Homocysteine and coronary heart disease

the role of polymorphic genes and hemostasis

Mariska Klerk

Promotoren
Prof. dr. ir. F.J. Kok
Hoogleraar Voeding en Gezondheid
Wageningen Universiteit

Prof. dr. E.G. Schouten
Hoogleraar in de Epidemiologie van de Voeding
Katholieke Universiteit van Leuven / Wageningen Universiteit

Co-promotor
Dr. ir. P. Verhoef
Projectleider
Wageningen Centre for Food Sciences / Wageningen Universiteit

Promotiecommissie
Dr. R. Clarke
Oxford University, Oxford, Verenigd Koninkrijk

Dr. J.C.M. Witteman
Erasmus Universiteit, Rotterdam

Prof. dr. C. Kluft
Gaubius Laboratorium, TNO Preventie en Gezondheid, Leiden

Prof. dr. M.B. Katan
Wageningen Centre for Food Sciences / Wageningen Universiteit

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Mariska Klerk

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Abstract

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Background Homocysteine is a sulfur-containing amino acid formed during catabolism of the essential amino acid methionine. Defects in genes encoding enzymes or sub-optimal intake of B-vitamins (e.g. folate) involved in homocysteine metabolism can lead to cellular accumulation of homocysteine resulting in elevated plasma levels. Numerous epidemiological studies have shown a positive association between elevated homocysteine levels and CVD risk. However, whether elevated homocysteine is a causal risk factor of CVD is still under debate. In addition, the pathogenic mechanism through which homocysteine would increase CVD risk is still unclear, but one of the proposed mechanisms is that homocysteine would impair hemostasis.

Methods The epidemiological studies described in this thesis explored the evidence with respect to a causal role of homocysteine in the occurrence of coronary heart disease (CHD) by investigating the association of polymorphic genes with risk of CHD. Two placebo-controlled intervention studies on homocysteine lowering in humans investigated the role of hemostasis as a potential mechanism.

Results In a meta-analysis we showed that homozygosity for the 677C>T mutation in the gene encoding methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in homocysteine remethylation, was associated with a higher risk of CHD, however only when folate status was low. This might explain why the mutation was associated with increased CHD risk in Europe, but not in North America, where folate intake is higher because of widespread use of vitamin supplements and folate fortification. In a case-control study, we observed an increased risk of CHD for individuals who were homozygous for the 2756A>G mutation in the gene encoding methionine synthase (MS), another enzyme involved in homocysteine remethylation. However, this could not be confirmed by the majority of other published case-control studies. In two trials, B-vitamin or folic acid supplementation had no clear beneficial effect on markers of hemostasis, despite a marked reduction in homocysteine concentrations was observed.

Conclusions The result of our meta-analysis on the MTHFR 677C>T polymorphism and CHD supports the hypothesis that impaired folate metabolism, leading to high homocysteine concentrations, is causally related to CHD risk. The meta-analysis could not reveal whether it is elevated homocysteine, low folate or impaired methylation that is the true cause of CHD. However, our results suggest that increasing population mean levels of folate would reduce the incidence of CHD. This could be achieved by improving the bioavailability of folate from foods and additional food fortification with folic acid. Provided the folate status is adequate, there is little additional value of screening for MTHFR 677C>T genotype. Homocysteine-lowering through B-vitamin supplementation does not seem to affect hemostasis effect in healthy volunteers. More research is needed to unravel the pathogenic mechanism.

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1

Introduction

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in the Netherlands and other Western countries. In the Netherlands, it accounted for 36% of total mortality in 1999.[1] Well-known risk factors for CVD, such as age, sex, body mass index, smoking, hypertension, hypercholesterolemia and diabetes only partly explain the occurrence of CVD. Therefore many other potential risk factors, including homocysteine, have been investigated.

In 1964, it was reported that people with a severe defect in homocysteine metabolism, leading to high concentrations of homocysteine in blood and urine, frequently experienced thromboembolic events at an early age.[2] In 1975, McCully and Wilson posed their theory on homocysteine causing arteriosclerosis.[3] Numerous epidemiological studies have subsequently confirmed the positive association between homocysteine and CVD risk. However, whether moderately elevated homocysteine concentrations are a cause of CVD, a consequence, or just a marker of some different underlying factor leading to CVD is still under debate. Studying the association between a genetic polymorphism, known to elevate homocysteine, and risk of CVD would help to elucidate whether homocysteine is causally involved in the occurrence of CVD. Furthermore, the pathogenic mechanism through which homocysteine would increase CVD risk is still unclear. One of the proposed mechanisms is that homocysteine causes endothelial damage and consequently lead to impaired hemostasis. In this thesis we investigated associations between genetic polymorphisms of homocysteine metabolism and risk of CVD on one hand, and effects of homocysteine lowering on hemostasis on the other hand.

Homocysteine metabolism

Homocysteine is a sulfur-containing amino acid formed during catabolism of the essential amino acid methionine as a product of numerous transmethylation reactions.[4] The intracellular homocysteine concentration is tightly regulated and any excess is transported to the plasma. Homocysteine can be metabolized by two major pathways. When methionine is in excess, homocysteine is directed to the transsulfuration pathway, where it is irreversibly converted to cysteine by cystathionine ß-synthase (CBS) which requires vitamin B_6 as a co-factor. This pathway is limited mainly to liver and kidneys. Under conditions of negative methionine balance, homocysteine is primarily metabolized through the methionine-conserving remethylation pathway. In the liver, a substantial portion of homocysteine is remethylated by betaine-homocysteine methyltransferase (BHMT), which uses betaine as a methyl donor. In most tissues, however, the remethylation of homocysteine is catalyzed by methionine synthase (MS), which uses vitamin B_{12} as a co-factor and 5-methyltetrahydrofolate (5-MTHF) as a methyl donor. The formation of 5-MTHF is catalyzed by methylenetetrahydrofolate reductase (MTHFR), which uses vitamin B_2 as a co-factor. An overview of the homocysteine metabolism is presented in figure 1.1.

In plasma about 70% of homocysteine is bound to albumin; about 25% is oxidized and occurs as the disulfide homocystine, and the remainder (< 5%) oxidizes with other thiols, including cysteine, to form disulfides or circulates as reduced 'free' homocysteine. In this thesis the term homocysteine refers to the total pool of

circulating homocysteine in plasma,[5] which is usually measured in epidemiological studies.

Defects in genes coding for enzymes involved in homocysteine metabolism or sub-optimal intake of the B-vitamins can lead to cellular accumulation of homocysteine and consequently elevated plasma levels. Disturbances in the remethylation pathway are merely reflected by elevated fasting plasma homocysteine concentrations. No clear cut-off points have been established yet, but fasting plasma concentrations of homocysteine < 15 μmol/L are generally regarded as normal. Plasma concentrations > 15-30 μmol/L are regarded as mildly elevated, concentrations between 30-100 μmol/L are regarded as intermediate hyperhomocysteinemia, and levels > 100 μmol/L are regarded as severe hyperhomocysteinemia.[6] Recently, the Netherlands Heart Foundation proposed to take 12 μmol/L as target concentration for treatment of hyperhomocysteinemia.[7] Disturbances in the transsulfuration pathway can be detected using a methionine loading test. Individuals receive a methionine load, which results in a peak in homocysteine concentrations after 4 to 8 hours. Post-load homocysteine concentrations of ≥ 55 μmol/L or an increase in homocysteine of > 30 μmol/L is regarded as abnormal.[8]

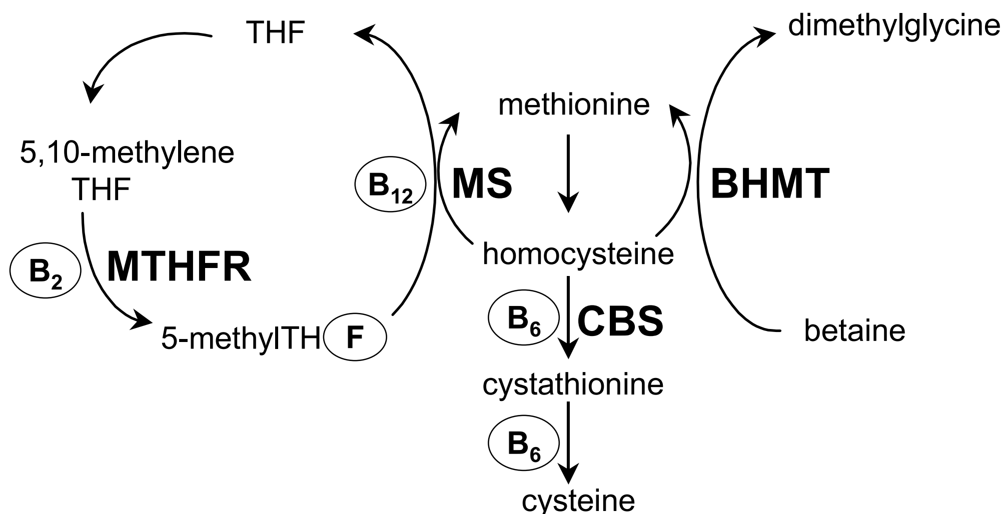

Figure 1.1 Homocysteine metabolism. Enzymes are indicated in bold capitals. Vitamin co-factors or co-substrates are indicated by ellipses. MS = methionine synthase; MTHFR = methylene tetrahydrofolate reductase; BHMT = betaine-homocysteine methyl transferase; CBS = cystathionine ß-synthase; THF = tetrahydrofolate; F = folate; B_2 = vitamin B_2 (riboflavin); B_{12} = vitamin B_{12} (methylcobalamin); B_6 = vitamin B_6 (pyridoxal 5'-phosphate).

Determinants of homocysteine concentrations

Important determinants of homocysteine are age, sex and renal function. Men have higher homocysteine concentrations than women, and homocysteine concentrations increase with age. After menopause, homocysteine concentrations in women increase and soon reach levels of men of similar age. Decreased renal function raises homocysteine concentration, because homocysteine clearance takes place via renal metabolism.[9]

Intervention trials have shown that coffee[10-12] and alcohol consumption increase homocysteine concentrations.[13] Cross-sectional studies have shown positive correlations of homocysteine with smoking, and with biological factors, like body mass index, serum total cholesterol or blood pressure, but whether these factors are really determinants of homocysteine remains to be established.[14]

B-vitamins

Other important determinants are B-vitamins required in homocysteine metabolism. Dietary intake and status of especially folate, but also the vitamins B_2, B_6 and B_{12} are inversely associated with plasma homocysteine levels.[15-21]

B-vitamin supplementation can effectively lower fasting plasma homocysteine levels. A meta-analysis of homocysteine-lowering trials and several dose-finding studies have shown that folic acid in dosages of 0.4 to 5 mg can reduce fasting homocysteine levels by 25%, even when plasma levels of B-vitamins are normal.[22-24] Vitamin B_{12} supplementation results in an additional reduction of 7%, whereas vitamin B_6 does not seem to have an additional effect.[22] However, vitamin B_6 might lower homocysteine after a methionine load.[25]

Inborn errors and polymorphisms in homocysteine metabolism

Several inborn errors may lead to accumulation of homocysteine in the cell, and consequently in the circulation. The most common genetic disorder in homocysteine metabolism is homozygous CBS deficiency. Subjects with this defect have elevated homocysteine concentrations in plasma (severe hyperhomocysteinemia) and excrete large amounts with the urine (homocystinuria).[2;26] Other examples of inborn errors leading to extreme homocysteine elevation are defects in cobalamin (vitamin B_{12}) metabolism and deficiency of MTHFR.[27] However, since these inborn errors are rare they are not important causes of elevations in homocysteine at a population level.

In addition to these rare inborn errors, several polymorphisms have been described. A polymorphism is a mutation that is present in a population at an allele frequency of ≥ 1.0%. Polymorphisms have less pronounced effects on the activity of the enzyme they encode than the rare inborn errors. The most studied polymorphism is the 677C>T polymorphism (an alanine to valine change) in the gene coding for MTHFR. In 1995, Frosst et al.[28] discovered this MTHFR 677C>T polymorphism and showed that it was associated with reduced enzyme activity. The prevalence of the TT genotype varies between 5.4% and 16.3% in control groups of different studies.[29] In the Netherlands the prevalence of the TT genotype is about 8-10%.[30-32] Individuals with the TT genotype have on average 2.5 µmol/L (≈ 25%) higher homocysteine levels than individuals with the CC genotype.[29]

Recently, a second polymorphism of the MTHFR gene, an A to C substitution (a glutamate to alanine change) at basepair 1298, was described. There are no data thus far to suggest that the 1298A>C polymorphism alone influences homocysteine, but combined heterozygosity for the 1298A>C and 677C>T polymorphism is associated with increased homocysteine concentrations.[33-36]

Another polymorphism that has been described is the 2756A>G variant (an aspartic acid by glycine change) in the gene coding for MS. The prevalence of the GG genotype is 2-4%. It is not known whether the variant results in changes in enzymatic activity or levels of MS.[33] Studies investigating the association between the MS 2756A>G polymorphism and homocysteine levels have shown inconsistent results.[36-46]

In addition, several other polymorphisms of genes coding for CBS and MTHFR have been detected. However an association between these polymorphisms and homocysteine has not consistently been shown yet. The prevalences of these variants are generally low, and in most cases it is not clear whether it concerns a functional polymorphism.[33]

Gene-nutrient interaction

The effect of a certain polymorphism on homocysteine concentration might depend on availability of the involved co-enzyme or substrate. For example, many studies have shown that the MTHFR 677TT genotype is associated with high homocysteine levels only when folate status is low.[29]

The interaction might be explained by the results of Guenther et al.[47], who showed in bacteria that a mutation homologous to the human MTHFR 677C>T mutation was associated with an enhanced dissociation of flavine adenine dinucleotide (FAD, i.e. cofactor form of vitamin B_2). An optimal folate supply prevented the loss of FAD binding and suppressed the inactivation of the enzyme.

Although individuals with the TT genotype generally have higher homocysteine levels, several trials have shown that in these individuals the reduction in homocysteine after folic acid supplementation is more pronounced compared to individuals with the CC genotype.[48-51] However, whether individuals with the TT genotype require a higher folate intake for regulation of homocysteine concentrations remains to be established.

