of folate in the prevention of CVD. Indeed, low blood or dietary folate is associated with increased blood tHcy concentration (12), and on the other hand, folate supplementation is an effective way to decrease blood tHcy (13). Several case-control and prospective epidemiological studies have demonstrated an association between low blood folate levels and risk of CVD (12), which would support the concept of tHcy as a risk factor. However, low blood folate concentration has been suggested to be a risk factor independent of tHcy concentrations, which would indicate that a folate deficiency rather than elevated tHcy concentration is the actual risk factor (12). The ongoing supplementation trials with folic acid (a synthetic form of folate) should clarify the question whether increase in blood folate concentration will reduce CVD risk (14).

The purpose of this work was to study serum/plasma tHcy and two compounds closely linked to its metabolism, folate and methionine, and their role in the development of CVD in middle-aged men from Eastern Finland.

2. REVIEW OF THE LITERATURE

2.1 Homocysteine

The story of Hcy (2-amino-4-mercaptobutyric acid) actually began already in 1932, when Hcy was isolated for the first time (15). Next year, in 1933, a mentally retarded 8-year old boy died of a stroke (16). It was concluded that he died of arteriosclerosis of the carotid arteries with thrombosis and cerebral infarct. The condition of his carotid arteries was described as "process one might expect to find in a very old man", but no definite explanation for the unusual appearance was given. Over 30 years later, in 1965, a niece of that boy with advanced arteriosclerosis was found to have homocystinuria, a disease that was discovered in 1962 by Mudd and co-workers in Ireland. They had observed that a deficiency in the enzyme cystathionine β-synthase (CBS) resulted in an accumulation of Hcy in blood and consequently in urine leading to homocystinuria, one possible result of which was death from arterial or venous thrombosis (2). When the parents of the patient told about the uncle whose case had been published in 1933, the old case was studied. Based on the signs and symptoms, it was concluded that the uncle most likely had died of homocystinuria. The correct etiological diagnosis had been hereby established because of the discovery of Mudd and co-workers in 1962. However, the biochemical and pathophysiological processes leading to arteriosclerosis in patients with homocystinuria were not yet known.

A few years later, in 1969, Kilmer McCully observed extensive atherosclerotic changes in a 2-month old boy with homocystinuria (3). However, this infant represented different type of homocystinuria compared with the earlier cases, since he excreted both Hcy and cystathionine. Also, plasma levels of methionine were low, unlike in the previous cases. These findings suggested that CBS could not be deficient, but the resynthesis of methionine from Hcy was impaired. Because of the evidence of Hcy accumulation in two different types of homocystinuria, McCully postulated that Hcy or one of its derivates was responsible for the arterial damage (3). The basis for the hypothesis that moderately elevated blood Hcy levels may be a risk factor for CVD in the general population was thus formed (17). Wilcken and Wilcken were the first to test this hypothesis. They showed in 1976 that the prevalence of hyperhomocysteinemia was higher in patients with CHD than in controls (4). Since then the number of studies about the effects of Hcy on CVD has increased exponentially.

2.1.1 Homocysteine metabolism

Hcy is a non-protein forming, sulfur containing amino acid that plays an important role in methionine and folate metabolism (Fig. 1) (18). Methionine is a dietary essential amino acid, of which considerable proportion is activated by adenosine triphosphate (ATP) to form S-adenosylmethionine (SAM) in a reaction catalyzed by SAM synthase [EC 2.5.1.6] (Fig. 2). SAM serves primarily as a universal methyl donor to a variety of acceptors, such as creatine, epinephrine, carnitine, phospholipids, proteins, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (19). It is a methyl donor for essentially all known biological methylation reaction, except for those involved in methylation of Hcy (19). In the transmethylation of SAM, S-adenosylhomocysteine

(SAH) is formed, which is subsequently hydrolyzed by SAH hydrolase [EC 3.3.1.1] to yield adenosine and Hcy.

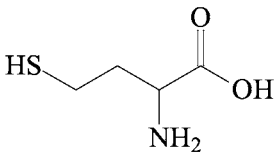

Figure 1. *The structure of homocysteine.*

Hcy can be either remethylated back to methionine by a methyl group from 5-methyltetrahydrofolate (MTHF) or betaine or irreversibly metabolized to cystathionine by transsulfuration with serine. Methionine synthase (MS) [EC 2.1.1.13], which is widely distributed in mammalian tissues, catalyzes the transfer of a methyl group from MTHF. Methylcobalamin (vitamin B_{12}) acts as an essential cofactor in this reaction. Serine donates nearly all of the one-carbon units for the *de novo* synthesis of the methyl group, and 5,10-methylene-tetrahydrofolate reductase (MTHFR) [EC 1.1.1.68] catalyzes the resynthesis of MTHF (Fig. 1).

The other remethylation pathway is catalyzed by betaine-Hcy methyltransferase [EC 2.1.1.5], which is present only in liver, kidney, and lens of humans (19). The enzyme uses betaine (trimethylglycine) as the methyl donor (Fig. 1). Betaine can be either of dietary origin or derived from choline. This remethylation pathway may be important when the folate-dependent pathway is impaired by ethanol ingestion, drugs, nutritional imbalances, or when betaine or choline levels are high (20-22).

The transsulfuration of Hcy is an irreversible reaction. Hcy first condenses with serine to form cystathionine in a reaction catalyzed by CBS [EC 4.2.1.22] (Fig. 2). The cystathionine is hydrolyzed to cysteine, α-ketobutyrate and ammonia by cystathionine γ-lyase [EC 4.4.1.1]. Both of these enzymes are dependent on pyridoxal 5'-phosphate (vitamin B_6). Excess cysteine that is not used in the synthesis of proteins or as a precursor of the antioxidant glutathione is then oxidized to taurine or inorganic sulfates or is excreted in the urine.

Unlike transmethylation and remethylation, not all tissues are capable of transsulfuration (19). In humans, the highest activity of CBS has been found in liver (23). The highest level of CBS messenger-RNA (mRNA) were found in liver in human adults and in liver and brain of human fetal tissues (24). Normal blood Hcy concentration may therefore represent its transit from the site of production to a site of catabolism (19). In summary, Hcy is located at an important regulatory point. It may be used for resynthesis of SAH, undergo remethylation to methionine or conversion to cystathionine or be exported from the cell (19).

In blood Hcy exists in reduced free (~1%) or oxidized disulfide forms (99%) (25). About 75% of this oxidized form is bound to proteins, mainly albumin. The remainder occurs in non-protein-bound forms: Hcy-Hcy disulfide (homocystine), Hcy-cysteine disulfide, and in minor amounts of other mixed disulfides (25). Plasma (or serum) tHcy refers to the combined pool of these forms. In patients with abnormally elevated tHcy (tHcy 150-400 μmol/L), the proportion of free form rises to about 10-25% (26). Normal

tHcy concentration varies between 5 and 15 µmol/L (27). The elevations in the tHcy can be roughly categorized as moderate (fasting tHcy concentration 15 to 30 µmol/L), intermediate (30 to 100 µmol/L) and severe hyperhomocysteinemia (>100 µmol/L) (28).

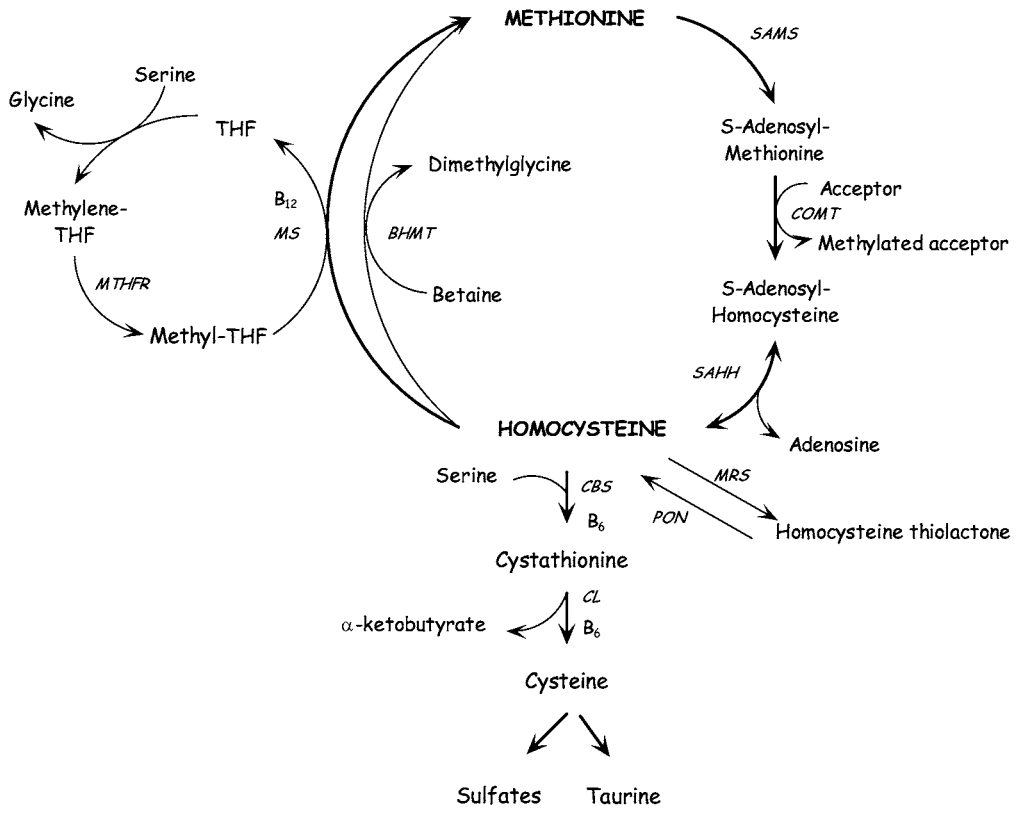

Figure 2. *Homocysteine metabolism. BHMT = betaine-homocysteine methyltransferase, CBS = cystathionine β-synthase, CL = cystathionine γ-lyase, COMT = Catechol-o-methyltransferase, PON = paraoxonase, MRS = methionyl-tRNA synthase, MS = methionine synthase, MTHFR = methylenetetrahydrofolate reductase, SAMS = S-adenosylmethionine synthase, SAHH = S-adenosylhomocysteine hydrolase, THF = tetrahydrofolate.*

2.1.2 Factors influencing blood homocysteine levels

Blood tHcy concentration is regulated by a multitude of factors (**Table 1**). They can be broadly classified into the following groups: genetic, physiological, lifestyle, pharmacological, and disease factors.

Table 1. Causes of hyperhomocysteinemia

	Effect[a]	Possible modes of action	Refs.
Genetic factors			
MTHFR C677T homozygosity	↑	Reduced remethylation	(29, 30)
Heterozygozity for CBS defects (uncommon)[b]	↑	Reduced transsulfuration	(31, 32)
Homocystinuria (very rare)[c]	↑↑↑	Inhibited transsulfuration	(33)
Down syndrome	↓	Over-expression of CBS	(34)
Physiological factors			
Increasing age	(↑)	Changes in renal function, menopause	(35, 36)
Male sex	(↑)	Androgens, larger muscle mass	(36, 37)
Pregnancy	↓	Hemodilution, reduced plasma albumin	(38, 39)
Postmenopausal state	(↑)	Hormonal changes	(36, 40)
Reduced GFR	(↑)	Compromised renal metabolism	(41, 42)
Increased muscle mass	(↑)	Creatine synthesis, androgens	(43)
Lifestyle factors			
High vitamin intake (folate, B_{12}, B_6, B_2)	↓	Normal Hcy remethylation or transsulfuration	(44-47)
Betaine intake	↓	Normal Hcy remethylation	(48)
Smoking	(↑)	Less healthy diet, cellular damage, changes in plasma thiol redux status, enzyme inhibition	(37, 49, 50)
Coffee	(↑)	Vitamin B_6 antagonist, chlorogenic acid in coffee	(50-52)
Alcohol consumption	↑↓	Changes in enzymes in the methionine cycle	(50, 53)
Physical inactivity	↑	Unhealthy lifestyle	(37, 54)
Clinical conditions			
Folate deficiency	↑↑	Reduced remethylation	(18, 55)
Vitamin B_{12} deficiency	↑↑	Reduced remethylation	(55, 56)
Vitamin B_6 deficiency[b]	↑	Reduced transsulfuration and remethylation	(57)
Renal failure	↑↑	Compromised renal metabolism	(41)
Hypothyroidism	↑	?	(58, 59)
Hyperthyroidism	↓	?	(58, 59)
Diabetes			
Early stage	↓	Hyperfiltration, regulation by insulin	(42, 60)
Late stage	↑	Compromised renal metabolism	(60, 61)
Hyperproliferative disorders	↑	Increased demand for methyl groups	(62, 63)
Intestinal diseases	↑	Deficiency of folate or vitamin B_{12}	(50)
Rheumatoid arthritis	↑	Drugs, vitamin deficiency, MTHFR C677T genotype, gastrointestinal dysfunction	(64)
Drugs			
Lipid lowering			
Cholestyramine, niacin, fibrates	(↑), ↑	Inhibition of folate absorption, inhibition of pyridoxal kinase, renal impairment	(65-67)
Anticonvulsants	↑	Inhibition of polyglutamation, folate depletion	(68)

Table 1. Causes of hyperhomocysteinemia, cont.

	Effect[a]	Possible modes of action	Refs.
Sex hormones			
Androgens	(↑)	Increased muscle mass/creatine synthesis	(69)
Estrogens (postmenopausal)	↓	Not known, interference with vitamin function	(40)
Anti-rheumatic drugs			
Methotrexate	↑	Inhibition of DHFR	(62, 63)
Other			
Cyclosporin, diuretics, L-dopa, theophylline, trimethoprim	↑	Impaired renal function, reduced GFR, inhibition of pyridoxal kinase, inhibition of DHFR	(57, 70-73)

Modified from (74-77).
[a] ↓ = decrease in tHcy, (↑) = increase within the reference interval, ↑, ↑↑, ↑↑↑ = moderate (15-30 μmol/L), intermediate (30-100 μmol/L) and severe hyperhomocysteinemia (> 100 μmol/L), respectively.
[b] In individuals with vitamin B_6 deficiency or mild defects in CBS, the fasting concentrations are usually within the reference interval, whereas the post-methionine-load tHcy concentrations are often increased.
[c] Homocystinuria includes deficiency in CBS, MTHFR, methionine synthase, or methionine synthase reductase and defects in intracellular cobalamin metabolism.
CBS = cystathionine β-synthase, DHFR = dihydrofolate reductase, GFR = glomerular filtration rate, MTHFR = 5-methyltetrahydrofolate reductase.

Genetic factors

In addition to the rare inborn errors in the Hcy metabolism that lead to very high tHcy levels (homozygous CBS deficiency, defects in MS, in MTHFR, or in cobalamin metabolism), there are genetic variations that lead to less pronounced elevations in the tHcy. The most common genetic determinant for tHcy is homozygosity for the MTHFR 677C→T polymorphism (29, 78). Individuals with the 677TT genotype usually have about 25% higher tHcy than in those with the wild genotype (78). This is especially seen in subjects with low folate status (29). Individuals heterozygous for CBS deficiency have normal fasting tHcy, but some respond to a methionine loading with an abnormal increase in tHcy (31). Paraoxonase (PON) is a high-density lipoprotein (HDL)-associated enzyme that converts Hcy thiolactone back to Hcy (79). Different polymorphisms in the PON1 gene has been shown to have different effects on the hydrolysis of thiolactones (80). Also several other polymorphisms have been associated with increased tHcy levels, including polymorphisms in MTHFR (other than 677C→T) (81), MS (82), MS reductase (83), transcobalamin-II (84), reduced folate carrier-1 (85) and thymidylate synthase (86).

Physiological factors

Hcy concentration increases with age in both sexes, and approximately double from childhood to old age (76). After puberty the mean tHcy concentration is about 2 μmol/L higher in men than in women, but the difference diminishes with increasing age (35, 36). The reason for the difference is most likely the increased muscle mass in men, since the muscle formation is associated with simultaneous formation of Hcy in connection with creatine/creatinine synthesis (43). Androgens and menopause may also explain part of the difference (36).

Dietary and lifestyle factors

The most important dietary determinant of tHcy is folate (12). Vitamin B_6 and vitamin B_{12} have only modest tHcy lowering effects. There is also some evidence that riboflavin (vitamin B_2), which acts as a precursor to flavin adenine dinucleotide (FAD), a cofactor for MTHFR, may also be a determinant of tHcy levels (46). Inadequate intake of folate and other B vitamins has been estimated to account for about 2/3 of all cases of hyperhomocysteinemia (44). Supplementation with folic acid was shown to reduce tHcy levels on the average by 25% in a meta-analysis of 12 randomized trials (87). The effect was similar in dosages from 0.5 to 5 mg/day. The reductions in blood tHcy were greater at higher pretreatment blood tHcy levels and at lower pretreatment folate levels. Furthermore, higher dietary intake of folate is associated with lower tHcy levels, independent of other dietary and lifestyle factors (45, 88, 89). It has also been suggested that the folic acid dose necessary to achieve the greatest reductions in tHcy is 0.8 mg/day (90). The differences between the effects of folate and other B vitamins may be due to their different metabolic roles (50). Folate is used as a substrate when it donates a methyl group in the remethylation of Hcy to methionine (Fig. 2). Vitamins riboflavin, B_6 and B_{12} act as cofactors of enzymes in the Hcy metabolism and are not utilized.