Homocysteine and hemostasis

There are several indications that homocysteine-induced oxidative stress causes functional abnormalities of blood vessels. Impairment of endothelium-dependent vasodilation due to experimentally induced hyperhomocysteinemia has been observed in several animal species, but also in humans.[52] Impairment of vascular function might lead to both atherogenesis and thrombogenesis. In inborn errors of metabolism resulting severe hyperhomocystinuria, the histological findings differ from the typical atheromatous changes related to hyperlipidemia, and thromboembolic events dominate the clinical picture.[26] Moreover, most animal models of severe hyperhomocysteinemia, such as the cysthathionine ß-synthase-null mouse, do not

spontaneously develop atherosclerotic lesions.[53] These findings suggest that homocysteine promotes thrombogenesis rather than atherogenesis. Therefore, in this thesis we haven chosen to focus on the effect of homocysteine on hemostasis.

Hemostasis

Hemostasis is a defense mechanism that serves to protect the integrity of the vascular system after injury of the endothelium of the vessel wall. The hemostasis process comprises platelet activation, blood coagulation and fibrinolysis; processes which are all mediated by the endothelium. Together with platelet activation, triggering of the coagulation system results in the formation of a blood clot which may lead to occlusion of the injured vessel. The fibrinolytic system dissolves the hemostatic plug in the course of vessel healing and repair. A schematic overview of these processes is shown in figure 1.2. If the balance between coagulation and fibrinolysis is disturbed, extensive clot formation can take place, which may influence the development of CVD.[54] There is evidence that high levels of circulating homocysteine may disturb this balance and predispose to thrombogenesis.[55]

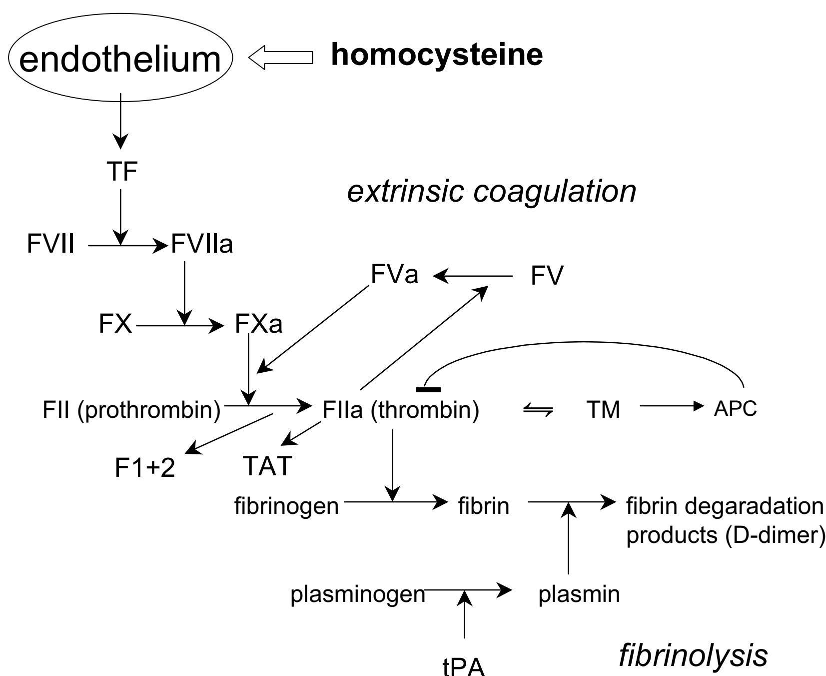

Figure 1.2 Simplified overview of coagulation cascade and fibrinolysis. After injury, blood coagulation is activated by the release of tissue factor (TF) from the vessel wall. Tissue factor binds to factor VII (FVII) and forms a complex with it. If factor VII is activated it will activate factor X (FX) to Xa (FXa). Factor Xa in complex with factor Va (FVa) activates prothrombin (FII = factor II) into thrombin (FIIa = factor IIa), releasing fragments 1 + 2 (F_{1+2}). Thrombin cleaves fibrinogen into monomeric fibrin, which then polymerizes to form the fibrin clot. Thrombin-antithrombin (TAT) is an enzyme-inhibitor complex, which reflects the inhibition of thrombin by antithrombin. Thrombin further initiates a negative feedback by activating protein C (APC = activated protein C) via thrombomodulin (TM). The fibrin clot can be dissolved by plasmin, which is activated by by tissue plasminogen activator (tPA), into fibrin degradation products (FbDP, among which D-dimer).

It has also been proposed that hyperhomocysteinemia might contribute to low-grade inflammation.[56;57] Other studies have shown that inflammation may lead to increased coagulation.[58;59] C-reactive protein (CRP) is an acute phase reactant and increased levels reflect a chronic low-grade inflammatory state.[60] Therefore, if homocysteine affects hemostasis through inflammation, this should be paralleled by an effect of homocysteine on CRP.

In vitro and animal studies

In vitro and animal studies have shown that homocysteine might affect anticoagulant functions by inhibition of antithrombin III activity and the thrombomodulin-protein C pathways. Homocysteine might also stimulate pro-coagulant factors, like factor V. Furthermore, homocysteine has been shown to increase transcription and activity of tissue factor, which would consequently lead to activation of the extrinsic coagulation cascade. Finally, homocysteine may impair fibrinolysis. Cells incubated with homocysteine showed a dose-related decrease in tissue-plasminogen activator activity and membrane binding sites. Homocysteine also promoted binding of lipoprotein(a) to fibrin, reducing the activation of plasminogen.[55;61]

In most of these studies supra-physiological homocysteine concentrations were used. The exposure period to homocysteine was limited in contrast to for example the lifelong exposure to homocysteine in patients with CBS-deficiency. Furthermore, we could question whether *in vitro* and animal models reflect human physiology. These limitations make the pathophysiological relevance of these experiments difficult to interpret. In addition, in some instances other thiols (such cysteine), not associated with CHD, exert similar effects.[55]

Human observational studies

Numerous studies have investigated the association between homocysteine and markers of hemostasis, in homocystinuric patients, vascular patients, and healthy populations. In homocystinuric patients with CBS-deficiency several abnormalities of coagulation, reflecting a hypercoagulable state, have been reported.[62] Studies with vascular patients or healthy volunteers did not consistently show a relation between homocysteine and hemostasis markers.[57;63-69] In addition, several studies did not show an association between homocysteine and CRP.[57;67;70;71] However, these were all cross-sectional analyses, and findings could have been biased due to confounding.

Human experimental studies

In three studies it was observed that acute hyperhomocysteinemia induced by methionine loading significantly increased levels of several hemostasis markers.[72-74] Since B-vitamin supplementation effectively lowers homocysteine, it was expected that B-vitamin supplementation would improve hemostasis. Studies investigating the effect of high dose folic acid supplementation on hemostasis in vascular patients and healthy volunteers have shown mixed results.[75-81] However, it should be noted that most studies did not include a placebo group, and sample sizes were generally small. Thus, there is a need for properly designed intervention studies to further elucidate whether homocysteine affects hemostasis.

Homocysteine and CHD; Cause, consequence or marker?

Since McCully postulated the homocysteine theory, many epidemiological studies investigated the association between homocysteine and CVD. In the studies reported in this thesis we have confined to the relation between homocysteine and coronary heart disease (CHD), since that relation has been most extensively studied. Although many studies have shown an association between elevated homocysteine levels and CHD risk, several observations have cast doubt on whether homocysteine is a causal risk factor for CHD.

In 1995, Boushey *et al.*[82] reviewed the available data, mainly retrospective case-control studies, in a meta-analysis. They showed that a 5 µmol/L increase in homocysteine was associated with a 60-80% increase in risk of CHD. A few years later, other meta-analyses showed that the association between homocysteine and CHD in prospective studies is much weaker. An increase in homocysteine of 5 µmol/L was estimated to be associated with an increase in CHD risk of only 20-30%.[83] However, the summary estimate of these prospective studies still reveals a higher risk of CHD associated with increased homocysteine. Several prospective studies had a long follow up. If homocysteine is a proximate risk factor, provoking an acute event, the association in prospective studies might be attenuated compared with retrospective (i.e. case-control) studies. Others suggested that homocysteine might be associated with increased CHD risk only among high risk groups, which might explain why the relationship between homocysteine and CHD risk seems to be stronger in patients with pre-existing disease such as in case-control studies, than in prospective studies of subjects who are initially healthy.[83] Nevertheless, some researchers have interpreted the weaker associations in prospective compared to retrospective studies as evidence of homocysteine being a consequence rather than a cause of CHD.

Although the MTHFR 677C>T polymorphism is consistently associated with homocysteine levels, studies investigating the relation between this polymorphism and CHD risk have shown inconsistent results. In 1998, Brattstrom *et al.*[29] published the results of a meta-analysis of 17 available studies that investigated the association between the MTHFR 677C>T polymorphism and CHD risk. The summary OR of CHD was 1.11 (95%CI 0.91 to 1.37). The authors concluded that the MTHFR 677C>T polymorphism did not increase CHD risk. However, presuming that an increase in plasma homocysteine of 2.5 µmol/L – i.e. the average difference in homocysteine concentration between the CC and the TT genotype - is associated with a 10-15% increase in CVD risk, the expected OR for the TT genotype compared with the CC genotype is 1.10-1.15. This is similar to the OR Brattstrom *et al.* found. The meta-analysis just included too few subjects to provide conclusive evidence for or against an association of this polymorphism and CHD risk. Therefore, the meta-analysis should not be considered as evidence against a causal role of homocysteine. In addition, interaction between MTHFR and folate might explain why the MTHFR 677C>T genotype was not significantly associated with CHD risk in the meta-analysis. Just like with homocysteine, the TT genotype might emerge as a risk factor for CHD mainly in populations with low-normal folate intake.[84]

Homocysteine is related to renal function, and increased homocysteine might reflect atherosclerosis due to impaired renal function.[85] However, this suggestion is not

supported by studies in inborn errors of metabolism in which renal metabolism is presumably normal.[86] There is also a possibility that impaired folate metabolism, and not elevated homocysteine levels per se, is the cause of CHD. For example, several observational studies have shown that individuals with a low folate status or intake have a higher risk for CHD.[87]

A plausible mechanism by which homocysteine would increase risk of CHD would help to accept homocysteine as a causal risk factor. Several potential mechanisms have been suggested, including homocysteine affecting hemostasis. However, no single biologically plausible mechanism has gained general acceptance yet.

Rationale, objectives and outline of the thesis

As stated before, several observations have cast doubt on the causal role of homocysteine to risk of CHD, among which the finding that the MTHFR 677C>T polymorphism was not significantly associated with CHD, and the lack of an unequivocal mechanism. The main goal of the research described in this thesis was to get more insight into the causal role of homocysteine in CHD. Establishing the existence of an association, if any, between a genetic polymorphism associated with homocysteine levels and CHD risk would be supportive for the hypothesis that the association between homocysteine and CHD risk is causal. In addition, we wanted to further explore the possibility that homocysteine affects hemostasis.

The specific research questions were:

1) Is the MTHFR 677TT genotype associated with increased CHD risk?

To study the association between the MTHFR 677C>T polymorphism and CHD risk with sufficient power, we have brought together and re-analyzed individual participant data in a meta-analysis of 40 observational studies. The results of this meta-analysis are described in chapter 2.

2) Is the MS 2756A>G polymorphism associated with homocysteine and/or CHD risk?

If the MS 2756A>G polymorphism would appear to be associated with homocysteine as well as CHD risk, this would give extra support for the hypothesis that homocysteine plays a causal role in the occurrence of CHD. To study this research question we used data from a Dutch case-control study, of which the results are described in chapter 3.

3) Do individuals with the MTHFR 677TT genotype have a higher folate requirement for adequate regulation of plasma homocysteine concentrations?

It has been suggested that subjects with the MTHFR 677TT genotype might have higher folate requirements for regulation of plasma homocysteine concentrations. To study this research question, we used data from a dose-finding study with 316 healthy volunteers who received 0, 50, 100, 200, 400, 600 or 800 µg folic acid per day for 3 months. Results are described in chapter 4.

4) Does homocysteine lowering by B-vitamin supplementation have a beneficial effect on hemostasis in healthy volunteers?

Whether homocysteine lowering by B-vitamin supplementation would have a beneficial effect on hemostasis has been studied in two different trials. In the first trial, we studied the effect of high-dose B-vitamin supplementation for 8 weeks on markers of clotting activation (F_{1+2}, TAT, and D-dimer). In the second trial, we studied the effect of a relatively low dose of folic acid (0.8 mg) for 1 year on various markers of hemostasis (TF, FVIIa, F_{1+2}, FbDP, tPA and vWF) and CRP. In addition, possible effect modification by the MTHFR 677C>T genotype was studied. The results of these trials are described in chapter 5 and 6, respectively.

Finally, in chapter 7 our findings are summarized and discussed by comparing them with findings from other studies. We discuss methodological issues, give recommendations for future research, and evaluate implications for public health.

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MTHFR 677C>T polymorphism and risk of coronary heart disease: a meta-analysis

Mariska Klerk, Petra Verhoef, Robert Clarke, Henk J. Blom,
Frans J. Kok, Evert G. Schouten,
and the MTHFR Studies Collaboration Group

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Abstract

Context In observational studies, individuals with elevated levels of plasma homocysteine tend to have moderately increased risk of coronary heart disease (CHD). The MTHFR 677C>T polymorphism is a genetic alteration in an enzyme involved in folate metabolism that causes elevated homocysteine concentrations, but its relevance to risk of CHD is uncertain.
Objective To assess the relation of MTHFR 677C>T polymorphism and risk of CHD by conducting a meta-analysis of individual participant data from all observational case-control studies with data on this polymorphism and risk of CHD.
Data Sources Studies were identified by searches of the electronic literature (MEDLINE and Current Contents) for relevant reports published before June 2001 (using the search terms *MTHFR* and *coronary heart disease*), hand searches of reference lists of original studies and review articles (including meta-analyses) on this topic, and contact with investigators in the field.
Study Selection Studies were included if they had data on the MTHFR 677C>T genotype and a case-control design (retrospective or nested case-control) and involved CHD as an endpoint. Data were obtained from 40 (34 published and 6 unpublished) observational studies, involving a total of 11,162 cases and 12,758 controls.
Data Extraction Data were collected on MTHFR 677C>T genotype, case-control status, and plasma levels of homocysteine, folate, and other cardiovascular risk factors. Data were checked for consistency with the published article or with information provided by the investigators and converted into a standard format for incorporation into a central data-base. Combined odds ratios (ORs) for the association between the MTHFR 677C>T polymorphism and CHD were assessed by logistic regression.
Data Synthesis Individuals with the MTHFR 677TT genotype had a 16% (OR 1.16; 95% confidence interval (CI) 1.05 to 1.28) higher risk of CHD compared with individuals with the CC genotype. There was significant heterogeneity between the results obtained in European populations (OR 1.14; 95% CI 1.01 to 1.28), compared with North American populations (OR 0.87; 95% CI 0.73 to 1.05), which might largely be explained by interaction between the MTHFR 677C>T polymorphism and folate status.
Conclusion Individuals with the MTHFR 677 TT genotype had a significantly higher risk of CHD, particularly in the setting of low folate status. These results support the hypothesis that impaired folate metabolism, resulting in high homocysteine levels, is causally related to increased risk of CHD.

Introduction

Homocysteine is a sulfur containing amino acid, that plays a pivotal role in methionine metabolism. Genetic defects of the enzymes or dietary deficiency of B-vitamin cofactors involved in this metabolism result in elevated homocysteine levels. Elevated homocysteine levels have been associated with increased risk of coronary heart disease (CHD),[1] but whether this association is causal is uncertain.[2] Observational studies have shown that individuals with a low folate levels or intake have a higher risk of CHD,[3-6] and it is possible that these associations are independent of homocysteine.[7]

A common polymorphism exists for the gene that encodes the methylenetetrahydrofolate reductase (MTHFR) enzyme, which converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate required for the conversion of homocysteine to methionine. Individuals who have a C>T substitution at base 677 of the gene (amino acid change A222V) have reduced enzyme activity and higher homocysteine[8] and lower folate levels than those without this substitution.[9-13] Elucidation of an association, if any, between this polymorphism and CHD risk might be informative regarding the hypothesis that impaired folate metabolism, resulting in high homocysteine concentrations, plays a causal role in the occurrence of CHD.

Individual studies and previous meta-analyses of such studies[8;14] included too few subjects to provide conclusive evidence for or against an association of this polymorphism and CHD risk.[15] The aim of this study was to assess the relation of the MTHFR 677C>T polymorphism with risk of CHD by conducting a meta-analysis of individual participant data from all observational case-control studies that had data on this polymorphism and risk of CHD.

Methods

Data Sources and Study Selection

Eligible studies were identified by searching the electronic literature (Medline and Current Contents) for relevant reports published before June 2001 (using the search-terms *MTHFR* and *coronary heart disease*), by hand-searching reference lists of original studies and review articles (including meta-analyses) on this topic, and by personal contact with investigators in the field. Studies were included if they had data on the MTHFR 677C>T genotype, had a case-control design (retrospective or nested case-control), and involved CHD as an endpoint.

Among a total of 53 published studies that examined the relation between the MTHFR 677C>T polymorphism and CHD risk, 6 studies were not included, because they did not have a proper case-control design[16-20] or they studied cardiovascular mortality[21] only. Data from 13 further studies were unavailable because the investigators were unable or unwilling to collaborate.[22-34] Data from 6 unpublished studies that fulfilled the eligibility criteria, were included after personal contact with the investigators. Among the 6 unpublished studies, 4 had previously reported on the relationship between homocysteine and CHD[35-38] whereas no data had been previously reported in 2 studies. Hence, data were available for these analyses from

40 studies (34 published[4;11;12;14;39-67] and 6 unpublished[35-38]) involving 11,162 cases and 12,758 controls (table 2.1).

Data Extraction

Data were collected on MTHFR 677C>T genotype, case-control status, and plasma levels of homocysteine, folate, and other cardiovascular risk factors, if available. Data were checked for consistency with the published article or with information provided by the investigators, and converted into a standard format for incorporation into a central database. In the majority of the studies that included cases with myocardial infarction, diagnosis was defined using World Health Organization criteria.[68] In most studies that included cases with coronary artery disease, diagnosis was based on angiographic confirmation of significant stenosis (≥ 50%) in at least 1 of the 3 major coronary arteries. However, 1 study included also cases with silent myocardial infarction and coronary revascularization.[4] If studies included both population-based controls and hospital-based controls, only data on population-based controls were included. All studies used a standardized method to determine MTHFR 677C>T genotype,[69] with 2 exceptions in which the method had been validated elsewhere.[70;71]

Data synthesis

Assuming that a prolonged increase in plasma homocysteine of 1 μmol/L (0.14 mg/L) is associated with a 5% increase in CHD risk,[1;72] and that the average homocysteine concentration is 2.5 μmol/L (0.34 mg/L) higher in TT genotype patients than CC genotype patients,[8] the expected odds ratio (OR) of CHD for the TT compared with the CC genotype would be about 1.13. With an average prevalence of the TT genotype of 12%,[8] more than 9,526 cases and an equal number of controls were required to have sufficient statistical power to estimate an OR in the expected range, using a two-sided α of 0.05 and 80% power.[72]

Plasma homocysteine and folate values were log-transformed to improve normality, and geometric means are shown. Differences between cases and controls, and between MTHFR 677C>T genotypes were assessed using analysis of variance (ANOVA) for continuous data and χ^2 tests for categorical data. We assessed whether the frequencies of CC, CT and TT genotypes among controls in individual studies were consistent with the expected distribution (i.e. in Hardy-Weinberg equilibrium) using Pearson's χ^2 test.

The OR and 95% confidence interval (CI) of CHD for the TT genotype or for the CT genotype compared with the CC genotype were assessed in each individual study using logistic regression. The analyses ignored matching of cases and controls on age and sex, which had been applied in some studies. The study-specific ORs were then pooled with adjustment for study. Possible heterogeneity between the results of individual studies or in groups defined by continent of origin or by study design was assessed using χ^2 tests.

To explore interaction between the MTHFR 677C>T genotype and folate status 6 subgroups were created, whereby folate status was defined as below or

above the median serum/plasma folate level. ORs were calculated for all subgroups, with the subgroup with CC genotype and 'high folate' as the reference group.

Complete data on age, sex, smoking, hypertension, and hypercholesterolemia were only available in a subset of studies, and analyses of the possible effects of confounding by these risk factors on the relationship between MTHFR and CHD risk was assessed using multivariate logistic regression in this subset.

A funnel plot was created by plotting the OR of CHD for TT vs CC genotype against the number of individuals in each study. A pattern resembling a symmetrical inverted funnel implied absence of significant selection or publication bias. All analyses were performed using Statistical Application Software for PC, version 6.12 (SAS institute Inc, Cary, NC, USA).

Results

Characteristics of included studies

Table 2.1 shows the number of cases and controls and selected characteristics for the controls of included studies. About half the data came from studies involving European populations and about a quarter from those of North American populations. The age distribution was similar in all studies. The prevalence of the TT genotypes among controls varied considerably among studies, ranging from 3.2 (in UK Indians)[52] to 30.2 (in an Italian population)[42] The MTHFR 677C>T genotype frequencies in controls were in Hardy-Weinberg equilibrium in all but 3 studies.[53;54;60]

Characteristics of cases and controls by genotypes

Table 2.2 shows the geometric mean plasma concentrations of homocysteine and folate and the presence of established cardiovascular risk factors for cases and controls, and within MTHFR 677C>T genotypes. Cases had a higher mean homocysteine concentration and a more adverse cardiovascular risk profile. There were no significant differences in plasma folate concentrations between cases and controls. Among both cases and controls, individuals with the TT and CT genotype had higher plasma homocysteine concentrations and lower folate concentrations than individuals with the CC genotype. Among controls, individuals with the CT genotype had a lower body mass index and individuals with the TT genotype had lower creatinine concentrations compared with individuals with the CC genotype. Among cases, there were significant differences in the prevalence of male sex, hypercholesterolemia and smoking between genotypes.

Table 2.1 Characteristics of included studies

Author, year of publication, country of origin	Number of cases/controls	Controls		
		age* (years)	prevalence TT (%)	homocysteine (μmol/L) †
Europe (22 studies)				
Kozich (-), Czech Republic‡	278 / 591	47 ± 11	10.0	9.7
Meleady (-), Europe‡	345 / 723	43 ± 10	10.8	9.7
Meisel (2001), Germany[39]	995 / 992	60 ± 10	9.7	9.7
Abbate (1998), Italy[40]	84 / 106	-	30.2	-
Ardissino (1999), Italy[41]	195 / 200	42 ± 8	18.5	-
Gemmati (1999), Italy[42]	107 / 200	47 ± 13	16.0	7.8
Girelli (1998), Italy[43]	440 / 137	57 ± 13	16.8	13.3
Kluijtmans (1997), Netherlands[14]	735 / 1250	-	8.5	-
Tanis (-), Netherlands‡	218 / 764	45 ± 8	9.0	11.9
Verhoef (1997), Netherlands[44]	131 / 100	50 ± 7	7.0	11.8
Verhoeff (1998), Netherlands[45]	258 / 272	-	14.0	-
Szczeklik (2001), Poland[46]	176 / 309	41 ± 13	4.9	10.8
Ferrer (1998), Portugal[47]	127 / 127	57 ± 16	3.9	-
Ferrer (-), Portugal‡	40 / 50	40 ± 13	16.0	7.8
Fernandez (1999), Spain[48]	272 / 213	61 ± 16	18.3	-
Thøgersen (2001), Sweden[49§]	69 / 129	54 ± 7	5.4	11.5
Todesco (1999), Switzerland[50]	75 / 224	57 ± 21	12.5	10.3
Adams (1996), UK[51]	310 / 222	57 ± 13	13.1	-
Chambers (2000), UK[52‖]	230 / 424	50 ± 7	9.7	10.2
Chambers (2000), UK[52¶]	224 / 381	49 ± 6	3.2	10.8
Fowkes (2000), UK[53§]	151 / 324	63 ± 6	6.5	-
McDowell (1998), UK[54]	747 / 605	-	12.1	-
Subtotal	**6207 / 8343**	**51 ± 13**	**10.2**	**10.3**

Table 2.1 *continued*

North America (10 studies)				
Chistensen (1997), Canada[55]	152 / 121	42 ± 5	10.7	8.6
Anderson (1997), USA[56]	549 / 145	61 ± 12	12.4	14.2
Folsom (1998), USA[57§]	274 / 505	56 ± 5	9.3	8.9
Hopkins (1995), USA[‡]	230 / 147	49 ± 6	10.9	9.8
Ma (1996), USA[11§]	294 / 291	60 ± 9	13.4	10.2
Malinow (1997), USA[57]	144 / 105	61 ± 9	10.5	8.8
Schmitz (1996), USA[58]	190 / 188	59 ± 9	14.4	9.3
Schwartz (1997), USA[12]	79 / 373	38 ± 5	12.6	10.6
Tsai (1998), USA[59]	734 / 157	44 ± 11	11.5	8.5
Verhoef (1998), USA[60§]	500 / 500	59 ± 8	14.4	-
Subtotal	**3146 / 2532**	**53 ± 11**	**12.2**	**9.8**
Other continents (8 studies)				
Van Bockxmeer (1997), Australia[61]	518 / 140	41 ± 6	11.4	-
Silberberg (-), Australia[‡]	274 / 112	-	9.8	11.8
Morita (1997), Japan[62]	227 / 778	48 ± 10	10.2	-
Nakai (2000), Japan[63]	230 / 199	60 ± 8	11.1	-
Ou (1998), Japan[64]	258 / 310	55 ± 6	13.6	-
Inbal (1999), Israel[65]	112 / 187	40 ± 5	10.7	-
Guleç (2001), Turkey[66]	96 / 100	37 ± 5	5.0	-
Tokgözoglu (1999), Turkey[67]	94 / 57	53 ± 10	5.3	14.7
Total	**11162 / 12758**	**51 ± 12**	**10.7**	**10.2**

*Mean ± SD; †geometric mean; ‡unpublished studies; §prospective studies; ||Caucasians; ¶India

Table 2.2 Distribution MTHFR 677C>T genotypes, homocysteine, folate and known cardiovascular risk factors for cases and controls, and by subgroups of MTHFR 677C>T genotype*

	Cases					Controls				
	N	All	CC	CT	TT	N	All	CC	CT	TT
MTHFR 677C>T genotype (%)	11162	-	44.3	43.4	12.3	12758	-	46.4	42.9	10.7
Homocysteine (µmol/L)	6031	11.5	11.2	11.4‡	13.4‡§	6720	10.2†	9.9	10.2‡	11.4‡§
Folate (nmol/L)	3242	11.1	11.7	10.8‡	9.8‡§	4472	11.2	11.7	11.0‡	9.4‡§
Age (years)	9004	56 ± 11	56 ± 11	56 ± 11	56 ± 11	10383	51 ± 12†	51 ± 12	51 ± 12	51 ± 13
BMI (kg/m²)	4062	26.7 ± 4.1	26.7 ± 4.1	26.7 ± 4.2	26.6 ± 4.1	4483	25.4 ± 3.8†	25.6 ± 3.8	25.3 ± 3.8‡	25.3 ± 3.8
Creatinine (µmol/L)	1788	88 ± 22	88 ± 21	88 ± 22	89 ± 22	2347	80 ± 16†	80 ± 16	80 ± 16	78 ± 16‡
Sex (% male)	9630	82	84	81‡	82	10706	69†	70	69	69
Hypertension (%)	7254	43	44	43	43	8364	19†	19	18	17
Hypercholestrolemia (mmol/L)	6510	29	31	29‡	24‡§	8074	16†	16	17	14
Diabetes (%)	6910	16	16	15	15	8144	5†	5	4	5
Smoking (%)	6477	39	38	39	42‡	8036	27†	27	28	29
Alcohol (%)	2576	67	66	68	72	3816	72†	71	73	71

*Data are given as mean ± SD or %. Homocysteine and folate are shown as geometric mean. Hypertension was defined as systolic blood pressure > 140-160 mmHg and/or diastolic blood pressure > 90-95 mmHg, and/or use of antihypertensives. Hypercholesterolemia was defined as total cholesterol > 5.7-6.5 mmol/L (or > 220-250 mg/dL) and/or use of lipid lowering medicine. Smoking was defined as currently smoking or not. Alcohol use was defined are current user or not. Cases and controls were matched on age and sex in nine studies,[39;41;47;48;49;59;60;67] on age only in two studies,[37,61] and on age+smoking in another study.[11] One study used frequency matching for age and sex,[57] and two studies for age only.[12;38]
$^{\dagger}P$<0.05 versus cases; $^{\ddagger}P$ <0.05 versus CC, $^{\S}P$<0.05 versus CT

MTHFR 677C>T polymorphism and risk of CHD

Figure 2.1 shows the OR of CHD for the TT genotype compared with the CC genotype in individual studies, and a summary estimate for the combined analysis of all studies with adjustment for study. Overall, individuals with the TT genotype had a significantly higher risk of CHD compared with individuals with the CC genotype (OR 1.16 ;95% CI 1.05 to 1.28). There was a trend toward an increased risk, for the CT genotype compared with the CC genotype (OR 1.04; 95% CI 0.98 to 1.10). There was significant heterogeneity between the results of individual studies (χ^2_{39}=63.8, P<0.01). The continent of origin appeared to account for most of this heterogeneity. Continent-specific ORs showed that CHD risk was significantly increased for individuals with the TT genotype compared with those with the CC genotype in Europe (OR 1.14; 95% CI 1.01 to 1.28), but not in North America (OR 0.87; 95% CI 0.73 to 1.05). There was no heterogeneity within European studies (χ^2_{21}=27.1, P=0.17) or North American studies (χ^2_9=4.2, P=0.90), but there was significant heterogeneity between the pooled estimates for Europe and North America (χ^2_1=6.6, P=0.01). Data on studies from other continents were too sparse to assess a continent-specific OR.