Since Hcy is formed of methionine, increased dietary methionine intake could lead to an increase in blood tHcy and thus increase the risk for CVD. Although a single high dose of methionine in a methionine-loading test (see below) increases tHcy concentration, chronic moderately high dietary methionine or protein intake has not been shown to have an effect on the tHcy concentration (45, 91-95). One hypothesis is that the enzymes in the methionine cycle (Fig. 2) adapt to the high methionine intake and thus maintain a normal concentration of tHcy in circulation (91).

Dietary betaine intake, either from foods or supplements, may have some effect on tHcy levels (48, 96-99). High doses ($\geq$6 g/day) have been used to reduce fasting tHcy levels in homocystinuric patients (48), but it has also reduced tHcy levels in healthy patients by 10-15% and post-methionine loading tHcy levels by 40% (97-99). Furthermore, also lower doses of betaine, in the range of dietary intake (about 0.5-2 g/day), have been shown to reduce fasting and post-methionine loading tHcy levels (96).

Smoking (37, 49), coffee (51, 52), alcohol (53, 100) and physical inactivity (37, 54) are also positively associated with tHcy concentration. The smoking effect remains after correction for important confounders, coffee consumption and folate intake (54, 88). The relationship between alcohol consumption and tHcy is complex, most probably following a J-shaped curve (53).

Clinical conditions

Several diseases and drugs are associated with elevated tHcy concentration (**Table 1**). Renal failure is the most common clinical cause for elevated tHcy, next to deficiencies in B vitamins (50). Although the mechanism is not entirely clear, decreased Hcy clearance has been suggested to cause hyperhomocysteinemia (101). Of the endocrine disorders, tHcy is positively associated with hypothyroidism and advanced stages of the Type I diabetes and negatively associated with hyperthyroidism and early stage diabetes (42, 58-61). Proliferating diseases, such as cancer and psoriasis, intestinal diseases and rheumatoid arthritis may also result in higher tHcy levels (50, 62-64).

Methionine loading test

Methionine loading test was developed to diagnose heterozygosity for CBS deficiency (102). It is currently used to identify individuals who have often normal fasting but increased post-methionine load tHcy concentration due to mild disturbances in the transsulfuration pathway (103, 104). In methionine-loading test, a high dose of methionine (0.1 g/kg body weight) is ingested, and serum tHcy is measured immediately before and usually 4-6 h after ingestion (104). In adults, the mean post-methionine load tHcy measured at 4 or 6 h is about 3 and 2 times the fasting value, respectively (104). The upper reference limit is 5 times the fasting tHcy value (76).

2.1.3 Homocysteine and CVD

Elevated tHcy has been suggested to be both atherogenic and thrombogenic (105). The strongest evidence that elevated tHcy is a causal risk factor for CVD comes from patients with inborn errors in Hcy metabolism. About 50% of the patients with genetically determined CBS deficiency will have a vascular event before age 30, if untreated (106). Other inherited defects, like MTHFR deficiency (107) and defects in cobalamin metabolism (3), also cause similar effects at a very young age. In all these conditions tHcy concentrations are >100 μmol/L. These patients develop extensive arterial intimal thickening and fibrous plaques rich in smooth muscle cells and collagen (3, 104). Venous, and to a lesser extent, arterial thrombosis are common (108).

Since the first study by Wilcken and Wilcken in 1976, who showed that the prevalence of hyperhomocysteinemia was higher in patients with CHD than in controls (4), the research on the effects of Hcy on the risk of CVD in general population has been active. Since 1995 several meta-analyses have been conducted about the subject (5-11), the four latest ones in 2002 (7-10). The first meta-analysis by Boushey et al. in 1995 summarized 27 studies relating Hcy to CVD (5). Retrospective and cross-sectional studies showed a significantly increased risk of CHD for each 5 μmol/L increase in tHcy concentration. Only two prospective studies were available at that time, and one found a positive association (109), while the other did not (110).

The meta-analysis by the Homocysteine Studies Collaboration included 18 retrospective and 12 prospective studies which involved a total of 5073 ischemic heart disease (IHD) events and 1113 stroke events (8). Eighty-five percent of the subjects were men. In this meta-analysis, 25% lower tHcy levels (about 3 μmol/L) were associated with 11% lower odds of IHD and 19% lower odds of stroke, after adjustment for known CVD risk factors.

In a concurrent meta-analysis of 20 prospective studies (3820 participants) of tHcy and CVD risk, a 5 μmol/L increase in tHcy was associated with a 32% increase in the odds of IHD and 59% increase in the odds of stroke (10). The authors also analyzed 72 studies, where the prevalence of the mutation in the MTHFR gene (677C→T) was determined in 16849 subjects (10). They found that a 5 μmol/L increase in tHcy was associated with a 42% increase in the odds of IHD, a 65% increase in the odds of stroke, and a 60% increase in the odds of venous thromboembolism. The authors estimated that lowering tHcy by 3 μmol/L from current levels would reduce the risk of IHD by 16% (95% CI 11-20%), deep vein thrombosis by 25% (95% CI 8-38%) and stroke by 24% (95% CI 15-33%), if the association is causal.

The third meta-analysis by Bautista et al. included only cohort studies (7). The 14 studies in the meta-analysis included data from 2529 cases and 7305 non-cases. The

median follow-up time was 9.0 years. According to their analysis, increments in tHcy concentration resulted in a significant increase in the risk of CVD by 33%, CHD by 49% and stroke by 37%.

One meta-analysis covered only ischemic strokes (9). Of the 14 studies included in the analyses of the risk associated with increased tHcy concentration, 11 were retrospective and 3 prospective. The total number of stroke and non-stroke cases was 1769 and 7400, respectively. The pooled odds ratio (OR) estimate of stroke risk associated with mild-to-moderate hyperhomocysteinemia was 1.79 (95% CI 1.61-2.00). The pooled mean tHcy concentration in the stroke cases (in 16 studies included in the analyses of the differences in the mean tHcy) was 2.32 μmol/L greater than in the non-cases (95% CI 1.60-3.04). The authors also determined the pooled estimate of stroke risk associated with the MTHFR 677TT genotype, and in the 19 studies that fulfilled the inclusion criteria, the risk for stroke associated with the TT genotype across all studies was 1.23 (95% CI 0.96-1.58).

There are also other meta-analyses concerning the effect of the MTHFR genotype on the risk of CVD. A meta-analysis by Klerk et al. included 40 observational (retrospective or nested case control) studies, which involved a total of 11162 CHD cases and 12758 control subjects (29). Their analysis showed that individuals with the MTHFR 677TT genotype had a 16% (95% CI 5-28%) greater risk of IHD than those with the CC genotype. They also stratified the analyses by the folate status and found that the TT genotype was associated with increased risk only when folate status was low, indicating an interaction between the MTHFR 677C→T polymorphism and folate status (29).

Similar results were obtained in another meta-analysis of 46 studies with 12193 cases in total, where the risk of IHD was 21% (95% CI 6-39%) higher in individuals with the 677TT genotype compared with the CC genotype (10). The summary OR for the heterozygous state (CT vs. CC) was 1.06 (95% CI 0.99-1.13). In the same meta-analysis the summary OR for the deep vein thrombosis for the TT genotype was 1.29 (95% CI 1.08-1.54) and for the CT genotype 1.05 (95% CI 0.94-1.19) compared with the CC genotype. These analyses included 26 studies with 3439 cases. In addition, in seven studies of stroke (1217 cases), the OR for the TT genotype was 1.31 (95% CI 0.80-2.15) and for the CT genotype 1.15 (95% CI 0.93-1.42) compared with the CC genotype.

Furthermore, there is some data that tHcy may be an independent risk factor in subjects with other CVD risk factors, such as increased serum total or low-density lipoprotein (LDL) cholesterol or plasma fibrinogen concentration or smoking (49, 111-114).

Despite this data, there is continuous debate about whether the association between tHcy and CVD is causal or is it only a marker of the degree of an underlying vascular disease or for a low folate status (50, 115). One aspect that speaks against causality is the less robust or consistent association between tHcy and CVD found in prospective studies compared to case-control studies (8). The magnitude of the risk of CVD varies from about 20% in prospective cohort studies to 80% in case-control studies (105). This discrepancy may arise in part because most prospective studies start with a healthy population and the data and blood are collected before the event. In retrospective studies data is collected after the event, which may have distorted the recall of certain lifestyle and dietary habits or the disease could have influenced blood levels of tHcy (50, 116). Furthermore, tHcy concentration is usually higher in subjects

with CVD, irrespective of whether the concentration is a cause or consequence of CVD. Thus, prospective studies which have included subjects with pre-existing CVD should show a greater association between tHcy and CVD. Some studies do support this assumption (117, 118). More consistent associations have also been found in prospective studies using elderly subjects or shorter follow-up (50).

Another aspect against causality is the lack of evidence from the controlled supplementation trials with folic acid and other B vitamins, that lowering tHcy concentration prevents "hard" vascular outcome events. There are several such trials currently underway (14). However, many of the trials will be underpowered due to the initiation of the folic acid fortification programme of foods in the USA and Canada that coincided with some of the trials (119). Thus, the effect of the supplementation with folic acid and other B vitamins on the risk of CVD may be revealed only in the future meta-analyses of these trials. Furthermore, it will be impossible to determine if the possible beneficial effects in these trials are caused by lowered tHcy concentrations or by independent effects of folate/folic acid on cardiovascular health (14).

2.1.4 Homocysteine and other diseases

In addition to CVD, Hcy has also been suggested to be associated with birth defects, pregnancy complications, renal disease, psychiatric disorders and cognitive impairment in the elderly.

There is a general agreement among several observational studies that hyperhomocysteinemia, folate deficiency and homozygosity for the MTHFR thermolabile variant are probable risk factors for placenta-mediated diseases, such as pre-eclampsia, spontaneous abortion and placental abruption (120). Maternal hyperhomocysteinemia has also been associated with birth defects (121).

Kidneys are an important organ in the Hcy metabolism. Hyperhomocysteinemia is very common in dialysis patients (>85% of patients) and tHcy levels usually remain elevated even after optimal treatment (41). Increased tHcy is also associated with increased risk of hemodialysis access thrombosis, which is a common complication in dialysis patients (122).

In a study with psychogeriatric patients plasma tHcy levels were significantly increased in both the demented patients and the non-demented patients with other psychiatric disorders, compared to controls (123). Demented patients also had lower blood folate levels. If patients with low serum cobalamin or folate levels were excluded, both groups of patients still exhibited significantly higher plasma tHcy levels than the control subjects. High tHcy concentration has also been related to schizophrenia (124), Alzheimer's disease (125) and depression (126).

2.1.5 Mechanisms of action

The mechanisms by which Hcy induces the development and progression of vascular disease have not been fully elucidated. However, current research supports the role for Hcy as a mediator for endothelial damage and dysfunction (127). This would be a key factor in subsequent impaired endothelial-dependent vasoreactivity and decreased endothelium thromboresistance. This in turn would predispose hyperhomocysteinemic vessels to atherogenesis.

Endothelial damage and dysfunction

One of the major theories about how Hcy induces its harmful effects involves endothelial cell injury via oxidative damage. One of the effects is the impairment of vasodilation (128), which is believed to be an early step in the formation of atherosclerotic lesions (129). Impairment of normal endothelium-dependent vasodilation has been shown in humans with mild-to-moderate hyperhomocysteinemia (128). Supplementation with folic acid and vitamin B_{12} has been effective in restoring normal function (130), as has been antioxidant supplementation, which suggest the involvement of reactive oxygen species (131). Hcy may induce oxidative stress through several mechanisms. Hcy may enhance intracellular production of superoxide anion (O_2^-), which in turn may decrease the availability of endothelial nitric oxide (NO), thereby limiting normal vasodilation responses (129, 132). Hcy may also decrease the availability of NO by elevating plasma levels of asymmetric dimethylarginine (ADMA), which is a potent inhibitor of NO synthase (NOS) (133). Hcy has also been shown to inhibit and impair activity of antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase, and it may increase lipid peroxidation and oxidized LDL cholesterol (105, 134, 135). In our Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study population we have shown that elevated plasma tHcy is associated with increased plasma F_2-isoprostane levels, marker of *in vivo* lipid peroxidation (136). These effects increase the oxidative burden of the endothelium.

An important stimulus for arterial occlusive disease is arterial thrombus. Hcy can promote thrombosis by increasing the activity of the clotting cascade and enhancing platelet activation and adhesion (105). Treatment with folate (5 mg/d) and vitamin B_6 (300 mg/d) and B_{12} (1 mg/d) has been shown to attenuate thrombin formation in hyperhomocysteinemic patients (137).

Hcy may also cause endothelial damage and dysfunction by increasing DNA synthesis and proliferation of vascular smooth muscle cells, by increasing endothelial permeability to expose underlying vascular smooth muscle and tissue, by upregulating the expression of cell death genes within endothelial cells and by inhibiting the production of proteins important in the endothelial cell growth (105).

Other mechanisms

One compound that has recently received attention is Hcy thiolactone. Hcy thiolactone is a cyclic thioester derivative of Hcy which is synthesized by specific aminoacyl-tRNA synthases (Fig. 2) (138). Hcy thiolactone is synthesized in several cell types and the synthesis increases with an increase in Hcy levels (139). Hcy thiolactone has unique properties that can lead to homocysteinylation of numerous cellular proteins, which results in the decreased biological activity and premature degradation (140). Hcy thiolactone reacts with lysine residues in proteins, damaging their structure and impairing physiological activities (140). Hcy thiolactone may also decrease HDL-associated PON activity, which may render HDL less effective against oxidative damage and against toxic effects of Hcy thiolactone (141).

Many of the studies investigating the effects of Hcy have been *in vitro* studies using non-physiological concentrations and commercial preparations containing racemic mixtures of D- and L-homocysteine, of which only L-homocysteine is physiologically relevant form (50). Also, most studies have used reduced form of Hcy, whereas about

99% of Hcy in blood is present in oxidized forms. Therefore, the relevance of most of these studies *in vivo* can be debatable.

2.2 Folate

Folate is a generic term for compounds that have a similar structure and functions to those of folic acid. Folate, a B vitamin, is an essential vitamin in the diet, since mammals lack the enzymes to synthesize it (12). Folate is found in a variety of foods, mainly in foodstuffs of plant origin. In Finland the major sources are bread and cereals (43% in men, 36% in women), vegetables including potato, fruits and berries (26% and 34%), meat and egg dishes (16% and 14%), and milk products (11% in both men and women) (142). In foods natural folates exist as reduced tetrahydrofolate species, mainly as polyglutamates. Synthetic and oxidized form folic acid is a monoglutamate. The bioavailability of food folates is about 50% of that of folic acid. This resulted in the introduction of the concept of "dietary folate equivalent" (DFE) as a way to express mixed intake of natural folate and folic acid (143). One μg of natural folate equals 1 μg of DFE, and 1 μg of folic acid equals 1.7 μg of DFE. Unlike folic acid, food folates are also chemically labile and for example cooking tends to destroy folate (12).

The recommended daily intake of folate is 400 μg/day in the USA and 300 μg/day in Finland, for both men and women. The recommendations are not often met, though, and folate deficiency is thought to be one of the most common nutritional deficiencies, especially in elderly, pregnant women or women using contraceptives, smokers and alcoholics (44, 144). In Finland the average daily intake is 273 μg in men and 224 μg in women (142). Similar values were reported from the USA before the start of the fortification of grain products with folic acid in 1998 (145). Recent analyses have suggested >200 μg/day net increase in average folate intake after the start of the fortification (146). There are no toxicities associated with elevated intakes of folic acid or food folates (147). However, there are some concerns regarding long-term high intake of folate or folic acid. One is the masking of vitamin B_{12} deficiency, which often presents as folate deficiency and is therefore commonly misdiagnosed. Elevated folate intake ameliorates vitamin B_{12} deficiency-induced megaloblastic anemia, but does not have an effect on the symptoms of vitamin B_{12} deficiency, such as irreversible progression of neurological dysfunction and cognitive decline (147).

2.2.1 Folate metabolism

In the gut dietary folate polyglutamates must be converted to monoglutamate forms prior to their absorption in the intestine, since only monoglutamates are transported effectively into cells (144). In cells the polyglutamate chain is restored. Folic acid from supplements and fortified foods is reduced to the active tetrahydrofolate form in cells and is chemically identical to food folates (148).

Folates act as coenzymes in the transfer and processing of one-carbon units (148). These are needed for the remethylation of Hcy to methionine, the synthesis of thymidylate and purines and the formation of methyl groups needed for many biological methylation reactions. Therefore, folates are vital for cell division and homeostasis, by producing DNA and regulating metabolism (12). The metabolism of folate and Hcy are

closely linked (**Figure 2**). Remethylation of Hcy to methionine requires the donation of a methyl group from MTHF in a reaction catalyzed by MS.