Effect modification by folate status

The heterogeneity between European and North American studies may be explained by an interaction between MTHFR 677C>T polymorphism and folate status. Table 2.3 shows ORs of CHD within strata of the MTHFR 677C>T genotype and folate status for a subset of studies for which data on folate status was available. The results show that the TT genotype is associated with increased CHD risk only when folate status is low, which indicates an interaction between the MTHFR 677C>T polymorphism and folate status.

Table 2.3 ORs (95% CI) of coronary heart disease by strata of MTHFR 677C>T polymorphism and folate status

	MTHFR 677C>T		
	CC	**CT**	**TT**
cases / controls (N)	1543 / 2180	1355 / 1847	364 / 445
high folate status	1.00†	0.91 (0.78 to 1.06)	0.99 (0.77 to 1.29)
low folate status	1.24 (1.06 to 1.44)	1.32 (1.13 to 1.54)	1.44 (1.12 to 1.83)

*Studies that had data on folate status available were: 4, 11, 12, 37, 38, 42, 43, 44, 46, 52, 54, 55, 57, 58, 61, 62, 67, Ferrer 2 and Hopkins (unpublished). Folate status was defined as below or above the median serum/plasma folate per continent.
†Reference category

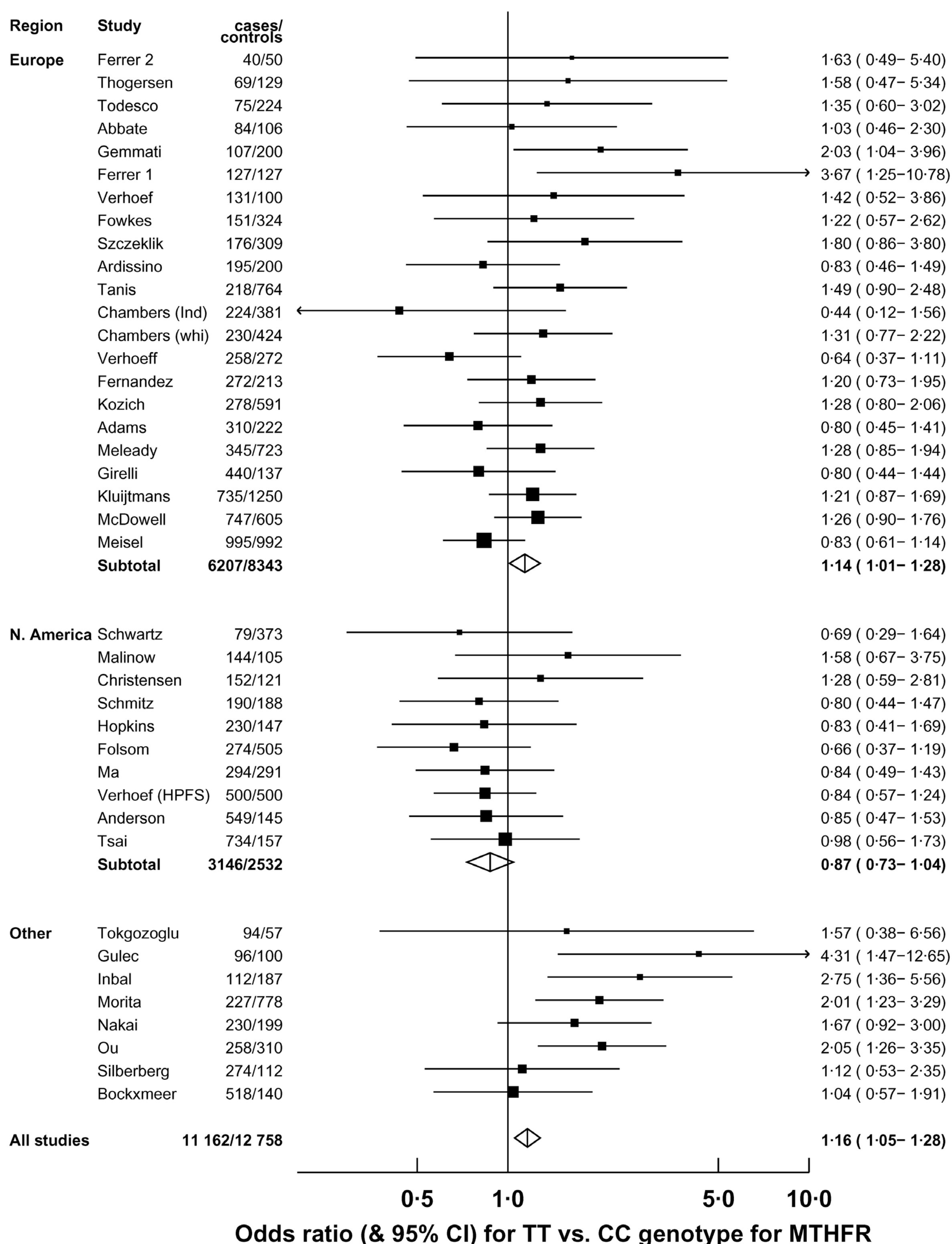

Figure 2.1 The odds ratio (& 95% CI) of CHD for MTHFR 677 TT vs CC genotype by region of origin. The size of the squares is inversely proportional to the variance of the log OR and the horizontal lines represent the 95% CI. Studies are ordered by the number of cases in each region. The combined OR and the sub-totals for each region and their 95% CI are indicated by the diamonds.

Prospective versus retrospective studies

To explore potential differences in the association between prospective and retrospective studies, we assessed pooled ORs for each study design. There was significant heterogeneity between the pooled estimates of prospective and retrospective studies (χ^2_1=16.1, *P*<0.0001). The pooled OR of CHD for the TT genotype compared with the CC genotype was 0.86 (95% CI 0.67 to 1.10) for prospective studies (5 studies involving 1,288 cases and 1,749 controls), and 1.21 (95% CI 1.10 to 1.33) for retrospective studies (35 studies involving 9,874 cases and 11,009 controls). However, since 3 of the 5 prospective studies were North American studies, it is likely that this sub-group analysis reflects a continent effect rather than an effect of the prospective study design.

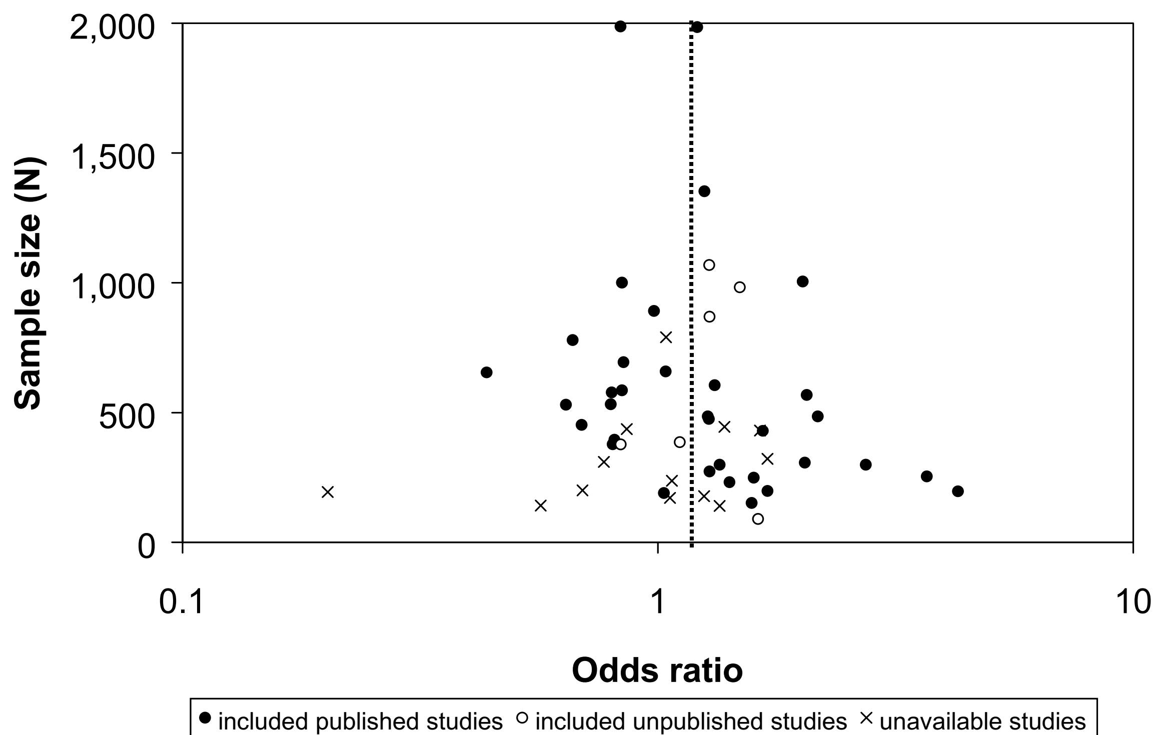

Figure 2.2 A funnel plot of the ORs of CHD for MTHFR TT versus CC for each study by the number of individuals studied. The plot shows the ORs for the 34 published and 6 unpublished studies and the 12 studies that were unavailable for inclusion in this analysis. Among the unavailable studies, one study was omitted[23] (because the OR could not be abstracted) and another study[29] was included twice (as the data was presented separately in 2 different populations). The summary estimate of the OR of CHD for TT compared with CC is represented by a vertical dashed line.

Possible confounding and bias

The effect of confounding was explored in a subgroup of studies with available data on age, sex, smoking, hypertension and hypercholesterolemia. Complete data on these cardiovascular risk factors were available for 5,343 cases and 7,308 controls. In this sub-group, the crude OR of CHD for the TT genotype versus the CC genotype was 1.15 (95% CI 1.02 to 1.30). After adjustment for these confounding factors, the OR of CHD was 1.21 (95% CI 1.06 to 1.38), thereby indicating that confounding is of little relevance to the overall results.

Figure 2.2 shows a funnel plot in which the OR of CHD for TT vs CC genotype was plotted against the number of individuals in each study. The figure includes data from published and unpublished studies and from studies for which data were not available. The shape of the funnel plot suggests that a few small studies finding an inverse association may not have been published. In addition, we calculated the average OR of CHD associated with TT compared with CC genotype among the 12 studies in which individual data were not provided for these analyses. The average OR for these studies was 1.15 (using the inverse of the variance as a weighting factor), suggesting that the present findings were probably not materially altered by the exclusion of studies for which data were unavailable.

Discussion

Importance of the genetic association

This study involving 11,162 CHD cases and 12,758 controls from 40 studies demonstrated that individuals with the MTHFR 677TT genotype have a 16% higher risk of CHD compared with individuals with the CC genotype. The results support the hypothesis that impaired folate metabolism, resulting in high homocysteine concentrations, plays a causal role in the occurrence of CHD. This meta-analysis illustrates the need to study a very large number of cases and controls to provide conclusive evidence for an association between genotype and disease, in a setting in which the disease risk associated with a genotype is moderate.

Effect modification by folate and other factors

The MTHFR 677TT genotype was significantly associated with a 14% increase in CHD risk in European populations, but not in North American populations. Previous studies had shown that the MTHFR 677C>T polymorphism is only associated with high homocysteine levels or increased CHD risk in the setting of low folate status.[11;12;43;44;67;73;74] Hence, at higher dietary intakes of folate, the effect of the MTHFR 677C>T genotype has no adverse effect on plasma homocysteine levels, or on subsequent risk of CHD. Our results confirm that a positive association between the MTHFR 677TT genotype and CHD risk is mainly present when folate levels are low. However, we think that these results should be interpreted with caution, since they are based on only part of the data, and there might be misclassification of folate status because of the different assays used. Therefore, the absolute estimates might not be completely valid. The average use of vitamin supplements has been

consistently higher for several years in North America (25-40%)[57;75-78] than in Europe (5-15%).[35;79] While the North American studies were carried out before the enhancement of folate fortification in 1998, fortification of breakfast cereals had been introduced several years before this. Hence, it is very likely that effect modification by dietary intake of folate may account for at least some of the difference in the ORs of CHD obtained for the European and North American populations.[44;72;80] In the present study, combined data from both cases and controls for each study, showed that the mean homocysteine concentration was higher in European studies (10.9 µmol/L [1.47 mg/L]) than in North American studies (10.5 µmol/L [1.42 mg/L]). Moreover, the differences between MTHFR TT and CC genotypes were greater in European studies compared with North American studies for both homocysteine (2.1 vs. 1.3 µmol/L [0.28 vs 0.18 mg/L]) and folate (2.5 vs. 1.7 nmol/L [1.1 vs 0.75 ng/mL]) concentrations, respectively.

Additional sources of heterogeneity between Europe and North America may include effect modification by other cardiovascular risk factors.[65;80-83] or linkage dis-equilibrium with other polymorphisms, such as the MTHFR 1298A>C polymorphism.[39;46;84] While the prevalence of hypercholesterolemia, smoking, and alcohol use was higher in European compared to North American studies (data not shown), these data were too sparse to examine possible effect modification by these factors.

Prospective versus retrospective studies

Studies on the association between the MTHFR 677C>T polymorphism and mortality or longevity have shown inconsistent results.[20;85-89] However, if individuals with TT have a higher case-fatality, then one might expect that the association in retrospective studies would be attenuated compared with that observed in prospective studies, because retrospective studies are restricted to survivors, whereas prospective studies can include fatal and non-fatal outcomes. The present study showed that the TT genotype was associated with increased CHD risk in retrospective studies, but not in prospective studies, but this is likely to reflect differences in populations rather than an effect of prospective studies, considering that that 3 of 5 prospective studies were North American studies.

Possible influence of bias

Although confounding is generally not anticipated in analyses of an association of a genotype with disease, there may be some imbalance in the distribution of cardiovascular risk factors by the MTHFR genotypes. Adjustment for the possible confounders in a subset of studies with available data did not attenuate the OR of CHD for the TT compared with CC genotype for MTHFR. However, the possibility of residual confounding cannot be completely excluded.

Another potential source of bias might be the inclusion of individuals from heterogeneous ethnic backgrounds. For example, the prevalence of the TT genotype is much lower in Blacks (~1%) than in Whites.[90] If the distribution of individuals with a specific ethnic background is unequal between cases and controls (so-called population stratification), this may bias an association between a genotype and risk

of CHD. In a recent study, however, bias from population stratification in case-control studies was quantified and it was concluded that its impact is likely to be small, even if ethnicity is ignored.[91] Furthermore, the risk of population stratification in this meta-analysis is small since adjustment for study ensured that cases from each study were compared with their own controls.

It is unlikely that publication bias accounted for the results obtained, as the funnel plot shows that only a few small negative studies may have been missed. Furthermore, selection bias is unlikely to have influenced the results, since the average OR of CHD associated with the TT genotype compared with the CC genotype of 12 studies that were unavailable for inclusion in these analyses was similar to our pooled OR.

Implications for public health

A meta-analysis of 30 studies involving 5000 cases with ischemic heart disease recently showed that among prospective studies, a 25% lower usual homocysteine was associated with 11% (OR=1.11; 95% CI 1.04 to 1.17) lower risk of ischemic heart disease.[92] The concordance between the risk estimates obtained in these studies provides support for a causal association between homocysteine and CHD. Several large trials are currently under way to assess if homocysteine lowering by supplementation with folic acid and other B-vitamins can reduce the risk of CHD.[93] Neither the meta-analyses nor these trials can solve the issue of whether high homocysteine levels per se or the accompanying low folate levels, which may operate via other mechanisms, are the cause of CHD. However, the present study provides some indirect evidence of the likely benefits of increasing population mean levels of folate, as the MTHFR genotype has no adverse effect on cardiovascular risk in the setting of normal folate status. Hence, provided that folate status is adequate, there is little clinical value of screening for MTHFR C677 C>T genotype in the general population for prediction of CHD risk.

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Appendix: Names and affiliations of remaining authors

*Abbate**: R.Abbate and R. Marcucci, Instituto di Clinica Medica Generale e Cardiologia, University of Florence, Florence, Italy. *Adams*: N.J. Samani, Dept. of Cardiology, Glenfield Hospital, Leicester, UK. *Anderson*: J.L. Anderson and J.S. Zebrack, University of Utah, Salt Lake City, USA. *Ardissino*: D. Ardissino**,** F.M. Merlini, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Milano, Italy. *Bockxmeer*: F.M. van Bockxmeer and L. Brownrigg, Dept. of Biochemistry, Royal Perth Hospital, Perth, Australia. *Chambers*: J. Chambers and J.S. Kooner, National Heart and Lung Institute, Hammersmith Hospital Campus, London, UK. *Christensen*: J. Genest, Dept. of Cardiology, McGill University Health Center, Royal Victoria Hospital, Montreal, Canada; R. Rozen, Montreal Children's Hospital, Montreal, Canada. *Ferrer*: C. Ferrer-Antunes and A. Palmeiro, Lab. de Hematologia, Hospitais

da Universidade de Coimbra, Coimbra, Portugal. *Fernandez*: N. Fernandez-Arcas and A. Reyes-Engel, Dept. of Biochemistry and Molecular Biology, Faculty of Medicine, University of Malaga, Spain. *Folsom*: A.R. Folsom, Div. of Epidemiology, School of Public Health, University of Minnesota, Minneapolis, USA. *Fowkes*: F.G.R. Fowkes and A.J. Lee, Wolfson Unit for Prevention of Peripheral Vascular Disease, Community Health Sciences, University of Edinburgh, Edinburgh, UK. *Gemmati*: D. Gemmati and G.L. Scapoli, Centre for Study of Haemostasis & Thrombosis, University of Ferrara, Ferrara, Italy. *Girelli*: D. Girelli and R. Corrocher, Dept. of Clinical & Experimental Medicine, Policlinico G.B. Rossi, Verona, Italy. *Güleç*: S. Güleç, Medical School of Ankara University, Ankara, Turkey. *Hopkins*: P.N. Hopkins, Cardiovascular Genetics, Salt lake City, USA. *Inbal*: A. Inbal and U. Selighson, Institute of Thrombosis and Hemostasis, Sheba Medical Center, Tel Hashomer, Israel. *Kluijtmans*: L.A.J. Kluijtmans, Lab. of Paediatrics & Neurlogy, Unversity Medical Center Nijmegen, Nijmegen, The Netherlands; J.W. Jukema, Div. of Cardiology, Leiden University Medical Center Leiden, The Netherlands. *Kozich*: V. Kozich and B. Janosikova, Institute of IMD, Charles University, 1st Faculty of Medicine, Prague, Czech Republic. *Ma*: J. Ma and M.J. Stampfer, the Channing Laboratory, Boston, USA. *Malinow*: M.R. Malinow, Oregon Regional Primate Research Center, Beaverton, USA. *McDowell*: P.A.L. Ashfield-Watt and Z.E. Clark, Wales Heart Research Institute, University of Wales College of Medicine, Cardiff, UK. *Meisel*: C. Meisel and K. Stangl, Institut für Klinische Pharmakologie, Universitätsklinikum Charité, Berlin, Germany. *Meleady:* I.M. Graham, Dept. of cardiology, the Adelaide and Meath Hospital, Dublin, Ireland. *Morita*: H. Morita, Dept. of Genetics, Harvard medical School, Boston, USA; R. Nagai, Dept. of Cardiovascular Medicine, University of Tokyo, Tokyo, Japan. *Nakai*: K. Nakai, Laboratory Medicine, Iwate Medical University, Morioka City, Japan. *Ou*: K. Yamakawa-Kobayashi, H. Hamaguchi, Dept. of Medical Genetics, University of Tsukuba, Institute of Basic Medical Science, Tsukuba, Japan. *Schmitz*: M. Gaziano, Div. of Preventive Medicine, Brigham and Women's Hospital, Boston, USA. *Schwartz*: S.M. Schwartz and D.S. Siscovick, Cardiovascular Health Unit, Seattle, USA. *Silberberg*: J.S. Silberberg, Cardiovascular Unit, John Hunter Hospital, New Castle, Australia. *Szczeklik*: A. Szczeklik and B. Domagala Teresa, Dept. of Medicine, Jagiellonian University School of Medicine, Crakow, Poland. *Tanis*: B.C. Tanis and F.M. Rosendaal, Div. Clinical Epidemiology, Leiden University Medical Center Leiden, the Netherlands. *Thøgersen*: A.M. Thøgersen and T.K. Nilsson, Dept. of Medicine, Umeå University Hospital, Umeå, Sweden. *Todesco*: L. Todesco, Div. of Clinical Pharmacology, University of Basel, Basel, Switzerland; P. Litynsky, University Children's Hospital Basel, Basel, Swizerland. *Tokgözoglu*: S.L. Tokgözoglu , Dept. of Cardiology, Hacettepe University Faculty Medicine, Ankara, Turkey. *Tsai*: M.Y. Tsai and N.Q. Hanson, Lab. of Medicine and Pathology, University of Minnesota, Minneapolis, USA. *Verhoef*: E.B. Rimm, Epidemiology and Nutrition, Harvard School of Public Health, Boston, USA. *Verhoeff*: B.J. Verhoeff and M.D. Trip, Div. Cardiology, Academic Medical Center, Amsterdam, The Netherlands.

3

The 2756A>G variant in the gene encoding methionine synthase: its relation with plasma homocysteine levels and risk of coronary heart disease in a Dutch case-control study

Mariska Klerk , Karin J.A. Lievers, Leo A.J. Kluijtmans, Henk J. Blom, Martin den Heijer, Evert G. Schouten, Frans J. Kok, and Petra Verhoef

Submitted

Abstract

Background Elevated plasma homocysteine levels have been associated with increased risk of cardiovascular disease. A 2756A>G polymorphism has been found in the gene coding for methionine synthase (MS), an enzyme catalyzing remethylation of homocysteine to methionine.

Methods In a Dutch case-control study comprising 123 cases with coronary heart disease (CHD) and 540 controls, we evaluated whether the MS 2756A>G polymorphism was associated with plasma homocysteine, folate and vitamin B_{12} concentrations, and CHD risk.

Results The polymorphism was not associated with fasting or post-methionine load homocysteine concentrations. Individuals with the GG genotype had 30% lower vitamin B_{12} concentrations than individuals with the AA or AG genotype ($P<0.05$). After adjustment for CHD risk factors, the odds ratio (OR) of CHD compared with the AA genotype was 4.0 (95% CI 1.4 to 11.6) for the GG genotype and 0.7 (95% CI 0.4 to 1.2) for the AG genotype. In a meta-analysis of five case-control studies, including the present one, the combined OR of CHD for the GG genotype was 1.0 (95% CI 0.6 to 1.7) and for the AG genotype 1.0 (95% CI 0.8 to 1.2), compared with the AA genotype.

Conclusions Despite the absence of an association with plasma homocysteine, the GG genotype showed a four-fold increased CHD risk. However, this was not confirmed by the meta-analysis. Before putting effort in additional epidemiological studies, it needs to be established first whether this polymorphism has functional consequences on enzyme activity.

Introduction

Homocysteine is a sulfur-containing amino acid, formed at demethylation of the essential amino acid methionine. Homocysteine can either be degraded by transsulfuration, involving the vitamin B_6-dependent enzyme cystathionine ß-synthase (CBS), or remethylated to methionine, involving the vitamin B_{12}-dependent enzyme methionine synthase (MS). The latter enzyme requires 5-methyltetrahydrofolate as methyl donor which is formed upon reduction of 5,10-methylenetetrahydrofolate by the enzyme methylenetetrahydrofolate reductase (MTHFR).[1] Many epidemiological studies have shown that elevated homocysteine concentrations are associated with increased risk of atherosclerosis and thrombosis.[2;3] Mild hyperhomocysteinemia can be caused by suboptimal intake of vitamins involved in the homocysteine metabolism, like folic acid, vitamin B_{12} and B_6. It may also be due to reduced function of one or more key-enzymes of its metabolism, as a result of variations in the genes encoding these enzymes.

The most extensively studied polymorphism in the homocysteine metabolism is the 677C>T polymorphism in the MTHFR gene. The MTHFR 677TT genotype is associated with a ~25% increase in homocysteine concentration,[4] and a 16% increase in coronary heart disease (CHD) risk.[5] Only recently, the role of polymorphisms of MS in hyperhomocysteinemia has attracted interest. In 1996, the human MS gene was cloned using cell lines from *cblG* patients who have an inborn error resulting in MS deficiency,[6] and sequencing analysis revealed a 2756A>G variant. Due to this transition, aspartic acid is substituted by glycine. Subsequent reports confirmed that this was a common variant, with allele frequencies ranging from 0.15-0.20.[7;8] Although it is not known whether the variant changes enzymatic activity or levels of MS, it was hypothesized that the MS 2756A>G variant might increase homocysteine levels and hence increase risk of cardiovascular disease.[6]

In the present case-control study we evaluated whether the MS 2756A>G polymorphism is associated with homocysteine concentrations and CHD risk in a Dutch population. Furthermore, the association between the polymorphism and serum vitamin B_{12} and folate concentrations was studied in a subgroup of the controls. To summarize the available data with respect to the MS 2756A>G polymorphism and CHD risk so far, we performed a meta-analysis of 5 international case-control studies, including the present one.

Methods

Study population

A total of 130 cases were selected from patients who underwent coronary angiography in the Zuiderziekenhuis Hospital in Rotterdam between 1992 and 1994. Reasons for angiography were myocardial infarction or angina pectoris. Cases were only selected if they met certain criteria for coronary stenosis:
$\geq$ 90% occlusion in one and $\geq$ 40% occlusion in one additional major coronary vessel. The control group consisted of 101 healthy subjects who were recruited from the general population in Rotterdam.[9] Exclusion criteria for both groups were

diabetic, renal, hepatic, thyroid or gastro-intestinal disease, cancer, alcohol or drug abuse, and psychiatric illness. To increase statistical power the control group was expanded with data of 440 healthy subjects who were recruited from a general practice in The Hague to take part in a health survey.[10]

Blood sampling and analyses

Venous blood samples were obtained from all subjects after an overnight fast. In addition, subjects were subjected to a standardized oral methionine loading test (0.1 g L-methionine/kg body weight). Total homocysteine was measured in plasma using a high-performance liquid chromatography (HPLC) procedure, with reverse phase separation and fluorescence detection as described by Te Poele-Pothoff et al.[11] In serum samples from the control subjects from The Hague, vitamin B_{12} and folate concentrations were analyzed by radioimmunoassay (Dualcount SPNB; Diagnostic Product Corporation, Los Angeles, USA).

MS 2756A>G genotyping

DNA was obtained from buffy coats by a standard method.[12] DNA for the analysis of the MS 2756A>G genotype (see below) was available for 123 cases and 540 controls. The polymorphism involves an A to G substitution (D919G) at basepair 2756. The transition creates a *Hae*III site, which was used for mutation analysis by PCR and restriction fragment length polymorphisms analysis. The PCR was carried out in a total volume of 50 µl containing 50 ng of the forward primer 5'-GGTGTGTTCCCAGCTGTTAGATG-3' and 50 ng of the reverse primer 5'-GACACTGAAGACCTCTGATTTGAAC-3', 200 µM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$ and 1 unit *Taq* polymerase (Life Technologies). PCR conditions were an initial denaturation step of 3 minutes at 92°C, followed by 35 cycles of 92°C/60 s (denaturation), 54°C/60 s (annealing), and 72°C/60 s (extension), and a final extension of 7 minutes at 72°C. The PCR amplification resulted in a fragment of 265 bp which was digested with the restriction enzyme *Hae*III, followed by gel electrophoresis analysis on a 2% agarose gel. After restriction enzyme analysis the 2756A allele shows one fragment of 265 bp, while the 2756G allele results in two fragments of 180 and 85 bp, respectively.