2.2.2 Folate and diseases

The interest in the potential CVD risk lowering effect of folate has stemmed from the observations that elevated tHcy levels can be effectively lowered by increased folate or folic acid intake (13). Furthermore, in many studies elevated tHcy has been associated with reduced levels of folate in circulation (12). Since folate is the most important determinant of tHcy, higher risk of CVD in subjects with low folate status can be speculated to be caused by increased tHcy levels. Alternatively, folate may have independent effects on the cardiovascular health and increased tHcy is only a marker of impaired folate status.

There are several case-control and prospective studies that have demonstrated an association between low dietary intake or low levels of folate in circulation and risk of CVD (12). For example, in the Nurses' Health Study 80802 women between 30 and 55 years of age were followed for a period of 14 years (145). The women in the highest folate intake fifth (median intake 696 μg/day) had an adjusted hazard rate ratio (RR) for CHD of 0.69 (95% CI 0.55-0.87), compared with the women in the lowest fifth (median intake 158 μg/day). The authors also found the strongest benefit in those women who consumed alcohol. In the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) study cohort, 1981 men 42-60 years of age were followed on the average for 10 years, during which 199 acute coronary events occurred (149). The RR comparing the extreme folate intake fifths, after adjustment for other risk factors, was 0.51 (95% CI 0.28-0.92). Interestingly, unlike in the Nurses' Health Study, in this cohort the association was stronger in light alcohol users compared to alcohol users. In the Physicians' Health Study the men in the lowest fifth of plasma folate had a RR for myocardial infarction (MI) of 1.4 (95% CI 0.9-2.3) compared to the highest fifth (150). There was no change in the RR when tHcy was added in the models. This could suggest that increased risk of CVD in subject with low folate status may be independent of tHcy. Increased folate intake has been shown to be associated also with decreased risk of ischemic stroke (151). 43732 men, aged 40 to 75 years, were followed from 1986 to 2000. The multivariate adjusted risk of ischemic stroke in the highest vs. lowest intake quarter was 0.71 (95% CI 0.52-0.96). In contrast to the other prospective studies, the Atherosclerosis Risk in Communities (ARIC) study did not find an association between plasma folate and risk of CHD (152).

The disadvantage of prospective studies is that folate status is assessed only once and most of the studies are based on serum/plasma measurements. This is likely to underestimate the role of folate in CVD, since circulating folate levels represent short-term body stores of folate. In addition, folate may be unstable in frozen plasma or serum samples (12). Furthermore, since the metabolisms of folate and Hcy are closely linked, epidemiological studies cannot fully exclude the possibility that folate is associated with the risk of CVD independently of Hcy.

In addition to CVD, impaired folate metabolism has been associated with risk for developmental anomalies and disease including neural tube defects (NTDs) and cancer (153, 154). The mechanisms that underlie the folate-NTD or folate-cancer relationships are not completely known. However, folate has an important role in the DNA synthesis, stability and integrity, and repair, which may explain its association with developmental

anomalies and cancer (153, 155). It is well documented that prophylactic supplementation with folic acid reduces the incidence of birth complications and NTDs (121). It is currently recommended that low-risk women take 400 μg and high-risk women (women with a history of NTD pregnancy) 4000 μg of folic acid daily periconceptually (156).

Folic acid supplementation studies

There are several international trials, involving over 50000 subjects, investigating the effects of supplementation with folic acid and other B vitamins on the risk of CVD (**Table 2**). The dose of folic acid in these trials ranges from 200 μg to 5000 μg/day. The trials were originally designed to investigate the hypothesis that lowering tHcy with folic acid may improve cardiovascular outcomes. At the time of the initiation of these trials it was not known that folic acid may have independent effects on CVD health. If a reduction in the risk of CVD is observed, it will be impossible to distinguish between the effects of tHcy lowering and independent effects of folic acid (14). If no reduction is observed, then it may indicate that tHcy is not a risk factor for CVD. Or if folate does have independent effects, then the dose used may have been too low to find any benefit in some studies (14). Furthermore, three large trials in the USA and Canada were started before the initiation of fortification of cereals and grains with folic acid in these countries, which reduces the power in these studies (119).

Table 2. Clinical trials assessing whether lowering of tHcy by folic acid (FA) and other B vitamins attenuates cardiovascular outcomes

Study	Country	Sample size	Intervention & study design	Disease group	Start year
Vitamins and Thrombosis (VITRO) trial	The Netherlands	700	FA (5 mg/d)+B_6 (50 mg/d)+B_{12} (0.4 mg/d) vs. placebo	Deep vein thrombosis or pulmonary embolism	1996
Vitamin Intervention for Stroke Prevention (VISP)	USA	3600	FA (2.5 mg/d)+B_6 (25 mg/d)+B_{12} (0.4 mg/d) vs. FA (0.2 mg/d)+B_6 (0.2 mg/d)+B_{12} (0.06 mg/d)	Stroke	1998
Women's Antioxidant and Cardiovascular Disease Study (WACS)	USA	6000-8000	FA (2.5 mg/d)+B_6 (50 mg/d)+B_{12} (1 mg/d) vs. placebo	Vascular disease and high risk for vascular disease	1998
Cambridge Heart Antioxidant Study (CHAOS-2)	UK	4000	FA (5 mg/d) vs. placebo	Myocardial infarction or unstable angina	1998
Norwegian Study of Homocysteine Lowering with B-vitamins in Myocardial Infarction (NORVIT)	Norway	3000	FA (5 mg/d) for 2 weeks, then FA (0.8 mg/d)+B_{12} (0.4 mg/d) vs. placebo in a 2x2 factorial design with B_6 (40 mg/d) vs. placebo	Myocardial infarction	1998
Vitamins to Prevent Stroke Study (VITATOPS)	Australia	8000	FA (2 mg/d)+B_6 (25 mg/d)+B_{12} (0.5 mg/d)	Stroke	1998
Western Norway B Vitamin Trial (WENBIT)	Norway	2000	FA (5 mg/d) for 2 weeks, then FA (0.8 mg/d) vs. placebo; B_6 (40 mg/d) vs. placebo in a 2x2 factorial design	Coronary heart disease	1999
Heart Outcomes Prevention Evaluation-2 (HOPE-2)	Canada	5000	FA (2.5 mg/d)+B_6 (50 mg/d)+B_{12} (1 mg/d) vs. placebo	Vascular disease	1999
Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH)	UK	12000	FA (2 mg/d)+B_{12} (1 mg/d) vs. placebo in a 2x2 factorial design with simvastatin (80 mg/d vs. 20 mg/d)	Myocardial infarction	1999
Prevention with a Combined Inhibitor and Folate in Coronary Heart Disease (PACIFIC)	Australia	10000	FA (0.2 mg/d or 2 mg/d) vs. placebo in a 2x2 factorial design with Omapatrilat (ACE-inhibitor) vs. placebo	High risk for vascular disease or previous history of vascular disease	2000

FA = folic acid, ACE = angiotensin converting enzyme. From (12).

2.2.3 Mechanisms of action

Until recently, the beneficial effects of folate were thought to be a consequence of tHcy lowering. However, recent evidence suggests that folate may have also other beneficial effects on vascular endothelial function. Some proposed mechanisms are summarized in **Figure 3**. The effect on tHcy has already been discussed in the chapter 2.1.2.

Folic acid or its derivative MTHF may reduce oxidative stress by reducing superoxide radical production or acting as a free radical scavenger and inhibitor of microsomal lipid peroxidation (157, 158). The relevance of these *in vitro* studies is questionable, however, because the levels of MTHF used in the studies are not attainable *in vivo* following ingestion of folic acid (12). Furthermore, the free radical scavenging capacity of MTHF is about 20 times lower than that of vitamin C (159).

Folate, as MTHF, may also reverse endothelial dysfunction through its effects on tetrahydrobiopterin (BH_4). BH_4 acts as a cofactor for endothelial nitric oxide synthase (eNOS). For example, increase in oxidative stress results in the oxidation of BH_4 to its inactive metabolite dihydrobiopterin (BH_2), which results in the production of superoxide radicals by eNOS (160). It has been suggested that MTHF may stabilize BH_4, and thus prevent its oxidation to BH_2 or it may regenerate BH_4 from BH_2 (12). However, folic acid therapy did not lead to a measurable increase in plasma biopterin levels in a study by Verhaar et al. (161), so the exact mechanisms remain to be investigated.

PON, as an important HDL-associated antioxidative enzyme, has an important role in the protection against lipid peroxidation (162). Since oxidized LDL is also known to inactivate serum PON activity (163), it is therefore possible that folate could reduce circulating tHcy concentration and LDL oxidation and in this way spare the PON activity of HDL. Folate could also induce PON production directly. However, no studies to date have confirmed these hypotheses.

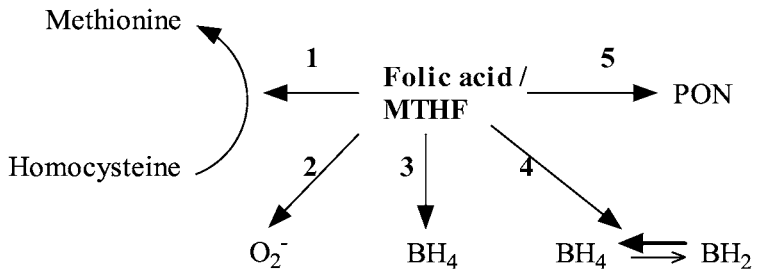

***Figure 3**. Possible mechanisms for the ameliorative effects of folates on vascular endothelial function. (1) Remethylation of homocysteine to methionine. (2) Scavenging of superoxide anions (O_2^-). (3) Stabilization of tetrahydrobiopterin (BH_4). (4) Regeneration of BH_4 from its oxidized and inactive form dihydrobiopterin (BH_2). (5) Sparing of PON activity. MTHFR = 5,10-methylene-tetrahydrofolate reductase, PON = paraoxonase. Modified in part from (12).*

3. AIMS OF THE STUDY

The aims of the present work were:

I To study the association between dietary methionine intake and the risk of acute coronary events in men.

II To examine the relations of both serum folate and tHcy concentrations with acute coronary events in men.

III To study the association between both serum tHcy and folate concentrations and the risk of stroke in men.

IV To explore the joint effect of increased serum tHcy concentration and other risk factors on the risk of CVD mortality in men.

4. MATERIALS AND METHODS

4.1 Study population

The KIHD Study is an on-going population-based study of risk factors for CVD, atherosclerosis, and related outcomes in men from Eastern Finland (164). The baseline examinations were carried out between March 1984 and December 1989. The study sample was composed of 3235 men aged 42, 48, 54, or 60 years at baseline examination. Of these, 2682 (82.9 %) participated. The study protocol was approved by the Research Ethics Committee of the University of Kuopio. All subjects gave their written informed consent for participation.

Data from the baseline examinations were used for the original work I. Men with prior CHD at baseline (n=677) were excluded from the analyses. Of the remaining 2005 men, food record data were available for 1981 men. The follow-up lasted until December 2002.

The original works II and IV are based on the KIHD 4-year re-examination data from 1991-93 and this period also formed the baseline for these studies. Out of the total of 1229 men eligible for the study, 52 had died, had had a severe illness, or had migrated from the region, and 139 could not be contacted or refused to participate. Thus, 1038 men were examined in 1991-93. Men with prior CHD (n=217) in the work II and men with prior CHD or stroke (n=225) in the work IV were excluded from the analyses, as the presence of clinical CHD could have influenced dietary habits. Prior CHD was defined as either a history of acute coronary event or angina pectoris or positive angina pectoris on effort in Rose interview or the use of nitroglycerin tablets once a week or more frequently. The follow-up extended until December 1999 (work II) or 2003 (work IV). Of the remaining 813 men in the work IV, data on serum tHcy concentration were available for 802 men. Work II included 810 men.

The original work III is also based on the KIHD 4-year re-examination data from 1991-93. Of the 1038 men examined in 1991-93, data on serum tHcy were available for 1027 men. Men with a history of stroke (n=12) were excluded, leaving 1015 men for the final analyses. The follow-up time extended until December 2002.

4.2 Ascertainment of follow-up events

The province of Kuopio participated in the multinational MONICA (Monitoring of Trends and Determinants of Cardiovascular Disease) project (165), in which detailed diagnostic information of all coronary deaths and non-fatal MIs that occurred by December 1992 was collected prospectively. The diagnostic classification was made by the FINMONICA coronary registry group (164).

Data on acute coronary events between 1993 and 2002 in the work I and between 1993 and 1999 in the work II were obtained via computer linkage to the national hospital discharge register and were classified by using diagnostic criteria, including symptoms, cardiac enzymes, and electrocardiographic findings, that were identical to those used in the MONICA project, as explained previously (165). The average follow-

up time was 14.0 years in the work I and 7.7 years in the work II. If multiple nonfatal events occurred during the follow-up, the first event for each subject was considered as the endpoint for the analyses. According to the diagnostic classification of the events, there were 156 definite and 79 possible acute myocardial infarctions and 57 typical prolonged chest pain episodes in the work I in the men who were free of prior CHD at baseline. The respective numbers were 37, 17 and 7 in the work II.

In the work III, incident strokes between 1984 and 1992 were ascertained through the FINMONICA stroke register (166). Information on stroke incidence between 1993 and December 31, 2002 was obtained by computerized linkage to the Finnish national hospital discharge register and death certificate register. Diagnostic information was collected from hospitals and classified by one neurologist using diagnostic criteria identical to the FINMONICA criteria. The sources of information on stroke were from hospital documents, death certificates, autopsy reports, and medicolegal reports. The diagnosis of stroke was based on the sudden onset of clinical signs or focal or global disturbance of cerebral function lasting >24 hours (except in the case of sudden death or when interrupted by surgical intervention) with no apparent cause other than a vascular origin. Each suspected stroke [International Classification of Diseases, Ninth Revision (ICD-9) codes 430 to 439 and Tenth Revision (ICD-10) codes I60-I68 and G45-G46) was classified as (1) definite stroke, (2) no stroke, or (3) an unclassifiable event. The data from the FINMONICA stroke register were annually rechecked with the data obtained from the computerized national hospital discharge and death registers. Definite strokes and unclassifiable events were included in the group of any stroke. Each definite stroke was classified as (1) an ischemic stroke (ICD-9 codes 433 to 434 or ICD-10 code I63) or (2) a hemorrhagic stroke (ICD-9 codes 430 to 431 or ICD-10 codes I60-I61). If the subject had multiple strokes during follow-up, the first event was considered the end point.

In the work IV the CVD deaths were ascertained by a computer linkage to the national death certificate register using the Finnish personal identification code (social security number). CVD deaths were coded according to the ICD-9 code numbers 390-459 or the ICD-10 code numbers I00-I99.

4.3 Laboratory measurements

In the KIHD study, both in the baseline and 4-year re-examinations, the subjects came to give venous blood samples between 8 and 10 a.m. They were instructed to abstain from ingesting alcohol for three days and from smoking and eating for 12 hours. After the subject had rested in supine position for 30 minutes, blood samples were obtained by venipuncture and collected into vacuum tubes (Venoject; Terumo, Leuven, Belgium). No tourniquet was used. In the 4-year re-examination visit blood for folate and cholesterol determination and for lipoprotein separation was drawn into serum tubes and blood for α-tocopherol, lycopene, β-carotene and retinol measurements was collected in tubes containing lithium heparin.

4.3.1 Determination of serum tHcy concentration

For the work I plasma tHcy measurements were available for 174 acute coronary event cases and 160 others. Cases and others were matched for age, examination year and

residence. Plasma was separated within 60 min and stored at -20°C until analysis. The plasma tHcy concentrations were analyzed in 1998 at the department of Clinical Pharmacology, Rigshospitalet, Copenhagen, Denmark by gas-chromatography mass spectrometry using an isotope dilution method (167, 168).

In the works II-IV the serum tHcy concentration was analyzed by High Performance Liquid Chromatography (HPLC) (169), with modifications described by Schwab et al (98), in 2001 at the National Public Health Institute, Helsinki, Finland. The results represent serum total homocysteine, which is referred as serum tHcy. The coefficient of variation (CV) between batches (n=30) for two pooled serum samples were 4.3% and 5.4% at levels 11.2 and 7.4 μmol/L, respectively. The mean bias in a quality-control program was 2.2% (98).

4.3.2 Determination of serum folate concentration

Serum folate concentration was measured by radio-immunoassay (Quantaphase II, Bio-Rad, Hercules, CA, USA). Folate measurements were carried out in 1998 in serum samples collected during 1991-1993 and kept frozen at -80°C. The between-batch CV of quality control serum (Lyphochek Immunoassay Plus Control levels 1, 2, 3, Bio Rad Laboratories, ECS Division, Anaheim, CA, USA) at levels 5.5, 13.4 and 23.6 nmol/L were 6.4, 6.7 and 6.7%, respectively (n=16).

4.3.3 Assessment of nutrient intakes

The consumption of foods in the work I was assessed at the time of blood sampling at the KIHD study baseline with an instructed 4-day food recording by household measures. The instructions were given and the completed food records were checked by a nutritionist. The intakes of nutrients were estimated using the NUTRICA® version 2.5 software. The content data of amino acids in Finnish food items was obtained from the Fineli® databank at the National Public Health Institute (Fineli-Food composition databank of Finland, www.fineli.fi) (170). Intakes of 350 foodstuffs containing the most methionine and recipes containing these foodstuffs were assessed. The intakes of nutrients used as covariates in the Cox models were energy adjusted by the residual method (171). Energy adjustment is based on the notion that a larger, more physically active person requires a higher caloric intake, which is associated with a higher absolute intake of all nutrients. Therefore energy adjustment takes into account differences in energy requirements among individuals. The residuals were standardized by the mean nutrient intake of a subject consuming 10 MJ/d, the approximate average total energy intake in this study population. The major methionine sources were calculated based on randomly selected food records from 10 cases and from 50 others. We replicated the baseline food record data by 4-day food records (n=40) used in the 1-year follow-up visit of the KIHD study.

4.3.4 Other laboratory measurements

The measurement of baseline serum lipids and lipoproteins (172), plasma fibrinogen (173), and 24-hour urinary excretion of nicotine metabolites (174) have been described in detail previously.

In the KIHD study 4-year re-examinations, heparin plasma for α-tocopherol, β-carotene, retinol and lycopene determinations was stored at -80°C until extracted with ethanol and hexane and measured by an HPLC method by using α-tocopherol acetate as an internal standard (175). Lipoproteins were separated from fresh serum samples by combined ultracentrifugation and precipitation (176). Serum total, LDL and HDL cholesterol and triglyceride concentrations were determined enzymatically with an automatic analyzer (Kone Specific, Kone Instruments, Espoo, Finland).

4.3.5 Other measurements

Resting blood pressure was measured by two trained nurses with a random-zero mercury sphygmomanometer (Hawksley, United Kingdom). The measuring protocol included, after supine rest of five minutes, three measurements in supine, one in standing and two in sitting position with five minutes' intervals. The mean of all six measurements was used as the systolic and diastolic blood pressure.

Assessments of medical history and medications (177), family history of disease (177), ischemic findings in exercise test (178), maximal oxygen uptake (178), alcohol consumption (177) and smoking (177) were carried out as described previously. Annual income was obtained from a self-administered questionnaire. Diabetes was assessed by previous diagnosis of diabetes or fasting blood glucose concentration ≥6.7 mmol/L. Body mass index (BMI) was computed as the ratio of weight in kilograms to the square of height in meters.

4.4 Statistical methods

In all works means were compared with analysis of variance (ANOVA). Categorical variables were compared with the chi-square test. Correlations were estimated with Pearson correlation coefficients (r). Cox proportional hazards models were used to analyze the associations between serum tHcy, serum folate or dietary methionine or protein and incidence of stroke, CVD death or acute coronary event. Risk factor adjusted RRs were estimated as the antilogarithms of coefficients from multivariable models. CIs were estimated on the basis of the assumption of asymptotic normality of the estimates. All tests of significance were two-sided. P-values <0.05 were considered statistically significant except in interaction analyses where P-values <0.20 were considered significant (179). Missing values in covariates were replaced by population means. Data were analyzed using SPSS 10.0, 11.0 or 11.5 for Windows (SPSS Inc., Chicago, IL, USA).

In the work I the subjects were classified into quarters according to the daily mean methionine or protein intake. The covariates used in the initial model were age, examination years, serum LDL and HDL cholesterol and triglyceride concentrations, BMI, diabetes, urinary nicotine metabolites, IHD in family, maximal oxygen uptake, systolic blood pressure, annual income, alcohol usage, serum ferritin, serum creatinine, plasma ascorbic acid, and dietary intakes of vitamin B_6, B_{12}, C and E, β-carotene, fiber, folate, saturated fatty acids and protein (excluding methionine; only for the analyses with methionine intake). The exclusion of a covariate from this initial model was based on a change in RR for methionine or protein intake quarters of less than 10%. Age and examination years were forced in the models. When this criterion was used, BMI, urine

nicotine metabolites and protein intake remained in the final models in addition to the age and examination years in the analyses with methionine intake. For the analyses with dietary protein intake, only age and examination years remained in the final models. An eigenanalysis of the predictor correlation matrix using the full model was carried out to detect multicollinearities among the predictor variables.

In the work II the subjects were classified into thirds according to their serum folate and tHcy concentrations. Four sets of covariates were used. Model 1 was adjusted for age and examination year; model 2 for the covariates in model 1 plus smoking, BMI, and systolic blood pressure; model 3 for the covariates in model 2 plus serum LDL and HDL cholesterol; and model 4 for the covariates in model 3 plus the following dietary factors: serum lycopene, α-tocopherol, and ß-carotene.

In the work III the subjects were divided into thirds according to the serum tHcy or folate concentrations. The men in the highest third were compared with the lowest third. The RRs according to serum tHcy and folate medians were also determined. Systolic blood pressure, smoking, diabetes, BMI, serum total and LDL cholesterol, alcohol intake, history of IHD, income, and total physical activity were considered as confounders. These factors were eliminated from the full model if their joint removal did not result in a change of the tHcy RR of 10% or more. Age and examination year were forced in the models. Only age and examination years remained in the analyses with serum tHcy. Age and examination years, alcohol intake and diabetes remained in the analyses with serum folate and any stroke and age and examination years, serum LDL cholesterol and diabetes remained in the analyses with serum folate and ischemic stroke.

In the work IV, due to a limited number of cases only a few covariates could be used without over-fitting the models. As in the work III, the inclusion of covariates was based on a change in RR of at least 10%. When this criterion was used, smoking, systolic blood pressure and serum LDL cholesterol in addition to age and examination years remained in the models examining the effects of tHcy on the risk of CVD mortality in the whole population. Only systolic blood pressure remained as a covariate in addition to the age and examination years in the models examining the effects of tHcy with other risk factor. Interactions of serum tHcy with fibrinogen, total and LDL cholesterol, apo-B and smoking were assessed by stratified analyses and the use of a product term with both risk factors as binary variables. Serum tHcy levels were categorized as low-to-moderate and high according to the highest tertile. Plasma fibrinogen, serum total and LDL cholesterol and apo-B were categorized as high or low according to the median value. Smoking was categorized as yes/no. A subject was defined as a smoker if he had ever smoked on a regular basis and had smoked cigarettes, cigars, or a pipe within the past 30 days. For the evaluation of joint effect, four categories were generated: 1) neither risk factor present, 2) increased serum level of tHcy alone, 3) other risk factor alone, and 4) increased serum level of tHcy and other risk factor present.

5. RESULTS

5.1 High Dietary Methionine Intake Increases the Risk of Acute Coronary Events in Middle-Aged Men (Work I)

The work I is based on the KIHD study baseline examinations for 1981 men, the average follow-up time was 14.0 years. The major dietary sources of methionine were meat and meat products (31.8%), milk and milk products (31.7%), cereal (17.7%) and fish (9.8%). The men in the highest quarter of dietary methionine intake compared with the lowest quarter had a higher BMI and annual income, higher prevalence of diabetes and IHD history in family, higher serum ferritin concentration and lower plasma tHcy concentration, as presented in the work I. The intakes of energy adjusted nutrients and foods in the quarters of energy adjusted methionine intake are also shown in the work I. When crude (not energy adjusted) values were used, the men in the highest methionine intake quarter consumed significantly more energy, protein, saturated fat, folate, vitamin B_6, vitamin B_{12}, vitamin E, vitamin C, fiber and β-carotene than men in the lowest quarter ($P<0.001$ for each nutrient, except for β-carotene $P=0.002$). In addition, the consumption of meat, fish, milk products, eggs, vegetables (excluding potatoes), fruits and berries and coffee was significantly higher in the highest quarter of not energy adjusted methionine intake ($P<0.001$ for each food, except for fruits and berries $P=0.002$).

Crude intake of dietary methionine correlated with intakes of dietary protein ($r=0.92$), vitamin B_6 ($r=0.68$), folate ($r=0.58$), saturated fatty acids ($r=0.53$), vitamin E ($r=0.50$), fiber ($r=0.42$), vitamin B_{12} ($r=0.27$), vitamin C ($r=0.18$) and β-carotene ($r=0.06$). The correlation between methionine and total energy intake was 0.71.

The median of the daily methionine intake for the acute coronary event cases was 2.01 g per day and for the others 1.97 g per day. For the cases the methionine intake represents 2.3% and for the others 2.2% of the daily total protein intake (P for difference <0.001). After adjustment for age, examination years, BMI, urine nicotine metabolites and protein intake (excluding methionine) the RRs of acute coronary events in the three highest quarters of energy adjusted methionine intake were 1.31 (95% CI 0.92-1.86), 1.31 (95% CI 0.88-1.96) and 2.08 (95% CI 1.31-3.29) when compared with the lowest quarter. Further adjustments for CVD risk factors did not change the result. **Figure 4** presents survival curves for the quarters of energy adjusted dietary methionine intake, adjusted for age, examination years, BMI, urine nicotine metabolites and protein intake (excluding methionine).

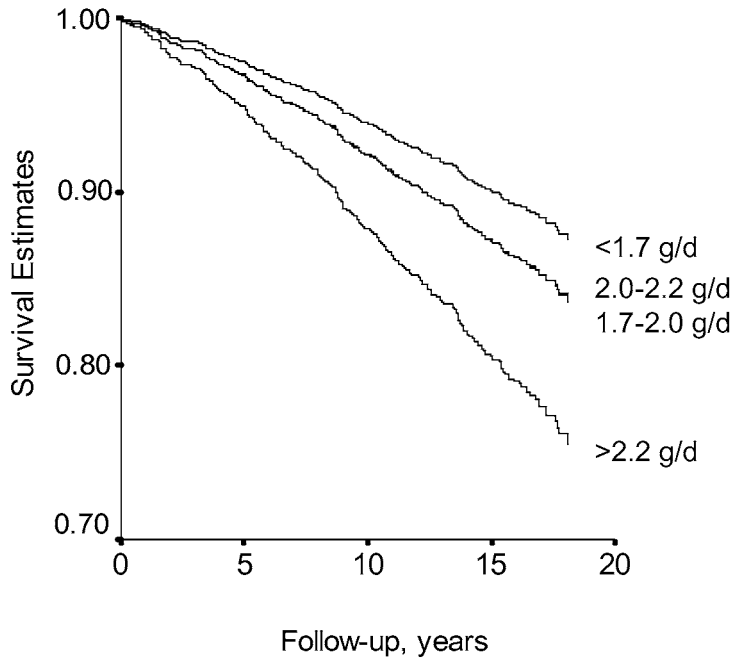

Figure 4. *Survival curves for quarters of energy adjusted dietary methionine intake, adjusted for age, examination years, body mass index, urine nicotine metabolites and protein intake (excluding methionine).*

We also assessed the effect of total protein intake on the risk of acute coronary events. The mean intake of protein was 90.7 g/day (SD 25.1) (15.5 ± 2.6% of energy). The age and examination year adjusted RRs of acute coronary event in quarters of energy adjusted protein intake were 1.0, 0.89 (95% CI 0.66-1.22), 0.86 (95% CI 0.63-1.18) and 0.78 (95% CI 0.56-1.09). If energy adjusted methionine intake was added in the models, the RRs for quarters of energy adjusted protein intake (excluding methionine) were 1.0, 0.76 (95% CI 0.55-1.05), 0.63 (95% CI 0.44-0.91) and 0.43 (95% CI 0.27-0.70). Further adjustments did not change the result.

We had plasma tHcy measurements available for 174 cases and 160 others. The mean plasma tHcy concentration in the cases was 11.2 μmol/L (SD 2.9) and in the others 11.3 μmol/L (SD 3.4), P for difference 0.867. The correlation between plasma tHcy and dietary methionine intake was -0.088 and between plasma tHcy and dietary protein intake -0.121.

5.2 Serum folate and homocysteine and the incidence of acute coronary events (Work II)

The work II is based on the KIHD study 4-year re-examinations for 1027 men, the average follow-up time was 7.7 years. At the beginning of the follow-up, the mean age of the men was 55.3 years. During the follow-up, 61 men with no previous CHD at baseline experienced an acute coronary event. Compared with the subjects who did not

experience an acute coronary event, those who did were significantly older and had significantly higher systolic blood pressure and serum total and LDL cholesterol concentrations and significantly lower serum lycopene concentration, as presented in the work II. The mean serum folate and tHcy concentrations in the study cohort were 10.4 nmol/L (SD 3.9, range 2.3–38.7 nmol/L) and 10.8 μmol/L (SD 3.3, range 3.3–51.2 μmol/L), respectively. The mean serum folate concentration was 10% lower in the men who experienced an acute coronary event than in those who did not (P=0.052). The two groups did not differ significantly in mean serum tHcy concentration.

The men with higher serum folate concentration (highest tertile) differed significantly from those with lower concentration in age, concentration of serum tHcy, BMI, systolic blood pressure, serum triglyceride concentration, and the concentrations of certain nutritional factors (e.g. serum α-tocopherol and lycopene), as shown in the work II. The men with higher serum tHcy concentration (highest tertile) differed significantly from those with lower concentration in age, and concentrations of serum folate, total and LDL cholesterol, triglyceride and lycopene concentrations.

During the follow-up, 10 (3.8%) men with higher serum folate concentration (highest tertile, serum folate concentration >11.3 nmol/L) and 28 (10.0%) men with lower serum folate concentration (lowest tertile, serum folate concentration <8.4 nmol/L) experienced an acute coronary event (P for difference 0.011) (**Figure 5**).

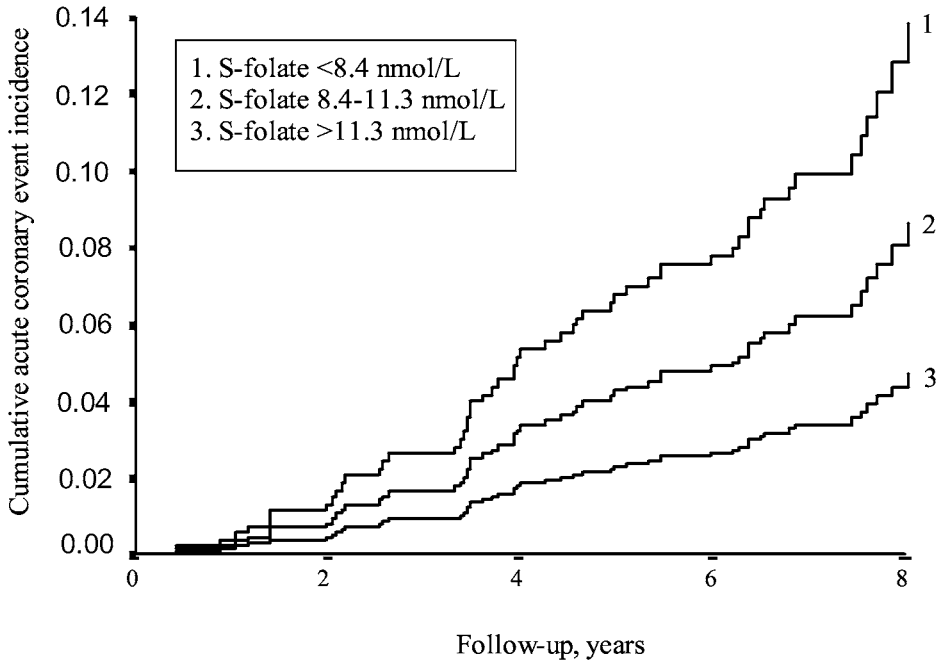

Figure 5. *Cumulative acute coronary event incidence in men according to thirds of serum folate levels adjusting for age and examination year.*

In a Cox proportional hazards model with adjustment for age and examination year, the men with serum folate concentration in the highest tertile had a RR of acute coronary events of 0.38 (95% CI 0.19-0.79) relative to that of the men with concentrations in the

lowest tertile. In a Cox model including examination year, age, and traditional CVD risk factors (BMI, smoking, systolic blood pressure, and serum LDL and HDL cholesterol), the RR for those with higher serum folate concentration remained almost unchanged (RR 0.35, 95% CI 0.17-0.73). Furthermore, adjustment for nutritional factors did not attenuate this association (**Table 3**). To check whether this effect was independent of tHcy concentration, we also adjusted models 1 and 3 for serum tHcy. This resulted in RRs of coronary events of 0.35 (95% CI 0.17-0.72) and 0.33 (95% CI 0.16-0.69) in models 1 and 3, respectively, when the men with serum folate concentration in the highest tertile were compared with those with concentration in the lowest tertile.