Statistics

Fasting and post methionine load homocysteine, vitamin B_{12} and serum and red blood cell folate concentrations were log-transformed prior to all statistical analyses to obtain normally distributed data. Geometric mean concentrations of fasting and post methioinine load homocysteine, vitamin B_{12} and folate were compared among genotypes. Vitamin B_{12} and folate values were available only for the subgroup of controls from The Hague. Since age and sex are important determinants of homocysteine and B-vitamin levels, mean concentrations were adjusted for age and sex using generalized linear models. Tests for trend were performed using linear regression analysis. All reported P-values are for two-tailed tests, with $\alpha = 0.05$.

Pearson's χ^2 test was used to determine whether the study population was in Hardy-Weinberg equilibrium and to study whether the frequency of the MS 2756A>G genotypes differed between cases and controls. We used logistic regression analysis to calculate odds ratios (OR) and 95% confidence intervals (CI) for subjects with the GG or the AG genotype, taking subjects with the AA genotype as a reference group.

For a summary quantitative risk assessment of the MS 2756A>G polymorphism in CHD, we evaluated 4 international case-control studies,[13-16] plus the present one. We confined the analysis to case-control studies on coronary artery disease or myocardial infarction, in which the MS 2756A>G genotype distributions among both CHD cases and controls were either given, or could be calculated from the data. Two studies were excluded since they investigated recurrent cardiovascular events[17] or severity of coronary artery disease.[18] We calculated the MS 2756A>G genotype distribution and ORs of CHD for the GG and AG genotype compared with the AA genotype in each study separately, and for all studies combined with adjustment for study. Heterogeneity between studies was assessed using Pearson's χ^2 tests. All data-analyses were performed with Statistical Application Software for PC, version 6.12 (SAS institute Inc., Cary, North Carolina, USA).

Results

Characteristics of cases and controls are shown in table 3.1. In the case group, mean age, body mass index, total cholesterol, and fasting homocysteine were significantly higher, and diastolic blood pressure lower compared with the control group. Among cases there were also significantly more males compared with controls.

Table 3.2 shows age- and sex-adjusted geometric mean fasting and post methionine load homocysteine concentrations in the three MS 2756A>G genotype groups. Since there were no differences in associations between cases and controls (data not shown) we combined both groups to increase statistical power. There were no significant differences in fasting or post-load homocysteine concentrations between genotypes; a trend test was also not significant (*P*=0.84 and *P*=0.78, respectively).

Table 3.3 shows vitamin B_{12} and folate concentrations for the subgroup of controls for which these data were available. Individuals with the GG genotype had ~30% lower serum vitamin B_{12} levels than individuals with the AA or AG genotype. This was also reflected by a trend of decreasing vitamin B_{12} concentrations with increasing number of G alleles (*P*=0.06). Mean concentrations of serum folate were not statistically significantly different between MS 2756A>G genotypes, although there was a trend of increasing folate concentrations with increasing number of G alleles (*P*=0.08). Mean concentrations of red blood cell folate were not significantly different between genotypes, nor showed a trend over the genotypes (*P*=0.80).

Table 3.1 Baseline characteristics of cases and controls (mean ± SD or %)

	Cases (n=123)	Controls (n=540)
Age	52.4 ± 7.6	50.4 ± 12.5[†]
Body mass index (kg/m2)	26.7 ± 3.0	25.9 ± 4.3[†]
Total cholesterol (mmol/L)	6.9 ± 1.4	6.1 ± 1.2[#]
Systolic blood pressure (mmHg)	135 ± 14	133 ± 21
Diastolic blood pressure (mmHg)	81 ± 8	83 ± 11[†]
Sex (% male)	85	46[#]
Smoking (% yes)	24	33
Alcohol (% yes)	84	75
Fasting homocysteine (µmol/L)*	14.3 (13.5 to 15.2)	13.1 (12.7 to 13.4)[#]
Post-load homocysteine (µmol/L)*	41.5 (39.0 to 44.1)	40.1 (39.0 to 41.2)

*age and sex adjusted geometric means (95% CI)
[†]*P*<0.05; [#]*P*<0.01

Table 3.2 Homocysteine concentrations (µmol/L)* within MS A2756G genotypes in cases and controls combined

	MS 2756A>G genotype			
	AA (n=457)	AG (n=185)	GG (n=21)	*P* trend
Fasting homocysteine	13.4	13.1	14.1	0.84
	(13.0 to 13.8)	(12.5 to 13.7)	(12.4 to 16.0)	
Post-load homocysteine	40.4	40.3	42.4	0.78
	(39.3 to 41.6)	(38.4 to 42.2)	(37.0 to 48.7)	

*age and sex adjusted geometric means (95% CI)

Table 3.3 B-vitamin concentrations* within MS 2756A>G genotypes in a subgroup of controls

	MS 2756A>G genotype			
	AA (n=318)	**AG (n=113)**	**GG (n=9)**	***P* trend**
Serum vitamin B_{12} (pmol/L)	225	217	152	0.06
	(214 to 236)	(201 to 236)	(114 to 201)†	
Serum folate (nmol/L)	12.7	13.6	15.6	0.08
	(12.0 to 13.4)	(12.4 to 14.9)	(11.3 to 21.6)	
Red blood cell folate (nmol/L)	904	922	759	0.80
	(859 to 951)	(847 to 1004)	(568 to 1015)	

*age and sex adjusted geometric means (95% CI)
†GG versus AA and AG; $P<0.05$

The distribution of the MS 2756A>G genotypes among cases and controls is shown in the left part of table 3.4. The genotype distribution was in Hardy-Weinberg equilibrium in controls (χ^2_1=0.55, *P*=0.46), but not in cases (χ^2_1=4.2, *P*=0.04). The genotype distribution was significantly different between cases and controls (χ^2_2=8.5, *P*=0.014). Crude analysis of the data showed that the GG genotype was associated with a 3.5-fold increase in risk of CHD compared with the AA genotype, whereas the AG genotype was not associated with CHD risk (table 3.4, right part). Although confounding is not to be expected in this type of analyses, there can be coincidental uneven distribution of CHD risk factors over the MS 2756A>G genotypes. Therefore, we adjusted our analyses for confounding by age, sex and other CHD risk factors. After adjustment for these factors, the OR for those with the GG genotype was associated with a 4-fold increase in risk, and there was a non-significant lower risk for those with the AG genotype (table 3.4).

Table 3.4 Frequencies of MS 2756A>G genotypes and ORs (95% CI) for CHD

	Cases	**Controls**	**OR (95% CI)**		
MS 2756A>G genotype	**n (%)**	**n (%)**	**crude**	**adjusted for age and sex**	**adjusted for age, sex, and CHD risk factors***
AA	81 (66)	376 (70)	1	1	1
AG	33 (27)	152 (28)	1.0 (0.7 to 1.6)	0.8 (0.5 to 1.3)	0.7 (0.4 to 1.2)
GG	9 (7)	12 (2)	3.5 (1.4 to 8.5)	3.3 (1.3 to 9.0)	4.0 (1.4 to 11.6)

*total cholesterol, systolic and diastolic blood pressure, current smoking and alcohol use

To summarize the available case-control data with respect to the MS2756A>G polymorphism and CHD risk so far, we combined data of 5 studies, including our own data, in a meta-analysis. Study specific risk estimates are shown in figure 3.1. There was significant heterogeneity between the results of individual studies for the risk associated with the GG genotype (χ^2_4=10.1, *P*=0.04), but not for the risk associated with the AG genotype (χ^2_4=0.25, *P*=0.99).The combination of all studies yielded a case group of 1174 individuals (789 AA, 352 AG and 33 GG) and a control group of 1746 individuals (1196 AA, 501 AG, 49 GG). After adjustment for study, the combined OR of CHD for the GG genotype was 1.0 (95%CI 0.6 to 1.7) and for the AG genotype 1.0 (95%CI 0.8 to 1.2), compared with the AA genotype.

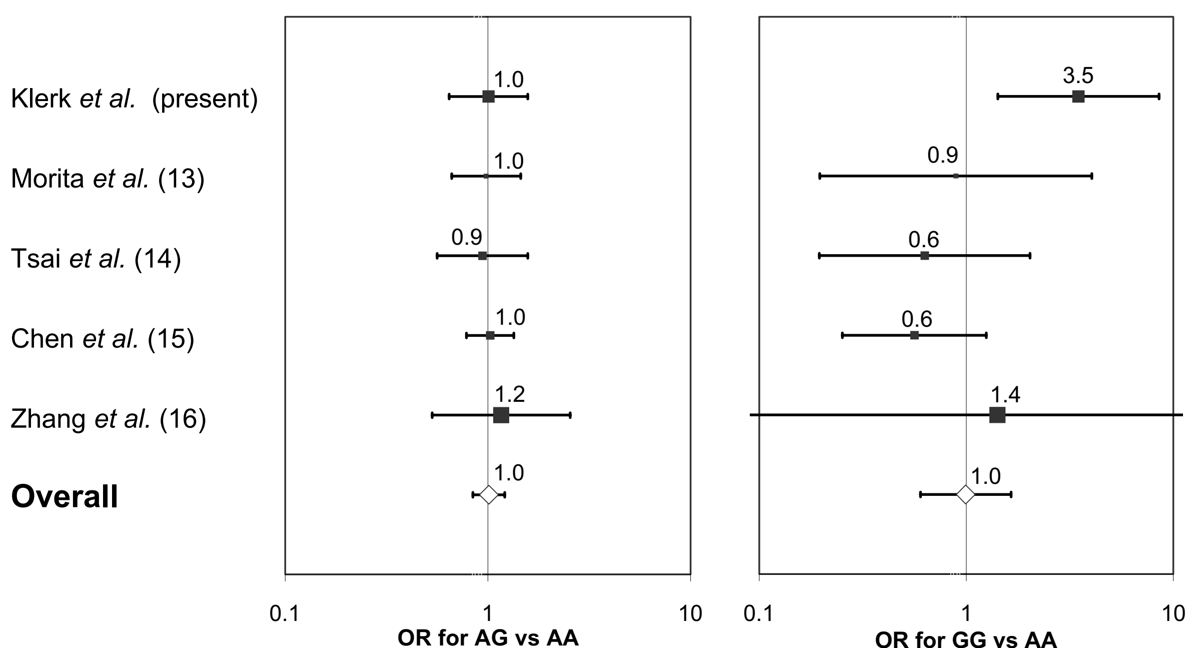

Figure 3.1 Crude ORs of CHD for MS 2756GG and AG vs AA genotype. Each square has an area proportional to the number of cases and controls and the horizontal lines represent the 95% CI. The combined ORs are indicated by diamonds.

Discussion

In this Dutch case-control study with 123 cases and 540 controls, the MS2756A>G polymorphism was not associated with fasting or post-methionine load homocysteine concentrations. However, individuals with the GG genotype had significantly lower vitamin B_{12} concentrations than individuals with the AA or AG genotype. The MS 2756GG genotype was significantly associated with a 4-fold increase in CHD risk, whereas the AG genotype was not associated with CHD risk.

Just like our study, several other studies did not show an association between the MS 2756A>G polymorphism and homocysteine concentrations.[7;13;17;19-21] Some studies, however, found that both fasting and post-methionine load homocysteine concentration decreased with increasing number of G alleles.[14;15;22-24]

In the present study, subjects with the GG genotype had significantly lower vitamin B_{12} concentrations. This is striking, because it is not expected that a polymorphism of an enzyme would affect the level of its co-factor. Two other studies did not find any association between the MS 2756A>G polymorphism and vitamin B_{12}[17] or methylmalonic acid concentration, which is an indicator of vitamin B_{12} status.[21] But even if the low vitamin B_{12} concentrations found in GG subjects are just a coincidence, this could have been responsible for the positive association between the GG genotype and CHD risk, provided that low B_{12} status is related to increased CHD risk. We could not control for confounding by B_{12} status, as we had only information on vitamin B_{12} status in control subjects. However, confounding of this type is not likely since an inverse association between vitamin B_{12} and CHD risk was observed in only one observational study.[25] Other observational studies observed no association or even a positive association between plasma vitamin B_{12} and CHD risk.[26-30]

Mean levels of serum or red blood cell folate were not significantly different among MS 2756A>G genotypes. However, serum folate levels showed a slight positive trend with increasing number of G alleles. Two other studies showed that plasma folate concentrations increased with increasing number of G alleles.[15;31] These results indicate that the MS 2756A>G variant might cause some methyl-trapping, hence resulting in higher plasma folate concentrations.

The overall result of our meta-analysis indicated that neither the GG nor the AG genotype is associated with CHD risk. However, the overall OR for the GG genotype seems to be strongly influenced by the quite discrepant positive association that was found in our own study. The majority of the case-control studies suggests that the MS 2756GG genotype has a protective rather than an adverse effect on CHD.[13-15]

The heterogeneity between studies might be explained by interaction between the MS 2756A>G polymorphism and vitamin B_{12}, folate or other cardiovascular risk factors. Wang *et al.*,[18] found that the MS 2756A>G variant enhanced the elevation in CHD risk related to smoking. A few other studies addressed possible interaction between the MS 2756A>G polymorphism and the polymorphisms of MTHFR on homocysteine concentration and homocysteine-related diseases. None of these studies showed a clear interaction between these polymorphisms.[13;19;23;24;31] Unfortunately, case-control studies investigating the association between a polymorphism and disease risk generally are much too small to investigate possible effect modification.

To improve the power of our study we a priori combined data of two control groups from Rotterdam and The Hague. We considered this a valid approach, as Rotterdam and The Hague are both large cities, close together in the Western part of Holland, and the populations were very similar with respect to CHD risk profile (data not shown). Nevertheless, subgroup analyses revealed there was a difference in MS 2756A>G genotype distribution between controls from Rotterdam (58% AA, 39% AG and 3% GG) and The Hague (72% AA, 26% AG and 2% GG). Both genotype distributions were in Hardy-Weinberg equilibrium. When the controls from the Hague were omitted from the analyses the adjusted OR for the GG genotype was 2.5 (95%CI 0.6 to 10.3), and for the AG genotype 0.5 (95%CI 0.3 to 1.0).