Table 3. Hazard rate ratios of acute coronary events by thirds of serum folate and total homocysteine (tHcy) concentration

Serum folate, nmol/L	**<8.4** (n=278)	**8.4 – 11.3** (n=267)	**>11.3** (n=265)	**P for trend**
Model 1[1]	1	0.77 (0.44 - 1.35)	0.38 (0.19 - 0.79)	0.009
Model 2[2]	1	0.76 (0.44 - 1.33)	0.35 (0.17 - 0.73)	0.005
Model 3[3]	1	0.76 (0.43 - 1.33)	0.35 (0.17 - 0.73)	0.005
Model 4[4]	1	0.78 (0.44 - 1.38)	0.39 (0.18 - 0.83)	0.016
Serum tHcy, μmol/L	**<9.55** (n=273)	**9.55 – 11.26** (n=268)	**>11.26** (n=269)	**P for trend**
Model 1[1]	1	0.78 (0.56 - 1.85)	1.02 (0.56 - 1.85)	0.926
Model 2[2]	1	0.78 (0.39 - 1.48)	1.00 (0.54 - 1.84)	0.958
Model 3[3]	1	0.73 (0.38 - 1.43)	1.04 (0.57 - 1.93)	0.854
Model 4[4]	1	0.74 (0.38 - 1.46)	1.01 (0.55 - 1.87)	0.925

[1] Adjusted for age and examination year.
[2] Adjusted for model 1 and smoking, body mass index and systolic blood pressure.
[3] Adjusted for model 2 and serum LDL and HDL cholesterol.
[4] Adjusted for model 3 and dietary factors: serum lycopene, α-tocopherol and β-carotene.

Serum tHcy concentration was not associated with acute coronary events (**Table 3**). With adjustment for age and examination year, the RR of acute coronary events in the men with higher serum tHcy concentration (highest tertile) was 1.02 (95% CI 0.56-1.85) relative to that of the men with the lowest serum tHcy concentration (lowest tertile). Subdividing the tHcy concentrations into quarters or adjustment for other dietary or risk factors did not uncover any association between tHcy and coronary events. We also studied the role of tHcy in the primary and secondary prevention of CHD. The mean serum tHcy concentration among 217 men with previous CHD at baseline (P=0.010 for the difference in tHcy concentration between the men with or without previous CHD at baseline) was 11.5 μmol/L (SD 3.4, range 2.7–32.6 μmol/L). Twenty-four percent (n=53) of these men had a coronary event during follow-up. Compared with the men with serum tHcy concentration in the lowest tertile, those with concentration in the highest tertile had RRs of 1.09 (95% CI 0.56-2.14) and 0.96 (95% CI 0.48-1.92) in models 1 and 3, respectively. Evaluation of the follow-up time suggested that there was no association for a short follow-up (3 or 5 y) in the men either with or without CHD at baseline.

We repeated the analyses in smokers and non-smokers. The mean serum folate concentrations in the smokers (n=215) and non-smokers (n=595) were 10.1 (SD 3.9) and 10.5 (SD 4.0) nmol/L, respectively (P=0.175). The mean serum tHcy concentrations

in the smokers and non-smokers were 10.9 (SD 3.5) and 10.5 (SD 3.0) μmol/L, respectively (P=0.194). The association between serum tHcy and the risk of acute coronary events appeared to be stronger among the smokers than among the non-smokers. In the non-smokers, with adjustment for examination year, age, BMI, systolic blood pressure, and serum LDL and HDL cholesterol, the RR of acute coronary events in the men with serum tHcy concentration in the highest tertile was 0.82 (95% CI 0.40-1.69) relative to that of the men with concentration in the lowest tertile. In the smokers, the RR was 2.34 (95% CI 0.78-7.01). In a similar analysis of the relation between serum folate and the risk of acute coronary events, the RRs were 0.37 (95% CI 0.15-0.90) and 0.38 (95% CI 0.10-1.42) in the non-smokers and smokers, respectively.

The cohort was also divided into two groups on the basis of their alcohol consumption [no or light alcohol consumption and heavy alcohol consumption (>30 g/wk)]. We found no effect of interaction between alcohol consumption and serum folate or tHcy on the risk of acute coronary events.

5.3 Serum homocysteine and folate and the risk of stroke (Work III)

The work III is based on the KIHD study 4-year re-examinations for 1015 men, the average follow-up time was 9.6 years. At baseline the mean serum tHcy concentration was 10.9 μmol/L (SD 3.4, range 2.8-51.2 μmol/L) and the mean serum folate concentration 10.4 nmol/L (SD 4.1, range 2.3-40.5 nmol/L). The baseline characteristics of the study population according to the serum tHcy and folate concentrations are presented in the work III. The men in the highest serum tHcy third were older, had lower serum folate and HDL concentrations and higher triglyceride concentration, higher systolic blood pressure and higher prevalence of symptomatic IHD or history of IHD compared to the lowest third. The men in the highest serum folate third were younger and had higher total and HDL cholesterol and triglyceride concentrations and lower serum tHcy concentration. They also had higher systolic and diastolic blood pressure, BMI and alcohol intake and were more physically active. In addition, they smoked less and had a higher prevalence of diabetes than men in the lowest third.

During a mean follow-up time of 9.6 years (SD 2.2) 49 men experienced a stroke, of which 34 were ischemic. The subjects were divided into thirds based on the serum tHcy concentration. The age and examination year adjusted RRs for any stroke in thirds of Hcy were 1, 2.16 (95% CI 0.94-4.96) and 2.77 (95% CI 1.23-6.24), P for linear trend across the thirds 0.014. The age and examination year adjusted RRs for ischemic stroke in thirds of tHcy were 1, 1.71 (95% CI 0.63-4.64) and 2.61 (95% CI 1.02-6.71), P for linear trend 0.039. Additional adjustment for stroke risk factors (systolic blood pressure, smoking, diabetes, BMI, serum total and LDL cholesterol, alcohol intake, history of IHD, income, and physical activity) or serum folate did not change the results. **Figure 6a** presents the age and examination year adjusted stroke survival curves for men in each serum tHcy third.

The age and examination year adjusted RRs for any stroke in the thirds of serum folate were 1, 1.04 (95% CI 0.57-1.90) and 0.43 (95% CI 0.18-1.05), P for linear trend 0.200. The respective RRs for ischemic strokes were 1, 0.81 (95% CI 0.38-1.72) and 0.49 (95% CI 0.18-1.31), P for linear trend 0.361. After additional adjustment for alcohol intake and diabetes the RRs for any stroke were 1, 1.00 (95% CI 0.55-1.83) and

0.35 (95% CI 0.14-0.87), P for linear trend 0.046. The RRs for ischemic stroke were 1, 0.79 (95% CI 0.37-1.68) and 0.40 (95% CI 0.15-1.09), P for linear trend 0.117, after additional adjustment for serum LDL cholesterol and diabetes. Further adjustment for other risk factors (systolic blood pressure, smoking, diabetes, BMI, serum total and LDL cholesterol, alcohol intake, history of IHD, income, and total physical activity) and serum tHcy did not change the results. Neither did adjustment for plasma lycopene and beta-carotene. **Figure 6b** presents the age, examination year, alcohol intake and diabetes adjusted stroke survival curves for men in each serum folate third.

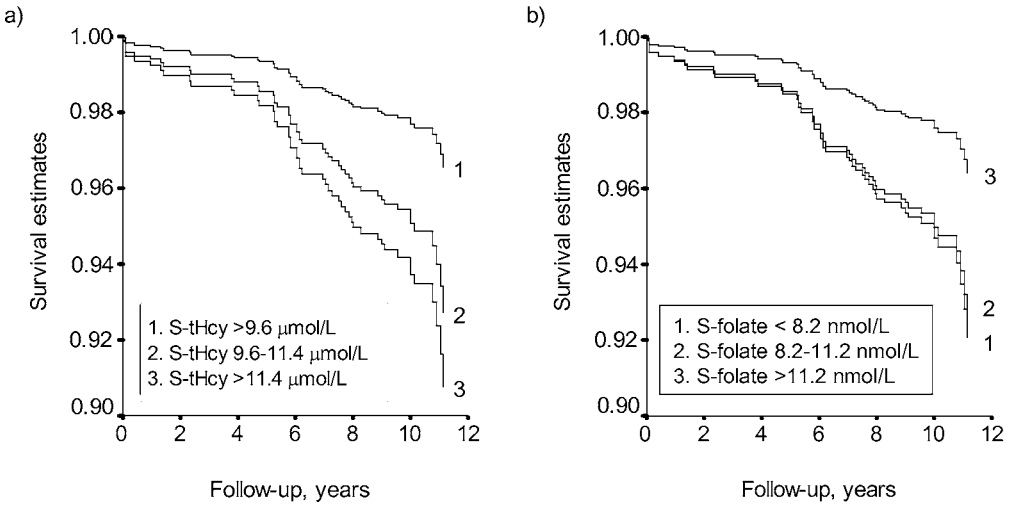

Figure 6. *Age and examination year adjusted stroke survival curves for thirds of serum total homocysteine (tHcy) (a) and age, examination year, alcohol intake and diabetes adjusted survival curves for thirds of serum folate (b).*

The RRs according to serum tHcy and folate medians were also determined. The risk of any stroke and ischemic stroke were increased in the group of men with both elevated serum tHcy and low serum folate concentrations compared with the group of men with low serum tHcy and high serum folate (P=0.048 for any strokes and P=0.052 for ischemic strokes). No significant increase in risk was observed in men with elevated serum tHcy when serum folate was above median. The correlation coefficient between serum tHcy and serum folate was -0.15 (P<0.001).

5.4 Hcy as a risk factor for CVD mortality in men with other CVD risk factors (Work IV)

This work is based on the KIHD study 4-year re-examination for 802 men, the average follow-up time was 10.8 years. The mean serum tHcy concentration was 10.8 µmol/L (SD 3.3), median 10.3 µmol/L. The subjects were divided into thirds based on the serum tHcy concentration. The baseline characteristics of the subjects in the thirds of serum tHcy are presented in the work IV. The men in the highest third of serum tHcy (>11.3

μmol/L) were significantly older, had lower serum total cholesterol concentration and higher systolic blood pressure and smoked less than men in the lower thirds.

During an average follow-up time of 10.8 years (~8700 person-years of follow-up) 50 men free of CHD and stroke at baseline experienced a CVD death. The RR for CVD mortality was 1.72 (95% CI 0.98-3.01, P for trend 0.11) in men in the highest tHcy third vs. lower thirds after adjusting for age and examination year in the whole population. After additional adjustments for smoking, serum LDL cholesterol and systolic blood pressure the RR was 1.80 (95% CI 1.02-3.19, P for trend 0.097).

The effect of increased serum tHcy concentration on CVD mortality risk was then studied separately in men with and without other risk factor present (**Table 4**). Increased serum tHcy (>11.3 μmol/L) was associated with an age, examination year and systolic blood pressure adjusted RR for CVD mortality of 2.25 (95% CI 1.10-4.59) in men with increased plasma fibrinogen, 1.93 (95% CI 0.93-3.97) in men with increased serum total cholesterol, 2.21 (95% CI 1.05-4.62) in men with increased serum LDL cholesterol, 1.91 (95% CI 0.94-3.86) in men with increased serum apo-B, and 2.80 (95% CI 1.12-6.99) among smokers. No association between elevated serum tHcy and CVD mortality was observed among men without these risk factors (**Table 4**). The age, examination year and systolic blood pressure adjusted P-value for the interaction between serum tHcy and plasma fibrinogen was 0.163, between serum tHcy and serum total cholesterol 0.619, between serum tHcy and serum LDL cholesterol 0.201, between serum tHcy and serum apo-B 0.759 and serum between tHcy and smoking 0.149.

Table 4. The age, examination year and systolic blood pressure adjusted hazard rate ratios (RR) in the highest serum total homocysteine third (>11.3 μmol/L) compared to the lower thirds in the risk factor groups

	RR (95% CI)	**P for trend**	**Number of cases (% in group)**
Plasma fibrinogen			
≤3.0 g/L	0.87 (0.32-2.34)	0.769	18 (4.3)
>3.0 g/L	2.25 (1.10-4.59)	0.052	32 (8.3)
Serum total cholesterol			
≤5.5 mmol/L	1.49 (0.61-3.65)	0.600	20 (5.0)
>5.5 mmol/L	1.93 (0.93-3.97)	0.139	30 (7.4)
Serum LDL cholesterol			
≤3.9 mmol/L	1.05 (0.43-2.56)	0.737	21 (5.3)
>3.9 mmol/L	2.21 (1.05-4.62)	0.043	29 (7.2)
Serum Apo-B			
≤0.93 g/L	1.47 (0.57-3.75)	0.443	18 (4.4)
>0.93 g/L	1.91 (0.94-3.86)	0.211	32 (8.1)
Smoker			
No	1.35 (0.66-2.76)	0.987	31 (5.3)
Yes	2.80 (1.12-6.99)	0.009	19 (8.8)

In **Table 5** are presented the RRs for subjects with both risk factors (increased serum tHcy and either increased serum total or LDL cholesterol or apo-B, plasma fibrinogen, or smoking), isolated risk factor or neither risk factor. The age and examination year adjusted risk of CVD death was significantly increased in subjects with both increased serum tHcy and either increased plasma fibrinogen, serum total cholesterol, serum apo-B or who were smokers compared to subjects with neither risk factor. Additional

adjustment for systolic blood pressure only slightly attenuated the associations, except in smokers where the risk was slightly increased. **Figure 7** presents the age, examination year and systolic blood pressure adjusted survival curves for tHcy in the whole population (**Fig. 7a**) and for the tHcy-fibrinogen group (**Fig. 7b**).

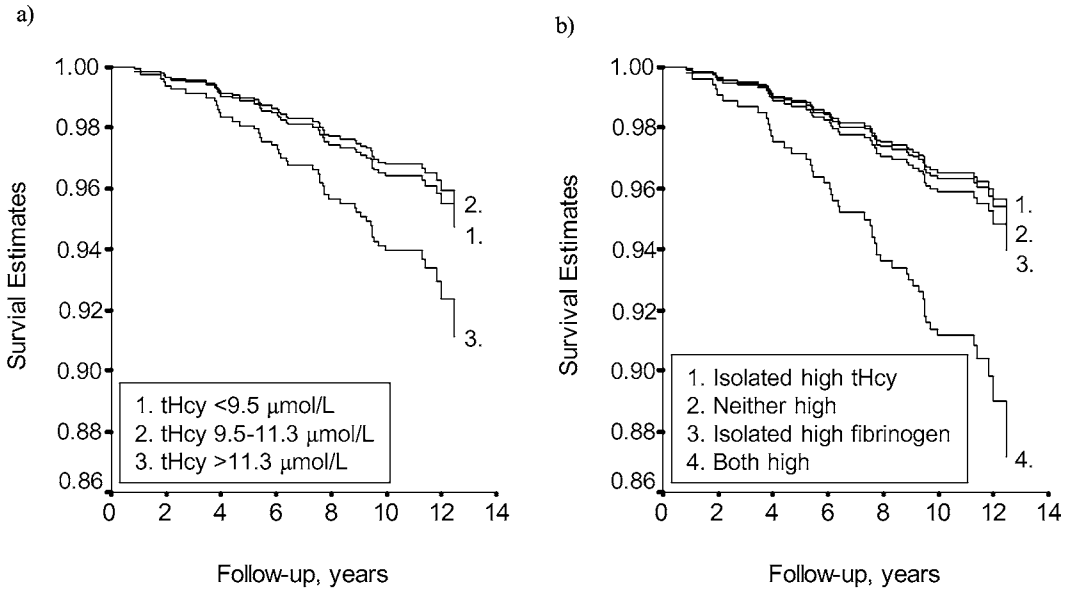

Figure 7. *Age, examination year, smoking, serum LDL cholesterol and systolic blood pressure adjusted survival curves for serum total homocysteine (tHcy) alone (a) and age, examination year and systolic blood pressure adjusted survival curves for tHcy-fibrinogen by group (b).*

Table 5. Hazard rate ratios (RR) for cardiovascular disease mortality models

			Age and examination year adjusted models		Age, examination year and systolic blood pressure adjusted models		Number of cases (% in group)
			RR (95% CI)	**P**	**RR (95% CI)**	**P**	
Group	tHcy, µmol/L	Fib, g/L					
1	≤11.3	≤3.0	1		1		12 (4.3)
2	≤11.3	>3.0	1.20 (0.55-2.62)	0.647	1.13 (0.52-2.47)	0.766	14 (5.4)
3	>11.3	≤3.0	0.92 (0.34-2.47)	0.870	0.95 (0.35-2.53)	0.814	6 (4.3)
4	>11.3	>3.0	2.96 (1.39-6.29)	0.005	2.48 (1.16-5.34)	0.020	18 (13.8)
	tHcy, µmol/L	Chol, mmol/L					
1	≤11.3	≤5.5	1		1		10 (3.9)
2	≤11.3	>5.5	1.51 (0.68-3.32)	0.310	1.52 (0.69-3.34)	0.302	16 (5.7)
3	>11.3	≤5.5	1.58 (0.65-3.81)	0.311	1.45 (0.60-3.51)	0.409	10 (6.9)
4	>11.3	>5.5	2.95 (1.30-6.68)	0.009	2.94 (1.30-6.65)	0.010	11 (11.3)
	tHcy, µmol/L	LDL, mmol/L					
1	≤11.3	≤3.9	1		1		12 (4.6)
2	≤11.3	>3.9	1.08 (0.50-2.34)	0.842	1.01 (0.46-2.19)	0.986	14 (5.1)
3	>11.3	≤3.9	1.27 (0.53-3.02)	0.594	1.10 (0.46-2.65)	0.829	9 (6.5)
4	>11.3	>3.9	2.41 (1.11-5.21)	0.026	2.33 (1.08-5.05)	0.032	15 (11.5)
	tHcy, µmol/L	Apo-B, g/L					
1	≤11.3	≤0.93	1		1		9 (3.4)
2	≤11.3	>0.93	1.98 (0.88-4.44)	0.099	1.77 (0.79-3.98)	0.169	17 (6.4)
3	>11.3	≤0.93	1.71 (0.68-4.33)	0.255	1.53 (0.60-3.89)	0.710	9 (6.3)
4	>11.3	>0.93	3.58 (1.57-8.26)	0.003	3.25 (1.41-7.50)	0.006	15 (11.8)
	tHcy, µmol/L	Smoker					
1	≤11.3	No	1		1		17 (4.5)
2	≤11.3	Yes	1.47 (0.65-3.32)	0.352	1.58 (0.70-3.58)	0.276	9 (5.8)
3	>11.3	No	1.35 (0.66-2.75)	0.412	1.28 (0.63-2.61)	0.499	14 (6.7)
4	>11.3	Yes	4.53 (2.06-9.95)	<0.001	4.69 (2.13-10.33)	<0.001	10 (16.7)

tHcy = serum total homocysteine, Fib = plasma fibrinogen, Chol = serum total cholesterol, LDL = serum low-density lipoprotein, Apo-B = serum Apo-B lipoprotein.

6. DISCUSSION

6.1 Study limitations

There are some limitations in our studies. First, the number of cases was relatively low, especially in the works III and IV, thus reducing the statistical power of the studies. For this reason we could not use multiple covariates in the statistical models and the results may occur due chance. It was also not reasonable to study hemorrhagic strokes separately in the work III. Secondly, the long sample storage time at -20°C could have weakened the reliability of serum determinations. However, it has been shown that long storage time of serum tHcy samples does not apparently lead to alterations in tHcy concentration (110). Also, all serum samples underwent the same freeze-thaw history and were analyzed within a short period of time. The analytical variation in tHcy determinations was also very low. Folate may be unstable in frozen serum or plasma samples. However, in this case our result would more likely be an underestimation than overestimation of the effect of folate on CVD.

In both of the works II and III serum folate was used as a marker of folate status, which may not reflect long-term folate status. Unfortunately, erythrocyte folate measurements, which is considered to be a marker of a long-term folate status (180), were not available. Finally, since folate is found mainly in foodstuffs of plant origin, other nutrients may also contribute to the benefit. However, plasma levels of lycopene or beta-carotene or dietary intakes of these nutrients or vitamin E, also markers of a healthy diet, did not attenuate the beneficial effect of folate in either study. Nonetheless, it is possible that the influence of a healthy lifestyle cannot be fully controlled for in multivariate models.

In the work I dietary methionine and protein intakes were assessed with a four-day food record. The number of days needed for a reliable estimation of protein intake is 5-7 days (181) and for single amino acids probably even longer. It is important that a method measuring dietary intake concerns an extended period of time, since the diet of most subjects is complex and varies over time. However, a sufficient sample size should reveal any important relationship between a dietary factor and disease (182). Although four days may be too short time to estimate the exact long-term methionine intake for each individual, we found a significant correlation between methionine intake assessed by a four-day food record at the KIHD study baseline and at one-year follow-up ($r=0.36$, $P=0.023$).

Unfortunately, the number of tHcy measurements available for the work I was low and the subjects were not randomly selected (a nested case-control design), which makes it difficult to draw any conclusions about relationships between methionine intake and tHcy.

6.2 Dietary methionine intake and the risk of acute coronary events

Although an elevation of blood tHcy occurs in the oral methionine loading test, in which a large dose of methionine is ingested to diagnose hyperhomocysteinemia, chronic moderately high methionine or protein intakes do not seem to increase tHcy (45, 91-95). The same was observed in our KIHD study cohort. It is not clear what causes the lack of association between methionine or protein intake and blood tHcy. One hypothesis is that the enzymes in the methionine cycle (**Figure 2**) adapt to the high methionine intake and thus maintain a normal concentration of Hcy in the circulation (91).

In the work I we found a positive association between dietary methionine intake and the risk of acute coronary events. The data from other prospective cohort studies is very limited. Rimm et al. studied 658 incident cases of nonfatal myocardial infarction and 281 cases of fatal CHD in a prospective Nurses' Health Study cohort of 80082 women (145). In this study population methionine intake was not associated with the risk of CHD.

Although the mechanisms by which methionine could cause its negative effects are still undetermined in humans, animal experiments offer some information. Feeding animals a methionine-rich diet has induced atherosclerosis independently of high plasma tHcy (183, 184). In mice fed a methionine-rich diet, a significant atheromatous pathology was seen even with normal plasma tHcy levels (183). In pigs a methionine-rich diet induced hyperhomocysteinemia and atherosclerosis and although folate supplementation normalized plasma tHcy levels, it did not prevent the methionine-induced arterial lesions (184). It has been suggested that the atherogenic effects are mediated by stimulation of free radical and lipid peroxidation processes (185).

In humans high methionine intake could also be a marker of an unhealthy diet that contains low amounts of fruits and vegetables and moderate to high amounts of animal products accompanied with saturated fat. In our study this is unlikely since, on one hand, adjusting for saturated fat did not change the results and on the other hand, consumption of vegetables, fruits and berries were highest in the highest methionine intake quarter, as were intakes of folate, vitamin E and C, β-carotene and fiber. Furthermore, in addition to the low number of tHcy measurements, the lack of relationship between methionine intake and plasma tHcy could also be explained by the higher intakes of vitamins B_6 and B_{12} in the highest methionine intake quarter.

Interestingly, total protein intake was not associated with increased risk of acute coronary events, but rather tended to modestly decrease the risk. The epidemiological data about protein intake and risk of CVD is limited, but there are studies that support our finding. Hu et al. studied the relation between protein intake and risk of IHD in a prospective Nurses' Health Study cohort study of 80082 women aged 34-59 years and without a previous diagnosis of IHD, stroke, cancer, hypercholesterolemia, or diabetes (186). They found that high protein intakes were associated with a low risk of IHD even after extensive adjustment for known risk factors. When extreme fifths of total protein intake (median intakes 14.7% vs. 24.0% of total energy) were compared, the RR was 0.74 (95% CI 0.59-0.94). Both animal and vegetable proteins contributed to the lower risk. In the same cohort very low levels of animal protein intake have also been associated with increased risk of hemorrhagic stroke (187). Interestingly, in this study

also dietary methionine intake was negatively associated with the risk. Similar results about the association between low animal protein intake and hemorrhagic stroke has also been found in a 14-year prospective study of middle-aged Japanese men and women (188). Furthermore, a significant inverse association between dietary protein and blood pressure in both sexes has been found in a meta-analysis of cross-sectional studies (189).

6.3 Serum folate and CVD

The results from the works II and III are similar to our previous studies where low serum folate concentration or low dietary intake of folate have been associated with higher risk of acute coronary events (149, 190). They are also in line with other previous studies that have studied either serum or dietary folate and risk of stroke (151, 191, 192) or CHD (145, 193, 194), although not all studies have found an association (152, 195, 196). In the Physicians' Health Study the increased risk of MI in men with low plasma folate concentration remained after adjustment for plasma tHcy (150). The same was observed in both our works II and III. As the authors of the Physicians' Health Study, as well as others (14, 115), suggest, folate may be beneficial independently of tHcy concentration.

The strength of our studies is that in the Finnish study populations the range of dietary folate intake and thus serum folate is large, which increases the power to find associations between folate and risk of events. Furthermore, in Finland there are no folate fortification programmes which would also potentially reduce the power.

6.4 Serum homocysteine and CVD

In our studies we found that increased serum tHcy was associated with the risk of stroke and CVD mortality, but not statistically significantly with acute coronary events. This may reflect different mechanisms by which Hcy induces stroke compared to acute coronary events. Also, the smaller diameter of cerebral arterioles may make them more susceptible to oxidative damage. Since strokes are included in the CVD mortality data, this may also explain the results in the work IV. On the other hand, we cannot fully exclude the possibility that tHcy is associated also with acute coronary events. In the recent analyses of the KIHD study population using acute coronary events that occurred during the follow-up period from the baseline examinations in 1991-93 until December 2002 (n=70), the risk in the highest tHcy third compared to the lower thirds after adjustment for age, examination years, serum LDL and HDL cholesterol and triglycerides, systolic blood pressure and smoking was 1.43 (95% CI 0.88-2.34, P=0.146) in 810 men free of previous CHD (unpublished results). A larger number of cases or a longer follow-up might reveal a more significant association.

The association between high blood tHcy concentration and increased risk of stroke has been found in previous meta-analyses of observational studies (8, 10). A very recent meta-analysis presented data about the association between MTHFR genotype and stroke and found that tHcy may be a causal risk factor for stroke (197). Their evidence was based on the so called Mendelian (natural) randomization. Since people are randomly allocated to MTHFR genotype groups with higher (TT) or lower (CC)

tHcy concentrations due to random assortment of alleles with polymorphism during gamete production and fertilization, the groups should not differ systematically in any other way. This randomization should minimize confounding and bias and establish causality (197). In this largest meta-analysis to date of studies about MTHFR and stroke (111 studies), the authors found that people with the TT genotype had a significantly greater mean tHcy (weighted mean difference 1.93 μmol/L, 95% CI 1.38-2.47) and risk of stroke (OR 1.26, 95% CI 1.14-1.40) than people with the CC genotype.

The finding of the work IV that elevated serum tHcy concentration is a risk factor for CVD mortality is in accordance with some previous cohort studies where the subject populations have consisted of both men and women (118, 198, 199). In the Hordaland Homocysteine Study a 5 μmol/L increase in plasma tHcy concentration was associated with a 49% increase in all-cause and 50% increase in CVD mortality after multiple adjustments for CVD risk factors in subjects aged 65 to 72 years, although after excluding the subjects with history of MI, stroke, angina pectoris, diabetes mellitus or ongoing treatment for hypertension, the result was not significant in case of CVD mortality anymore (118). Positive results were found also in 1933 elderly Framingham men and women, who were divided into quarters based on the plasma tHcy concentration (198). Subjects in the highest tHcy quarter had 2.2 times the risk of CVD death compared to the three lower quarters. The RR estimate was attenuated after adjustment for other risk factors, but remained significant. The third study, a recent nested case-control study done in 110 subjects and 154 controls in a French population with a low risk of CVD, revealed a significant 29% increased adjusted risk of CVD mortality in the highest plasma tHcy third compared to the lowest third (199). In this study population 5% of the cases and 6% of the controls had a prevalent CHD. The significantly increased risk in these studies may, at least in part, be explained by the inclusion of patients with prevalent CHD or by having elderly subjects, who may have more silent preclinical CHD compared to younger subjects.

6.5 Serum homocysteine as a risk factor in men with other risk factors

The increased risk found when tHcy is present with another CVD risk factor has also been observed in some other studies. In a study with 482 hypercholesterolemic subjects, the occurrence of an atherosclerotic vascular event was 72% in subjects with high plasma tHcy compared to 44% in those without high tHcy (111). The risk of an event was also almost three times higher in the highest tHcy fifth compared to the lowest fifth. In another study with 27 subjects even mildly increased plasma tHcy levels were of crucial importance for deterioration of endothelial function in subjects with elevated LDL cholesterol concentration (112).

Similar findings have also been reported in case of increased plasma fibrinogen levels. Subjects with increased fibrinogen concentration have been shown to have 2.3 times and subjects with both increased fibrinogen and tHcy concentrations a 3.3 times the risk of CHD mortality, compared to the subjects with neither high fibrinogen nor tHcy concentrations (113). The total number of subjects in this study was 2084. Compared to our study, this study included both men and women. It also included subjects with prevalent CHD, which may have had an effect on the results.

The observation that elevated serum tHcy concentration appeared to increase the risk of CVD mortality in smoking men is similar to a recent case-control study with 750 cases and 800 controls, where smokers were at increased risk of CVD and the risk was greatly increased in the presence of a raised plasma tHcy concentration when compared with non-smokers (49).

The increased risk observed when tHcy is present with high serum cholesterol or plasma fibrinogen can be explained by their roles in the deterioration of endothelial health. Both hyperhomocysteinemia and hypercholesterolemia have been shown to deteriorate endothelial function, in a recent study LDL cholesterol and tHcy levels predicted brachial flow-mediated vasodilatation, internal carotid mean intima-media thickness, and intima-media thickening in patients with hypercholesterolemia (200). Fibrinogen and tHcy, in turn, have complementary roles in the platelet activation-aggregation cascade (105, 201). However, an alternative explanation for the findings, which cannot be forgotten, is that tHcy could be just a marker for the severity of the disease. The increased risk observed when high blood tHcy concentration is present with other CVD risk factors could represent an overall deterioration of vascular health.

7. SUMMARY

The results of this work can be summarized as follows:

1. Increased serum tHcy levels were associated with increased risk of CVD mortality and stroke, but not with acute coronary events.

2. Increased serum folate levels were associated with decreased risk of acute coronary events and stroke.

3. High dietary methionine intake was associated with increased risk of acute coronary events and the association may be independent of plasma tHcy.

8. CONCLUSIONS

The results of this work suggest that high serum tHcy concentration may slightly increase the risk of CVD mortality and stroke in healthy middle-aged men, mortality especially when also other risk factors are present. On the other hand, high serum folate concentration, depicting high intake of plant-derived foods, seems to protect against acute coronary events and stroke. And lastly, high dietary intake of methionine, an important amino acid in the Hcy metabolism, may increase the risk of acute coronary events by other means than by increasing blood tHcy concentration.

However, we cannot rule out the possibility that Hcy is just a marker of the disease and folate a marker of a healthy diet. Increased tHcy concentration may also be a marker of a deficiency of folate. Even the ongoing folate supplementation trials may not be able to distinguish between the effects of decreased tHcy concentration and possible independent effects of folate on cardiovascular health. However, based on these and previous results, folate seems to be beneficial to cardiovascular health, irrespective of its mechanism(s) of action.

9. REFERENCES

1. StatFin - Online Services. http://statfin.stat.fi.

2. Mudd SH, Finkelstein, J.D., Irreverre, F., Laster, L. Homocystinuria: An enzymatic defect. Science 1964;143:1443-1445.

3. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 1969;56:111-128.

4. Wilcken DE, Wilcken, B. The pathogenesis of coronary artery disease. A possible role for methionine metabolism. J Clin Invest 1976;57:1079-1082.

5. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995;274:1049-1057.

6. Christen WG, Ajani UA, Glynn RJ, Hennekens CH. Blood levels of homocysteine and increased risks of cardiovascular disease: causal or casual? Arch Intern Med 2000;160:422-434.

7. Bautista LE, Arenas IA, Penuela A, Martinez LX. Total plasma homocysteine level and risk of cardiovascular disease: A meta-analysis of prospective cohort studies. J Clin Epidemiol 2002;55:882-887.

8. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. JAMA 2002;288:2015-2022.

9. Kelly PJ, Rosand J, Kistler JP, et al. Homocysteine, MTHFR 677C->T polymorphism, and risk of ischemic stroke: Results of a meta-analysis. Neurology 2002;59:529-536.

10. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ 2002;325:1202-1206.

11. Cleophas TJ, Hornstra N, van Hoogstraten B, van der Meulen J. Homocysteine, a risk factor for coronary artery disease or not? a meta-analysis. Am J Cardiol 2000;86:1005-1009.

12. Moat SJ, Lang D, McDowell IFW, et al. Folate, homocysteine, endothelial function and cardiovascular disease. J Nutr Biochem 2004;15:64-79.

13. Homocysteine Lowering Trialists' Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. BMJ 1998;316:894-898.

14. Doshi SN, Moat SJ, McDowell IFW, Lewis MJ, Goodfellow J. Lowering plasma homocysteine with folic acid in cardiovascular disease: what will the trials tell us? Atherosclerosis 2002;165:1-3.

15. Finkelstein JD, Martin JJ. Homocysteine. Int J Biochem Cell Biol 2000;32:385-389.

16. McCully KS. Homocysteine theory of arteriosclerosis: development and current status. In: Gotto AM, Paoletti, R., editor. Atherosclerosis Reviews. New York: Raven Press; 1983. p. 157-246.

17. McCully KS, Wilson, R.B. Homocysteine theory of arteriosclerosis. Atherosclerosis 1975;22:215-227.

18. Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217-246.

19. Stipanuk MH. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. Annu Rev Nutr 2004;24:539-577.

20. Finkelstein JD, Harris BJ, Martin JJ, Kyle WE. Regulation of hepatic betaine-homocysteine methyltransferase by dietary methionine. Biochem Biophys Res Commun 1982;108:344-348.

21. Finkelstein JD, Martin JJ, Harris BJ, Kyle WE. Regulation of hepatic betaine-homocysteine methyltransferase by dietary betaine. J Nutr 1983;113:519-521.