In conclusion, in the present case-control study a strong positive association was found between the MS 2756GG genotype and CHD risk. In contrast, the majority of the other observational studies suggests that the MS2756GG genotype has a protective rather than an adverse effect on CHD. Before putting effort in additional epidemiological studies, it needs to be established first whether this polymorphism has functional consequences for enzyme activity.

Acknowledgements

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4

The effect of different doses of folic acid on plasma homocysteine concentrations within MTHFR 677C>T genotypes

Mariska Klerk, Alida Melse-Boonstra, and Petra Verhoef

Submitted

Abstract

Background An elevated plasma concentration of homocysteine is an established risk factor for cardiovascular disease. The TT genotype for the MTHFR 677C>T polymorphism is associated with about 25% higher homocysteine levels compared with the CC genotype. Plasma homocysteine levels can be lowered by folic acid supplementation, but the response might be influenced by the MTHFR 677C>T polymorphism. The objective of this study was to evaluate whether individuals with the MTHFR TT genotype have higher folate requirement for regulation of plasma homocysteine concentrations.

Methods We used data of 307 healthy subjects aged 50-70 years who participated in a dose-finding study. Subjects were randomized to placebo, 50, 100, 200, 400, 600 or 800 µg folic acid daily for twelve weeks. To increase the number of TT subjects per treatment group, we combined data of consecutive dose-groups, i.e. 50 and 100, 200 and 400, and 600 and 800 µg/day.

Results For all MTHFR 677C>T genotypes, the reduction in plasma homocysteine concentrations relative to baseline concentrations increased with increasing dose of folic acid. However, at the dose of 600-800 µg/day there was only little additional lowering compared with 200-400 µg/day, and the relative reduction in homocysteine reached about 25% for all genotypes. However, even in the dose-group of 600-800 µg/day, homocysteine concentration after supplementation was still about 10% higher in subjects with the TT genotype compared with CC and CT genotype.

Conclusions Our findings suggest that individuals with the MTHFR 677TT genotype do not need more folic acid than individuals with the CC and CT genotype to achieve at least the same maximal relative homocysteine reduction (e.g. velocity of homocysteine reduction). However, TT subjects might need to be supplemented for longer than 12 weeks to reach final homocysteine concentrations similar to CC and CT subjects. In any event, the required dose of folic acid appears to be no more than 800 µg per day.

Introduction

Elevated plasma concentrations of homocysteine are associated with increased risk of cardiovascular disease. Many studies have shown that plasma homocysteine levels can be lowered by about 25% through folic acid supplementation.[1] A common 677C>T polymorphism exists for the gene that encodes methylenetetrahydrofolate reductase (MTHFR), which converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The latter is required as a methyl donor for the conversion of homocysteine to methionine. Individuals with the TT genotype, and to a lesser extent those with the CT genotype, have reduced activity of MTHFR. Many studies have shown that total plasma homocysteine concentrations are higher and serum folate concentrations are lower with increasing number of T alleles. In a meta-analysis involving data of 40 case-control studies (11,162 cases and 12,758 controls), we have recently shown that individuals with the TT genotype have a 16% increased risk of coronary heart disease (CHD). This finding provides support for the hypothesis that impaired folate metabolism leading to increased homocysteine concentrations plays a causal role in the occurrence of CHD (see also chapter 2).[2]

Jacques *et al*.[3] were the first who reported that the MTHFR 677C>T polymorphism is associated with elevated homocysteine levels mainly when folate status is low. They suggested that individuals with the MTHFR 677C>T mutation may have higher folate requirement for regulation of plasma homocysteine concentrations. At present, many studies have confirmed the interaction between the MTHFR 677C>T polymorphism and folate status or intake in determining the homocysteine concentration.[4-21] In addition, several studies examined whether the MTHFR 677C>T polymorphism modulated the extent of homocysteine reduction in response to folic supplementation, and showed that the reduction in homocysteine relative to initial concentrations increased with increasing number of T alleles.[22-26] Based on cross-sectional data from a large survey in the Netherlands, De Bree *et al*.[27] recently suggested that TT subjects would need a mean folate intake of more than 300 µg/day, i.e. at least 100 µg/day more than CC and CT subjects, to obtain comparable folate and homocysteine concentrations.

In 2001, we conducted a dose-finding study in which we searched for the lowest dose that reduces plasma homocysteine concentrations adequately in elderly subjects. We concluded that supplementation with 400 µg/day, on top of a normal diet providing about 200 µg/day of folate, was needed to achieve maximal homocysteine reduction.[28] The present article presents results of additional analyses, in which we investigated whether the dose-response relation of homocysteine to folic acid and the final achieved homocysteine level differ between the three MTHFR 677C>T genotypes. In this way we wanted to evaluate whether individuals with the MTHFR TT have higher folate requirement for regulation of plasma homocysteine concentrations.

Methods

Subjects and design

We used data of 307 healthy subjects aged 50-70 years who participated in a dose-finding study carried out in 2001.[28] Exclusion criteria were: history of cardiovascular disease, any chronic disease or use of medication known to interfere with folate or homocysteine metabolism, use of B-vitamin containing supplements or yeast extracts within three months prior to the study. Individuals with plasma homocysteine concentration > 26 µmol/L, serum vitamin B_{12} concentration < 160 pmol/L, or serum creatinine > 125 µmol/L were also excluded. All women were post-menopausal. Individuals started with a run-in period of 3-4 weeks, during which they used placebo-capsules to assess compliance with study procedures. Immediately after this run-in period, subjects were randomized to receive one out of seven treatments, namely placebo, or 50, 100, 200, 400, 600 or 800 µg folic acid daily for twelve weeks. The randomization procedure took account of the pre-treatment plasma homocysteine concentrations at screening. Subjects were asked to maintain their regular diet. All participants gave written informed consent to the protocol that was approved by the Medical Ethical Committee of Wageningen University.

Blood collection and analysis

Venous blood samples were taken at baseline and after 12 weeks of intervention into EDTA-containing evacuated tubes. Samples for the determination of plasma homocysteine concentrations were immediately placed on ice and the plasma was separated from blood cells within 30 minutes. Samples for folate determination were placed in the dark and stored at room temperature for at least 30 minutes before centrifugation for 10 minutes at 2600g at 4°C. We assessed hematocrit values immediately and diluted whole blood with four volumes of sodium ascorbate (10 g/L), for the determination of folate concentrations in red blood cells. All samples were stored at -80°C.

Samples collected from one subject at baseline and after 12 weeks of intervention were analyzed in the same batch to minimize variability. Total plasma homocysteine concentrations were measured by HPLC with fluorimetric detection at the Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands (intra- and interassay coefficient of variation 2% and 7%, respectively).[29; 30] We measured folate concentrations in serum and in red blood cells with a commercial chemiluminescent immunoassay analyzer (Immulite 2000, Diagnostic Products Company, Los Angeles, USA). The samples for red blood cell folate were further diluted with a concentrated human protein-based matrix (Immulite 2000 diluent) before measurement. Folate concentrations were measured at the clinical laboratory of the University Medical Centre St Radboud, Nijmegen, The Netherlands. The intra-assay coefficient of variation of the serum folate assay was < 10% and for red blood cell folate < 9%. Genomic DNA was isolated from whole blood. The presence of the 677C>T mutation in the MTHFR gene was investigated by PCR of genomic DNA and restriction enzyme digestion with *Hin*FI, according to Frosst *et al*.[31] All laboratory staff was blinded for the treatment allocation.

Statistical analysis

Baseline and 12-week values of serum folate, red blood cell folate and total plasma homocysteine were log-transformed to improve normality, and geometric means are shown. Treatment effects were expressed as percentage change relative to baseline values. The present study was a sub-analysis within existing data and therefore the number of TT subjects per dose-group was only small. To increase the number of TT subjects per treatment group we combined data of consecutive dose-groups, i.e. 50 and 100, 200 and 400, and 600 and 800 µg/day. Data-analyses were performed with the Statistical Application Software for PC, version 6.12 (SAS institute Inc., Cary, North Carolina, USA).

Results

Complete data were available for 307 participants. The mean age of the participants was 60 (SD 6) years, 59% (n=182) were male and 16% (n=48) were smokers. Mean body mass index was 27 (SD 4) kg/m2. In the entire study population 130 (42%) individuals had the CC, 134 (44%) the CT and 43 (14%) the TT genotype. Mean baseline geometric plasma homocysteine, serum and red blood cell folate concentration within MTHFR 677C>T genotypes are shown in table 4.1. The mean homocysteine concentration was higher and the serum folate concentration was lower with increasing number of T alleles. Mean red blood cell folate concentrations were higher in individuals with the TT genotype compared with individuals with the CC or CT genotype. There were no differences in plasma homocysteine, serum folate and red blood cell folate concentrations between dose-groups (data not shown). The intervention groups were also comparable with respect to age, sex distribution, BMI, creatinine, smoking, and intake of folate, vitamin B_6 and vitamin B_{12}, also within MTHFR 677C>T genotypes (data not shown).

Table 4.1 Geometric mean (95% CI) baseline homocysteine, serum and red blood cell concentrations within MTHFR 677C>T genotypes

	MTHFR 677C>T genotype		
	CC	**CT**	**TT**
N (%)	130 (42)	134 (44)	43 (14)
Homocysteine (µmol/L)	10.8 (10.4 to 11.3)	11.4 (10.9 to 11.9)	12.1 (11.3 to 12.9)
Serum folate (nmol/L)	12.5 (11.8 to 13.2)	12.2 (11.4 to 12.9)	11.3 (10.3 to 12.5)
Red blood cell folate (nmol/L)	682 (643 to 724)	662 (625 to 700)	812 (735 to 898)

Figure 4.1 and table 4.2 show the effects of the different doses of folic acid on homocysteine among MTHFR 677C>T genotypes. For all MTHFR 677C>T genotypes the reduction in plasma homocysteine concentration relative to the baseline concentration increased with increasing dose of folic acid, but there was little additional homocysteine reduction at 600-800 µg/day of folic acid compared with 200-400 µg/day, especially among those with the CT and TT genotype. The relative reduction in homocysteine at low doses was larger in CT and TT subjects compared with CC subjects. The high relative decrease among TT subjects at the dose of 50-100 µg/day was most likely due to the regression to the mean effect, as TT subjects in this dose-group started off at particularly high concentrations (see figure 4.1). With each dose of folic acid the TT subjects still had higher absolute homocysteine concentrations than the other two genotypes after 12 weeks intervention. In the 600-800 µg/day dose-group, the 12-week geometric mean homocysteine concentration was 9.6 µmol/L (95% CI 8.5 to 10.8) among TT subjects, and 8.8 (95% CI 8.3 to 9.4) and 8.6 µmol/L (95% CI 8.0 to10.3) among CT and CC subjects, respectively.

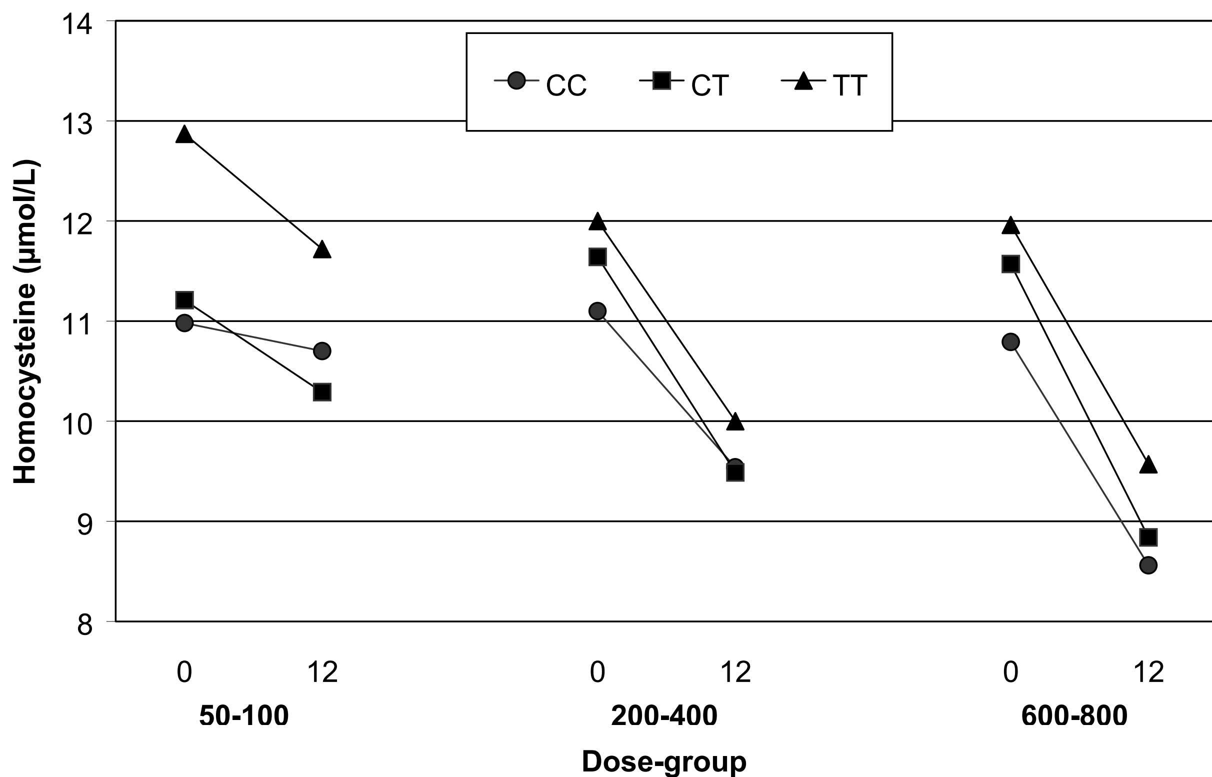

Figure 4.1 Concentrations of homocysteine within dose-groups for the MTHFR 677CC, CT and TT genotype at baseline, and after 12 weeks intervention

In all three genotypes, relative changes in serum folate concentrations increased with increasing doses of folic acid (table 4.2). The data suggest that at 600-800 µg/day the TT subjects had a larger relative increase in serum folate than the other two genotypes. After 12 weeks supplementation with 600-800 µg folic acid per day the geometric mean serum folate concentration was 59 nmol/L (95% CI 52 to 67) in CC, 55 nmo/L (95% CI 47 to 66) in CT and 62 nmo/L (95% CI 43 to 90) in TT subjects. Also the relative increase in red blood cell folate concentration became greater with increasing dose of folic acid in all three MTHFR 677C>T genotypes, but there were no marked differences in the dose-response relation between the genotype groups. Like at baseline, the TT subjects still had higher red blood cell folate concentrations after 12 weeks intervention than the other two genotypes. In the 600-800 dose-group, the 12-week geometric mean red blood cell folate concentration was 1452 nmol/L (95% CI 1338 to 1575) in CC, 1379 (95% CI 1260 to 1509) nmol/L in CT and 1575 nmol/L (95% CI 1362 to 1821) in TT subjects.