22. Townsend JH, Davis SR, Mackey AD, Gregory JF, 3rd. Folate deprivation reduces homocysteine remethylation in a human intestinal epithelial cell culture model: role of serine in one-carbon donation. Am J Physiol Gastrointest Liver Physiol 2004;286:G588-595.

23. Mudd SH, Finkelstein JD, Irreverre F, Laster L. Transsulfuration in mammals. Microassays and tissue distributions of three enzymes of the pathway. J Biol Chem 1965;240:4382-4392.

24. Quere I, Paul V, Rouillac C, et al. Spatial and temporal expression of the cystathionine beta-synthase gene during early human development. Biochem Biophys Res Commun 1999;254:127-137.

25. Ueland PM. Homocysteine species as components of plasma redox thiol status. Clin Chem 1995;41:340-342.

26. Mansoor MA, Ueland PM, Aarsland A, Svardal AM. Redox status and protein binding of plasma homocysteine and other aminothiols in patients with homocystinuria. Metabolism 1993;42:1481-1485.

27. Hankey GJ, Eikelboom JW. Homocysteine and vascular disease. Lancet 1999;354:407-413.

28. Kang SS, Wong PW, Malinow MR. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. Annu Rev Nutr 1992;12:279-298.

29. Klerk M, Verhoef P, Clarke R, et al. MTHFR 677C-->T polymorphism and risk of coronary heart disease: a meta-analysis. JAMA 2002;288:2023-2031.

30. Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci 2001;22:195-201.

31. Guttormsen AB, Ueland PM, Kruger WD, et al. Disposition of homocysteine in subjects heterozygous for homocystinuria due to cystathionine beta-synthase deficiency: relationship between genotype and phenotype. Am J Med Genet 2001;100:204-213.

32. Tsai MY, Garg U, Key NS, et al. Molecular and biochemical approaches in the identification of heterozygotes for homocystinuria. Atherosclerosis 1996;122:69-77.

33. Mudd SH, Levy, H.L, Skovby, F. Disorders of transsulfuration. New York: McGraw-Hill; 1995.

34. Chadefaux B, Ceballos I, Hamet M, et al. Is absence of atheroma in Down syndrome due to decreased homocysteine levels? Lancet 1988;2:741.

35. Jacques PF, Rosenberg IH, Rogers G, et al. Serum total homocysteine concentrations in adolescent and adult Americans: results from the third National Health and Nutrition Examination Survey. Am J Clin Nutr 1999;69:482-489.

36. Andersson A, Brattstrom L, Israelsson B, et al. Plasma homocysteine before and after methionine loading with regard to age, gender, and menopausal status. Eur J Clin Invest 1992;22:79-87.

37. Nygard O, Vollset SE, Refsum H, et al. Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. JAMA 1995;274:1526-1533.

38. Walker MC, Smith GN, Perkins SL, Keely EJ, Garner PR. Changes in homocysteine levels during normal pregnancy. Am J Obstet Gynecol 1999;180:660-664.

39. Kang SS, Wong PW, Zhou JM, Cook HY. Total homocyst(e)ine in plasma and amniotic fluid of pregnant women. Metabolism 1986;35:889-891.

40. Mijatovic V, van der Mooren MJ. Homocysteine in postmenopausal women and the importance of hormone replacement therapy. Clin Chem Lab Med 2001;39:764-767.

41. Bostom AG, Culleton BF. Hyperhomocysteinemia in chronic renal disease. J Am Soc Nephrol 1999;10:891-900.

42. Wollesen F, Brattstrom L, Refsum H, et al. Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. Kidney Int 1999;55:1028-1035.

43. Dierkes J, Jeckel A, Ambrosch A, et al. Factors explaining the difference of total homocysteine between men and women in the European Investigation Into Cancer and Nutrition Potsdam study. Metabolism 2001;50:640-645.

44. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 1993;270:2693-2698.

45. Jacques PF, Bostom AG, Wilson PW, et al. Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. Am J Clin Nutr 2001;73:613-621.

46. Hustad S, Ueland PM, Vollset SE, et al. Riboflavin as a determinant of plasma total homocysteine: effect modification by the methylenetetrahydrofolate reductase C677T polymorphism. Clin Chem 2000;46:1065-1071.

47. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr 2001;73:1027-1033.

48. Craig SA. Betaine in human nutrition. Am J Clin Nutr 2004;80:539-549.

49. O'Callaghan P, Meleady R, Fitzgerald T, Graham I, European COMAC group. Smoking and plasma homocysteine. Eur Heart J 2002;23:1580-1586.

50. De Bree A, Verschuren WMM, Kromhout D, Kluijtmans LAJ, Blom HJ. Homocysteine determinants and the evidence to what extent homocysteine determines the risk of coronary heart disease. Pharmacol Rev 2002;54:599-618.

51. Nygard O, Refsum H, Ueland PM, et al. Coffee consumption and plasma total homocysteine: The Hordaland Homocysteine Study. Am J Clin Nutr 1997;65:136-143.

52. Grubben MJ, Boers GH, Blom HJ, et al. Unfiltered coffee increases plasma homocysteine concentrations in healthy volunteers: a randomized trial. Am J Clin Nutr 2000;71:480-484.

53. Halsted CH. Lifestyle effects on homocysteine and an alcohol paradox. Am J Clin Nutr 2001;73:501-502.

54. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Lifestyle factors and plasma homocysteine concentrations in a general population sample. Am J Epidemiol 2001;154:150-154.

55. Stabler SP, Marcell PD, Podell ER, et al. Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. J Clin Invest 1988;81:466-474.

56. Lindenbaum J, Rosenberg IH, Wilson PW, Stabler SP, Allen RH. Prevalence of cobalamin deficiency in the Framingham elderly population. Am J Clin Nutr 1994;60:2-11.

57. Ubbink JB, van der Merwe A, Delport R, et al. The effect of a subnormal vitamin B-6 status on homocysteine metabolism. J Clin Invest 1996;98:177-184.

58. Nedrebo BG, Ericsson UB, Nygard O, et al. Plasma total homocysteine levels in hyperthyroid and hypothyroid patients. Metabolism 1998;47:89-93.

59. Diekman MJ, van der Put NM, Blom HJ, Tijssen JG, Wiersinga WM. Determinants of changes in plasma homocysteine in hyperthyroidism and hypothyroidism. Clin Endocrinol (Oxf) 2001;54:197-204.

60. Audelin MC, Genest J, Jr. Homocysteine and cardiovascular disease in diabetes mellitus. Atherosclerosis 2001;159:497-511.

61. Hultberg B, Agardh E, Andersson A, et al. Increased levels of plasma homocysteine are associated with nephropathy, but not severe retinopathy in type 1 diabetes mellitus. Scand J Clin Lab Invest 1991;51:277-282.

62. Refsum H, Wesenberg F, Ueland PM. Plasma homocysteine in children with acute lymphoblastic leukemia: changes during a chemotherapeutic regimen including methotrexate. Cancer Res 1991;51:828-835.

63. Refsum H, Helland S, Ueland PM. Fasting plasma homocysteine as a sensitive parameter of antifolate effect: a study of psoriasis patients receiving low-dose methotrexate treatment. Clin Pharmacol Ther 1989;46:510-520.

64. van Ede AE, Laan RF, Blom HJ, et al. The C677T mutation in the methylenetetrahydrofolate reductase gene: a genetic risk factor for methotrexate-related elevation of liver enzymes in rheumatoid arthritis patients. Arthritis Rheum 2001;44:2525-2530.

65. Tonstad S, Refsum H, Ose L, Ueland P. The C677T mutation in the methylenetetrahydrofolate reductase gene predisposes to hyperhomocysteinemia in children with familial hypercholesterolemia treated with cholestyramine. Journal of Pediatrics, The 1998;132:365-368.

66. Garg R, Malinow M, Pettinger M, Upson B, Hunninghake D. Niacin treatment increases plasma homocyst(e)ine levels. Am Heart J 1999;138:1082-1087.

67. Jankowski P, Kawecka-Jaszcz K. Lipid-lowering drugs and homocysteine. A comparison between statins and other lipid-lowering drugs. Atherosclerosis 2004;172:191-194.

68. James GK, Jones MW, Pudek MR. Homocyst(e)ine levels in patients on phenytoin therapy. Clin Biochem 1997;30:647-649.

69. Giltay EJ, Hoogeveen EK, Elbers JM, et al. Effects of sex steroids on plasma total homocysteine levels: a study in transsexual males and females. J Clin Endocrinol Metab 1998;83:550-553.

70. Arnadottir M, Hultberg B, Wahlberg J, Fellstrom B, Dimeny E. Serum total homocysteine concentration before and after renal transplantation. Kidney Int 1998;54:1380-1384.

71. Morrow LE, Grimsley EW. Long-term diuretic therapy in hypertensive patients: effects on serum homocysteine, vitamin B6, vitamin B12, and red blood cell folate concentrations. South Med J 1999;92:866-870.

72. Rogers JD, Sanchez-Saffon A, Frol AB, Diaz-Arrastia R. Elevated plasma homocysteine levels in patients treated with levodopa: association with vascular disease. Arch Neurol 2003;60:59-64.

73. Smulders YM, de Man AM, Stehouwer CD, Slaats EH. Trimethoprim and fasting plasma homocysteine. Lancet 1998;352:1827-1828.

74. Hankey GJ, Eikelboom JW, Ho WK, van Bockxmeer FM. Clinical usefulness of plasma homocysteine in vascular disease. Med J Aust 2004;181:314-318.

75. De Bree A, Verschuren WM, Kromhout D, Kluijtmans LA, Blom HJ. Homocysteine determinants and the evidence to what extent homocysteine determines the risk of coronary heart disease. Pharmacol Rev 2002;54:599-618.

76. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. Clin Chem 2004;50:3-32.

77. House JD, Jacobs RL, Stead LM, Brosnan ME, Brosnan JT. Regulation of homocysteine metabolism. Adv Enzyme Regul 1999;39:69-91.

78. Brattstrom L, Wilcken DEL, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease : the result of a meta-analysis. Circulation 1998;98:2520-2526.

79. Jakubowski H. Homocysteine thiolactone: metabolic origin and protein homocysteinylation in humans. J Nutr 2000;130:377S-381S.

80. Billecke S, Draganov D, Counsell R, et al. Human serum paraoxonase (pon1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. Drug Metab Dispos 2000;28:1335-1342.

81. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998;64:169-172.

82. Harmon DL, Shields, D.C., Woodside, J.V., McMaster, D., Yarnell, J.W., Young, I.S., Peng, K., Shane, B., Evans, A.E., Whitehead, A.S. Methionine synthase D919G polymorphism is a significant but modest determinant of circulating homocysteine concentrations. Genet Epidemiol 1999;17:298-309.

83. Gaughan DJ, Kluijtmans LAJ, Barbaux S, et al. The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. Atherosclerosis 2001;157:451-456.

84. Namour F, Olivier J-L, Abdelmouttaleb I, et al. Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: relation to transcobalamin and homocysteine concentration in blood. Blood 2001;97:1092-1098.

85. Chango A, Emery-Fillon N, de Courcy GP, et al. A polymorphism (80G->A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. Mol Genet and Metab 2000;70:310-315.

86. Kealey C, Brown KS, Woodside JV, et al. A common insertion/deletion polymorphism of the thymidylate synthase (TYMS) gene is a determinant of red blood cell folate and homocysteine concentrations. Hum Genet 2005.

87. Clarke R, Armitage J. Vitamin supplements and cardiovascular risk: review of the randomized trials of homocysteine-lowering vitamin supplements. Semin Thromb Hemost 2000;26:341-348.

88. Rasmussen LB, Ovesen L, Bulow I, et al. Folate intake, lifestyle factors, and homocysteine concentrations in younger and older women. Am J Clin Nutr 2000;72:1156-1163.

89. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr 2001;73:1027-1033.

90. Wald DS, Bishop L, Wald NJ, et al. Randomized trial of folic acid supplementation and serum homocysteine levels. Arch Intern Med 2001;161:695-700.

91. Haulrik N, Toubro S, Dyerberg J, et al. Effect of protein and methionine intakes on plasma homocysteine concentrations: a 6-mo randomized controlled trial in overweight subjects. Am J Clin Nutr 2002;76:1202-1206.

92. Mennen LI, de Courcy GP, Guilland JC, et al. Homocysteine, cardiovascular disease risk factors, and habitual diet in the French Supplementation with Antioxidant Vitamins and Minerals Study. Am J Clin Nutr 2002;76:1279-1289.

93. Stolzenberg-Solomon RZ, Miller ER, 3rd, Maguire MG, Selhub J, Appel LJ. Association of dietary protein intake and coffee consumption with serum homocysteine concentrations in an older population. Am J Clin Nutr 1999;69:467-475.

94. Shimakawa T, Nieto FJ, Malinow MR, et al. Vitamin intake: a possible determinant of plasma homocyst(e)ine among middle-aged adults. Ann Epidemiol 1997;7:285-293.

95. Ward M, McNulty H, Pentieva K, et al. Fluctuations in dietary methionine intake do not alter plasma homocysteine concentration in healthy men. J Nutr 2000;130:2653-2657.

96. Olthof MR, van Vliet T, Boelsma E, Verhoef P. Low dose betaine supplementation leads to immediate and long term lowering of plasma homocysteine in healthy men and women. J Nutr 2003;133:4135-4138.

97. Brouwer IA, Verhoef P, Urgert R. Betaine supplementation and plasma homocysteine in healthy volunteers. Arch Intern Med 2000;160:2546-a-2547.

98. Schwab U, Torronen A, Toppinen L, et al. Betaine supplementation decreases plasma homocysteine concentrations but does not affect body weight, body composition, or resting energy expenditure in human subjects. Am J Clin Nutr 2002;76:961-967.

99. Steenge GR, Verhoef P, Katan MB. Betaine supplementation lowers plasma homocysteine in healthy men and women. J Nutr 2003;133:1291-1295.

100. De Bree A, Verschuren WM, Blom HJ, Kromhout D. Alcohol consumption and plasma homocysteine: what's brewing? Int J Epidemiol 2001;30:626-627.

101. van Guldener C, Stehouwer, C.D. Homocysteine metabolism in renal disease. Clin Chem Lab Med 2003;41:1412-1417.

102. Brenton DP, Cusworth DC, Gaull GE. Homocystinuria: metabolic studies on 3 patients. J Pediatr 1965;67:58-68.

103. Bostom AG, Jacques PF, Nadeau MR, et al. Post-methionine load hyperhomocysteinemia in persons with normal fasting total plasma homocysteine: initial results from the NHLBI Family Heart Study. Atherosclerosis 1995;116:147-151.

104. Refsum H, Ueland PM, Nygard O, Vollset SE. Homocysteine and cardiovascular disease. Annu Rev Med 1998;49:31-62.

105. Splaver A, Lamas GA, Hennekens CH. Homocysteine and cardiovascular disease: biological mechanisms, observational epidemiology, and the need for randomized trials. Am Heart J 2004;148:34-40.

106. Mudd SH, Skovby F, Levy HL, et al. The natural history of homocystinuria due to cystathionine beta-synthase deficiency. Am J Hum Genet 1985;37:1-31.

107. Rosenblatt DS. Inherited disorders of folate transport and metabolism. New York: McGraw_hill; 1989.

108. Brattstrom L, Wilcken DE. Homocysteine and cardiovascular disease: cause or effect? Am J Clin Nutr 2000;72:315-323.

109. Stampfer MJ, Malinow MR, Willett WC, et al. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. JAMA 1992;268:877-881.

110. Alfthan G, Pekkanen J, Jauhiainen M, et al. Relation of serum homocysteine and lipoprotein(a) concentrations to atherosclerotic disease in a prospective Finnish population based study. Atherosclerosis 1994;106:9-19.

111. Glueck CJ, Shaw P, Lang JE, et al. Evidence that homocysteine is an independent risk factor for atherosclerosis in hyperlipidemic patients. Am J Cardiol 1995;75:132-136.

112. Schlaich MP, John S, Jacobi J, Lackner KJ, Schmieder RE. Mildly elevated homocysteine concentrations impair endothelium dependent vasodilation in hypercholesterolemic patients. Atherosclerosis 2000;153:383-389.

113. Acevedo M, Pearce GL, Kottke-Marchant K, Sprecher DL. Elevated fibrinogen and homocysteine levels enhance the risk of mortality in patients from a high-risk preventive cardiology clinic. Arterioscler Thromb Vasc Biol 2002;22:1042-1045.

114. Nygard O, Refsum H, Ueland PM, Vollset SE. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr 1998;67:263-270.

115. Verhaar MC, Stroes E, Rabelink TJ. Folates and cardiovascular disease. Arterioscler Thromb Vasc Biol 2002;22:6-13.

116. Evans RW, Shaten BJ, Hempel JD, Cutler JA, Kuller LH. Homocyst(e)ine and risk of cardiovascular disease in the Multiple Risk Factor Intervention Trial. Arterioscler Thromb Vasc Biol 1997;17:1947-1953.

117. Stehouwer CD, Weijenberg MP, van den Berg M, et al. Serum homocysteine and risk of coronary heart disease and cerebrovascular disease in elderly men: a 10-year follow-up. Arterioscler Thromb Vasc Biol 1998;18:1895-1901.

118. Vollset SE, Refsum H, Tverdal A, et al. Plasma total homocysteine and cardiovascular and noncardiovascular mortality: the Hordaland Homocysteine Study. Am J Clin Nutr 2001;74:130-136.

119. Bostom AG, Selhub J, Jacques PF, Rosenberg IH. Power Shortage: clinical trials testing the "homocysteine hypothesis" against a background of folic acid-fortified cereal grain flour. Ann Intern Med 2001;135:133-137.

120. Ray JG, Laskin, C.A. Folic acid and homocyst(e)ine metabolic defects and the risk of placental abruption, pre-eclampsia and spontaneous pregnancy loss: A systematic review. Placenta 1999;20:519-529.

121. Scholl TO, Johnson WG. Folic acid: influence on the outcome of pregnancy. Am J Clin Nutr 2000;71:1295S-1303.

122. Shemin D, Lapane KL, Bausserman L, et al. Plasma total homocysteine and hemodialysis access thrombosis: a prospective study. J Am Soc Nephrol 1999;10:1095-1099.

123. Nilsson K, Gustafson L, Faldt R, et al. Hyperhomocysteinaemia--a common finding in a psychogeriatric population. Eur J Clin Invest 1996;26:853-859.

124. Levine J, Stahl Z, Sela BA, et al. Elevated homocysteine levels in young male patients with schizophrenia. Am J Psychiatry 2002;159:1790-1792.

125. Seshadri S, Beiser A, Selhub J, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;346:476-483.

126. Tolmunen T, Hintikka J, Voutilainen S, et al. Association between depressive symptoms and serum concentrations of homocysteine in men: a population study. Am J Clin Nutr 2004;80:1574-1578.

127. Cook JW, Taylor J, Lloyd M., Orloff SL, et al. Homocysteine and arterial disease: experimental mechanisms. Vasc Pharmacol 2002;38:293-300.

128. Tawakol A, Omland T, Gerhard M, Wu JT, Creager MA. Hyperhomocyst(e)inemia is associated with iImpaired endothelium-dependent vasodilation in humans. Circulation 1997;95:1119-1121.

129. Lang D, Kredan MB, Moat SJ, et al. Homocysteine-induced inhibition of endothelium-dependent relaxation in rabbit aorta: role for superoxide anions. Arterioscler Thromb Vasc Biol 2000;20:422-427.

130. Chambers JC, Ueland PM, Obeid OA, et al. Improved vascular endothelial function after oral B vitamins : an effect mediated through reduced concentrations of free plasma homocysteine. Circulation 2000;102:2479-2483.

131. Chambers JC, McGregor A, Jean-Marie J, Obeid OA, Kooner JS. Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: An effect reversible with vitamin C therapy. Circulation 1999;99:1156-1160.

132. Gryglewski RJ, Palmer RMJ, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 1986;320:454-456.

133. Stuhlinger MC, Tsao PS, Her J-H, et al. Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. Circulation 2001;104:2569-2575.

134. Upchurch G.R. J, Welch GN, Fabian AJ, et al. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 1997;272:17012-17017.

135. Yamamoto M, Hara H, Adachi T. Effects of homocysteine on the binding of extracellular-superoxide dismutase to the endothelial cell surface. FEBS Letters 2000;486:159-162.

136. Voutilainen S, Morrow JD, Roberts LJ, II, et al. Enhanced in vivo lipid peroxidation at elevated plasma total homocysteine levels. Arterioscler Thromb Vasc Biol 1999;19:1263-1266.

137. Undas A, Domagala TB, Jankowski M, Szczeklik A. Treatment of hyperhomocysteinemia with folic acid and vitamins B12 and B6 attenuates thrombin generation. Thromb Res 1999;95:281-288.

138. Jakubowski H. Metabolism of homocysteine thiolactone in human cell cultures. Possible mechanism for pathological consequences of elevated homocysteine levels. J Biol Chem 1997;272:1935-1942.

139. Jakubowski H. Calcium-dependent human serum homocysteine thiolactone hydrolase. A protective mechanism against protein N-homocysteinylation. J Biol Chem 2000;275:3957-3962.

140. Jakubowski H, Ambrosius WT, Pratt JH. Genetic determinants of homocysteine thiolactonase activity in humans: implications for atherosclerosis. FEBS Letters 2001;491:35-39.

141. Ferretti G, Bacchetti T, Marotti E, Curatola G. Effect of homocysteinylation on human high-density lipoproteins: a correlation with paraoxonase activity. Metabolism 2003;52:146-151.

142. Männistö S, Ovaskainen, M-L., Valsta, L. eds. Finravinto 2002 -tutkimus. The National FINDIET 2002 Study. Kansanterveyslaitoksen julkaisuja B3/2003. Helsinki: Kansanterveyslaitos, Ravitsemusyksikkö. 2003.

143. Choumenkovitch SF, Selhub J, Wilson PWF, et al. Folic Acid Intake from Fortification in United States Exceeds Predictions J Nutr 2002;132:2792-2798.

144. Suh JR, Herbig AK, Stover PJ. New perspectives on folate catabolism. Annu Rev Nutr 2001;21:255-282.

145. Rimm EB, Willett WC, Hu FB, et al. Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women. JAMA 1998;279:359-364.

146. Quinlivan EP, Gregory JF, III. Effect of food fortification on folic acid intake in the United States. Am J Clin Nutr 2003;77:221-225.

147. Stover PJ. Physiology of folate and vitamin B12 in health and disease. Nutr Rev 2004;62:S3-12; discussion S13.

148. Gregory JF, III. Case study: folate bioavailability. J Nutr 2001;131:1376S-1382.

149. Voutilainen S, Rissanen TH, Virtanen J, Lakka TA, Salonen JT. Low dietary folate intake is associated with an excess incidence of acute coronary events : The Kuopio Ischemic Heart Disease Risk Factor Study. Circulation 2001;103:2674-2680.

150. Chasan-Taber L, Selhub J, Rosenberg IH, et al. A prospective study of folate and vitamin B6 and risk of myocardial infarction in US physicians. J Am Coll Nutr 1996;15:136-143.

151. He K, Merchant A, Rimm EB, et al. Folate, vitamin B6, and B12 intakes in relation to risk of stroke among men. Stroke 2004;35:169-174.

152. Folsom AR, Nieto FJ, McGovern PG, et al. Prospective study of coronary heart disease incidence in relation to fasting total homocysteine, related genetic polymorphisms, and B vitamins: the Atherosclerosis Risk in Communities (ARIC) study. Circulation 1998;98:204-210.

153. van der Put NMJ, Blom HJ. Neural tube defects and a disturbed folate dependent homocysteine metabolism. Eur J Obstet Gyn R B 2000;92:57-61.

154. Choi S-W, Mason JB. Folate and carcinogenesis: an integrated scheme1-3. J Nutr 2000;130:129-132.

155. Kim Y-I. Will mandatory folic acid fortification prevent or promote cancer? Am J Clin Nutr 2004;80:1123-1128.

156. Ritvanen A. Neural tube defects and folic acid. In Finnish. Duodecim 1996;112:975-982.

157. Verhaar MC, Wever RMF, Kastelein JJP, et al. 5-Methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. Circulation 1998;97:237-241.

158. Joshi R, Adhikari S, Patro BS, Chattopadhyay S, Mukherjee T. Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. Free Radical Bio Med 2001;30:1390-1399.

159. Stroes ESG, van Faassen EE, Yo M, et al. Folic acid reverts dysfunction of endothelial nitric oxide synthase. Circ Res 2000;86:1129-1134.

160. Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, Kalyanaraman B. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. Biochem J 2002;362:733-739.

161. Verhaar MC, Wever RMF, Kastelein JJP, et al. Effects of oral folic acid supplementation on endothelial function in familial hypercholesterolemia : a randomized placebo-controlled trial. Circulation 1999;100:335-338.

162. Mackness MI, Durrington, P.N., Mackness, B. How high-density lipoprotein protects against the effects of lipid peroxidation. Curr Opin Lipidol 2000;11:383-388.

163. Navab M, Hama-Levy S, Lenten BJV, et al. Mildly oxidized LDL induces an increased apolipoprotein J/paraoxonase ratio. J Clin Invest 1997;99:2005-2019.

164. Salonen JT. Is there a continuing need for longitudinal epidemiologic research? The Kuopio Ischaemic Heart Disease Risk Factor Study. Ann Clin Res 1988;20:46-50.

165. Tuomilehto J, Arstila, M., Kaarsalo, E., Kankaanpaa, J., Ketonen, M., Kuulasmaa, K., Lehto, S., Miettinen, H., Mustaniemi, H., Palomaki, P., et al. Acute myocardial infarction (AMI) in Finland--baseline data from the FINMONICA AMI register in 1983-1985. Eur Heart J 1992;13:577.

166. Tuomilehto J, Rastenyte D, Sivenius J, et al. Ten-year trends in stroke incidence and mortality in the FINMONICA stroke study. Stroke 1996;27:825-832.

167. Moller J, Rasmussen, K. Homocysteine in plasma: stabilization of blood samples with fluoride. Clin Chem 1995;41:758-759.

168. Rasmussen K. Solid-phase sample extraction for rapid determination of methylmalonic acid in serum and urine by a stable-isotope-dilution method. Clin Chem 1989;35:260-264.

169. Ubbink JB, Hayward Vermaak, W.J., Bissbort, S. Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr 1991;565:441-446.

170. Salo-Väänänen P. Determination of protein content in foods by the amount of total nitrogen and amino acids (Elintarvikkeiden proteiinipitoisuuden määrittäminen niiden typpi- ja aminohappopitoisuuksien avulla). In Finnish: University of Helsinki, Department of applied chemistry and microbiology. EKT-series 1050; 1996.

171. Willett WC, Stampfer, M. Implications of total energy intake for epidemiological analyses. In: Willett, W: Nutritional epidemiology. New York, NY: Oxford University Press; 1998. p. 273-301.

172. Salonen JT, Salonen R, Seppanen K, Rauramaa R, Tuomilehto J. HDL, HDL2, and HDL3 subfractions, and the risk of acute myocardial infarction. A prospective population study in eastern Finnish men. Circulation 1991;84:129-139.

173. Salonen JT, Seppanen K, Nyyssonen K, et al. Intake of mercury from fish, lipid peroxidation, and the risk of myocardial infarction and coronary, cardiovascular, and any death in eastern Finnish men. Circulation 1995;91:645-655.

174. Puhakainen EV, Barlow RD, Salonen JT. An automated colorimetric assay for urine nicotine metabolites: a suitable alternative to cotinine assays for the assessment of smoking status. Clin Chim Acta 1987;170:255-262.

175. Porkkala-Sarataho E, Nyyssonen M, Kaikkonen J, et al. A randomized, single-blind, placebo-controlled trial of the effects of 200 mg alpha-tocopherol on the oxidation resistance of atherogenic lipoproteins. Am J Clin Nutr 1998;68:1034-1041.

176. Salonen JT, Nyyssonen K, Tuomainen T-P, et al. Increased risk of non-insulin dependent diabetes mellitus at low plasma vitamin E concentrations: a four year follow up study in men. BMJ 1995;311:1124-1127.

177. Salonen JT, Nyyssonen K, Korpela H, et al. High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. Circulation 1992;86:803-811.

178. Lakka TA, Venalainen JM, Rauramaa R, et al. Relation of leisure-time physical activity and cardiorespiratory fitness to the risk of acute myocardial infarction in men. N Engl J Med 1994;330:1549-1554.

179. Greenland S. Modelling and variable selection in epidemiologic analysis. Am J Public Health 1989;79:340-349.

180. Wu A, Chanarin I, Slavin G, Levi AJ. Folate deficiency in the alcoholic--its relationship to clinical and haematological abnormalities, liver disease and folate stores. Br J Haematol 1975;29:469-478.

181. Karkeck JM. Improving the use of dietary survey methodology. J Am Diet Assoc 1987;87:869-871.

182. Willett W. Nutritional epidemiology: issues and challenges. Int J Epidemiol 1987;16:312-317.

183. Troen AM, Lutgens E, Smith DE, Rosenberg IH, Selhub J. The atherogenic effect of excess methionine intake. PNAS 2003;100:15089-15094.

184. Ambrosi P, Rolland PH, Bodard H, et al. Effects of folate supplementation in hyperhomocysteinemic pigs. J Am Coll Cardiol 1999;34:274-279.

185. Toborek M, Kopieczna-Grzebieniak E, Drozdz M, Wieczorek M. Increased lipid peroxidation as a mechanism of methionine-induced atherosclerosis in rabbits. Atherosclerosis 1995;115:217-224.

186. Hu FB, Stampfer MJ, Manson JE, et al. Dietary protein and risk of ischemic heart disease in women. Am J Clin Nutr 1999;70:221-227.

187. Iso H, Stampfer MJ, Manson JE, et al. Prospective study of fat and protein intake and risk of intraparenchymal hemorrhage in women. Circulation 2001;103:856-863.

188. Iso H, Sato S, Kitamura A, et al. Fat and protein intakes and risk of intraparenchymal hemorrhage among middle-aged Japanese. Am J Epidemiol 2003;157:32-39.

189. Liu L, Ikeda K, Sullivan DH, Ling W, Yamori Y. Epidemiological evidence of the association between dietary protein intake and blood pressure: a meta-analysis of published data. Hypertens Res 2002;25:689-695.

190. Voutilainen S, Lakka TA, Porkkala-Sarataho E, et al. Low serum folate concentrations are associated with an excess incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. Eur J Clin Nutr 2000;54:424-428.

191. Bazzano LA, He J, Ogden LG, et al. Dietary intake of folate and risk of stroke in US men and women: NHANES I Epidemiologic Follow-Up Study. National Health and Nutrition Examination Survey. Stroke 2002;33:1183-1189.

192. Giles WH, Kittner SJ, Anda RF, Croft JB, Casper ML. Serum folate and risk for ischemic stroke: First National Health and Nutrition Examination Survey epidemiologic follow-up study. Stroke 1995;26:1166-1170.

193. Morrison HI, Schaubel D, Desmeules M, Wigle DT. Serum folate and risk of fatal coronary heart disease. JAMA 1996;275:1893-1896.

194. de Bree A, Verschuren WMM, Blom HJ, et al. Coronary heart disease mortality, plasma homocysteine, and B-vitamins: a prospective study. Atherosclerosis 2003;166:369-377.

195. Giles WH, Kittner SJ, Croft JB, et al. Serum folate and risk for coronary heart disease: results from a cohort of US adults. Ann Epidemiol 1998;8:490-496.

196. Hung J, Beilby JP, Knuiman MW, Divitini M. Folate and vitamin B-12 and risk of fatal cardiovascular disease: cohort study from Busselton, Western Australia. BMJ 2003;326:131-.

197. Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: evidence on a causal link from mendelian randomisation. The Lancet 2005;365:224-232.

198. Bostom AG, Silbershatz H, Rosenberg IH, et al. Nonfasting plasma total homocysteine levels and all-cause and cardiovascular disease mortality in elderly Framingham men and women. Arch Intern Med 1999;159:1077-1080.

199. Blacher J, Benetos A, Kirzin JM, et al. Relation of plasma total homocysteine to cardiovascular mortality in a French population. Am J Cardiol 2002;90:591-595.

200. Vaudo G, Marchesi S, Siepi D, et al. Relevance of homocysteine on brachial flow-mediated vasodilatation and carotid and femoral intima-media thickness in patients with hypercholesterolemia. Am J Cardiol 2004;93:1413-1416.

201. Mannucci PM. Recent progress in the pathophysiology of fibrinogen. Eur Heart J 1995;16 Suppl A:25-30.

ORIGINAL PUBLICATIONS

I Virtanen JK, Voutilainen S, Rissanen TH, Happonen P, Mursu J, Laukkanen JA, Poulsen H, Lakka TA, Salonen JT. High dietary methionine intake increases the risk of acute coronary events in middle-aged men. Nutrition, Metabolism & Cardiovascular Diseases 2005. In press.

II Voutilainen S, Virtanen JK, Rissanen TH, Alfthan G, Laukkanen J, Nyyssönen K, Mursu J, Valkonen V-P, Tuomainen T-P, Kaplan GA, Salonen JT. Serum folate and homocysteine and incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. American Journal of Clinical Nutrition 2004;80:317-23.

III Virtanen JK, Voutilainen S, Happonen P, Alfthan G, Kaikkonen J, Mursu J, Rissanen TH, Kaplan GA, Korhonen MJ, Sivenius J, Salonen JT. Serum homocysteine, folate and risk of stroke: Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. European Journal of Cardiovascular Prevention and Rehabilitation 2005. In press.

IV Virtanen JK, Voutilainen S, Alfthan G, Korhonen MJ, Rissanen TH, Mursu J, Kaplan GA, Salonen JT. Homocysteine as a risk factor for CVD mortality in men with other CVD risk factors: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. Journal of Internal Medicine 2005; 257:255-262.

Kuopio University Publications D. Medical Sciences

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2004. 97 p. Acad. Diss.

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