Discussion

In this study with healthy elderly volunteers we addressed the issue whether individuals with the MTHFR TT genotype have higher folate requirement for regulation of plasma homocysteine concentrations. Subjects with the TT genotype showed relative reductions in homocysteine that were at least as large as those among the other two genotypes, at any of the folic acid doses. This relative reduction in homocysteine leveled off above 600-800 µg/day, reaching about 25% for all genotypes. However, even in the dose-group of 600-800 µg/day, the homocysteine concentration after supplementation was still about 10% higher in subjects with the TT genotype compared with CC and CT genotype.

In the main analyses of this dose-finding study it was shown that the relative reduction in homocysteine describes an exponential dose-response curve, and that 90% of the maximal reduction in homocysteine concentration was reached at a daily dose of 400 µg.[28] Results of other studies confirm that there is a maximum for homocysteine reduction, however the suggested adequate doses of folic acid to achieve this maximum vary from 400 to 800 µg.[1; 32; 33] Unfortunately, our sample size was too small to fit these dose-response curves for the three MTHFR 677C>T genotypes separately. However, it appeared that within all three genotypes, but especially in the TT genotype, the additional reduction in homocysteine by increasing the dose of folic acid from 200-400 to 600-800 µg was small. In the end, all genotypes reached a mean relative reduction in homocysteine of about 25%. Thus, we conclude that individuals with the MTHFR 677TT genotype did not require more folate to achieve a maximal percentage reduction in plasma homocysteine concentrations.

Table 4.2 Percentage change from baseline (95% CI)* in homocysteine, serum and red blood cell folate within MTHFR 677C>T genotypes

	MTHFR 677C>T genotype							
	All		CC		CT		TT	
	N	**change (%)**	**N**	**Change (%)**	**N**	**Change (%)**	**N**	**Change (%)**
Homocysteine								
50 - 100 µg	84	-8.3 (11.1 to -5.4)	45	-4.9 (-9.2 to -0.5)	29	-11.8 (-15.7 to -8.0)	10	-13.2 (-22.3 to -4.0)
200 - 400 µg	86	-19.7 (-22.4 to -17.1)	30	-16.7 (-20.7 to -12.7)	42	-21.5 (-25.7 to -17.3)	14	-21.0 (-27.6 to -14.3)
600 - 800 µg	86	-24.9 (-27.6 to -22.3)	35	-23.2 (-27.0 to -19.5)	39	-26.7 (-31.2 to -22.2)	12	-24.2 (-31.9 to -16.5)
Serum folate								
50 - 100 µg	83	37 (30 to 44)	45	35 (25 to 44)	28	43 (28 to 58)	10	32 (8 to 56)
200 - 400 µg	86	160 (135 to 185)	30	154 (106 to 202)	42	155 (126 to 185)	14	188 (104 to 272)
600 - 800 µg	86	452 (373 to 532)	35	414 (295 to 533)	39	450 (330 to 570)	12	573 (294 to 851)
Red blood cell folate								
50 - 100 µg	81	10 (6 to 13)	44	5 (1 to 10)	28	19 (12 to 25)	10	4 (-9 to 18)
200 - 400 µg	85	54 (48 to 61)	29	49 (37 to 62)	42	53 (45 to 62)	14	68 (48 to 88)
600 - 800 µg	80	111 (100 to 121)	32	108 (92 to 124)	39	116 (98 to 134)	11	103 (76 to 129)

*corrected for placebo-effect

We focused on the maximal relative reduction in homocysteine, but one could also be interested in lowering homocysteine below a certain target level. In our study, TT subjects still had about 10% higher homocysteine concentrations after intervention, even at the highest dose of folic acid. Based on the fact that TT subjects also seemed to reach a plateau with respect to homocysteine lowering at doses above 400 µg/day, we consider it unlikely that they would have reached similar final homocysteine concentrations as CT and CC genotypes at higher doses than 800 µg/day. In this context, it should be noted that there is no consensus yet with regard to target values for 'healthy' homocysteine levels. The results of most studies suggest that the relationship between homocysteine and risk of cardiovascular disease is gradual, and that even at low concentrations of homocysteine any elevation in homocysteine is associated with an increase in risk.[34]

In line with our results, several other trials also showed that doses of 400-500 µg folic acid per day caused a larger relative reduction in homocysteine in TT subjects compared with CC subjects.[23;25;26] One study showed no difference in response between MTHFR 677C>T genotypes after supplementation with 1 mg folic acid plus additional vitamin B_{12} and B_6[24], which would confirm that homocysteine reduction levels off below 1 mg in all three genotypes. One study observed a larger relative reduction in homocysteine concentrations in TT subjects compared with CC and CT subjects with folic acid doses of 1 or 2 mg per day for three weeks.[22] However, these differences between genotypes might have disappeared with a longer intervention period. Results of these studies with regard to final homocysteine concentrations within the three MTHFR 677C>T genotypes were not consistent. Even the studies that used similar doses of folic acid showed opposite results.

Our trial had a longer intervention period than most of the other trials. However, we do not know what the effect would have been if we had supplemented for a longer period. We have data from another trial in which we supplemented healthy volunteers with 800 µg/day folic acid for a period of one year (see chapter 6). In this trial, TT subjects showed a 40% reduction in homocysteine whereas the reduction in CC and CT subjects was 21% and 24%, respectively. In addition, the absolute homocysteine concentration after 1 year supplementation was lower in TT subjects (9.1 µmol/L) compared with CC (10.1 µmol/L) and CT subjects (9.6 µmol/L). This could indicate that 800 µg folic acid per day is sufficiently high for TT subjects to reach homocysteine concentrations similar to those of CC and CT subjects when supplemented for a longer period than 12 weeks. However, we do not know whether a similar effect could be achieved if TT subjects would be supplemented for a longer period with a lower dose of folic acid.

In our study, baseline mean plasma homocysteine concentrations were higher and mean serum folate concentrations were lower with increasing number of T alleles, which is in line with findings from other studies.[8;11;23-25;35] Baseline concentrations of red blood cell folate were higher in subjects with the TT genotype compared with the other two genotypes. Results of other studies investigating the relation between the MTHFR 677C>T polymorphism and red blood cell folate concentrations have shown inconsistent results.[23;26;36] The discrepancies between the different studies might be explained by the different assays used to determine red blood cell folate concentrations.[37] Due to the mutation, individuals with the TT

genotype have reduced production of 5-methyltetrahydrofolate (5-methyl-THF), and consequently less 5-methyl-THF in their red blood cells. However, in contrast to individuals with the CC genotype, their red blood cells contain a considerable proportion of formyl-THF.[38] We used an immuno-assay which also measures formyl-THF, in contrast to the microbiological assay which only measures 5-methyl-THF.

In the present study, at the dose of 600-800 µg/day TT subjects reached levels of red blood cell folate that were at least as high as those in the other two genotypes. In addition, TT subjects reached about similar serum folate levels as CC and CT subjects, despite the fact they still had 10% higher homocysteine concentrations. This discrepancy between effects of homocysteine and folate status may indicate that folate measured in serum or red blood cells may not fully reflect the folate available for homocysteine remethylation at a cellular level.

Only one other study reported on the effect of folic acid supplementation (400 µg/day) on red blood cell folate concentration within MTHFR 677C>T genotypes. Although a similar assay was used, they observed final red blood cell folate levels of 975 nmol/L for TT, 920 nmol/L for CT and 775 nmol/L for CC subjects.[23] Trials that looked at the effect of folic acid supplementation on serum folate within MTHFR 677C>T genotypes have shown mixed results.[23-25;39]

In conclusion, our findings suggest that individuals with the MTHFR 677TT genotype do not need more folic acid than individuals with the CC and CT genotype to achieve at least the same maximal relative homocysteine reduction (e.g. velocity of homocysteine reduction). However, TT subjects might need to be supplemented for longer than 12 weeks to reach final homocysteine concentrations similar to CC and CT subjects. In any event, the required dose of folic acid appears to be no more than 800 µg per day. A well-designed dose-finding study with equally large groups of the three MTHFR 677C>T genotypes, and a relatively long intervention period would be required to give the final answer to the question if TT subjects require more folate to regulate homocysteine metabolism.

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5

Effect of homocysteine reduction by B-vitamin supplementation on markers of clotting activation

Mariska Klerk, Petra Verhoef, Bert Verbruggen, Evert G. Schouten, Henk J. Blom, Gerard M.J. Bos, and Martin den Heijer

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Abstract

Background Homocysteine may have an effect on risk of cardiovascular disease by stimulating procoagulant factors and/or impair anti-coagulant mechanisms or fibrinolysis. However, data in humans of such effects are sparse. In this intervention study, we examined the effect of homocysteine lowering by B-vitamin supplementation on prothrombin fragments 1 and 2 (F_{1+2}), thrombin-antithrombin complex (TAT), and fibrin degradation products (D-dimer).

Methods The study comprised 118 healthy volunteers, 50 with homocysteine > 16 µmol/L and 68 with homocysteine ≤ 16 µmol/L, who were randomized to placebo or high-dose B-vitamin supplements (5 mg folic acid, 0.4 mg hydroxycobalamin, and 50 mg pyridoxine) daily for 8 weeks.

Results Although homocysteine concentrations were 27.7% (P<0.0001) reduced in the B-vitamin group compared to the placebo group, no effect on F_{1+2} and TAT concentrations was observed. A 10.4% reduction was observed for D-dimer (P=0.08).

Conclusion It appears that in healthy subjects homocysteine reduction by B-vitamin supplementation has a modest beneficial effect on clotting activation.

Introduction

Mild hyperhomocysteinemia is associated with increased risk for arteriosclerosis[1-3] and venous thrombosis,[4] although it is still unclear whether it is a cause or a consequence of the disease.[3; 5] Mild hyperhomocysteinemia is often caused by suboptimal intake of vitamins involved in the homocysteine metabolism, like folic acid, vitamin B_{12} and B_6. It may also be due to reduced function of one or more key-enzymes of the homocysteine metabolism, as a result of a mutation in the encoding gene.[6]

Still little is known about the mechanism by which hyperhomocysteinemia could increase risk of arteriosclerosis and thrombosis. *In vitro* and animal studies suggested that homocysteine might stimulate procoagulant factors and/or impair anti-coagulant mechanisms or fibrinolysis; mechanisms which are all mediated by the vascular endothelium.[7-10] Only a few studies investigated the relationship between homocysteine and coagulation or fibrinolysis in humans, but they all suggest that homocysteine may affect coagulation and fibrinolysis.[11-16]

This is the first randomized placebo controlled intervention study in humans that investigated the effect of homocysteine lowering on markers of coagulation. Our prior hypothesis was that homocysteine damages the endothelium and consequently leads to a prethrombotic state. This should be characterized by increased concentrations of clotting activation markers, like prothrombin fragments 1 and 2 (F_{1+2}), thrombin-antithrombin complex (TAT) and fibrin degradation products (D-dimer). Studies have shown that the combination of these intercorrelated markers[17-20] is a good reflection of clotting activation.[18; 21; 22] High homocysteine levels can be effectively reduced by treatment with vitamin supplements containing folic acid, B_{12} and/or B_6.[23] The objective of this trial was to investigate whether homocysteine reduction by B-vitamin supplementation would reduce the plasma concentrations of these clotting activation markers.

Methods

Subjects and Design

The study group consisted of subjects who had participated in a case-control study[24] and subsequently in a trial. The details of the study design of the trial have been described previously.[25] There were no exclusion criteria, except pregnancy. The study on the effect of homocysteine lowering on clotting activation markers was primarily intended in hyperhomocysteinemic volunteers because in this group the largest effects on homocysteine were observed (due to high initial values and maximal B-vitamin therapy). To answer the question whether a possible effect on clotting activation markers might be attributed to homocysteine lowering itself or to vitamin supplementation in general the study was extended to volunteers with normal homocysteine concentrations. At the time of the case-control study, there were 50 hyperhomocysteinemic (homocysteine > 16 µmol/L) and 70 normohomocysteinemic (homocysteine ≤ 16 µmol/L) participants. Plasma for measuring clotting activation markers was not available for two participants. Therefore, the present study

comprised 50 hyper- and 68 normohomocysteinemic healthy volunteers who were randomized to placebo or high-dose B-vitamin supplements (5 mg folic acid, 0.4 mg hydroxycobalamin, and 50 mg pyridoxine) daily for 8 weeks. The trial was conducted in a double blind manner. Before and after the intervention period, blood was collected after an overnight fast. The study was approved by the medical ethics committee of Leyenburg Hospital. All participants gave their written informed consent.

Blood Collection, Storage and Analyses

For homocysteine and vitamin measurements, fasting blood samples were taken from an antecubital vein and collected into EDTA-containing tubes. Blood for measurement of clotting activation markers were collected atraumatically into citrate containing tubes. Whole blood was stored at -70°C for pyridoxal-5'phosphate determination. For the other determinations, samples were immediately placed on ice and centrifuged within half an hour at 2000g at 4°C for 10 minutes. The EDTA-treated samples for folate and vitamin B_{12} measurements were stored at -70°C and analyzed within two months. Plasma samples for homocysteine determination were stored at -20°C. Plasma samples for determination of clotting activation markers were stored at -80°C.

Folate and vitamin B_{12} concentrations were measured with a Dualcount SPNB (solid phase no boil) radioassay kit (Diagnostic Products Corp). Determination of pyridoxal-5'phosphate was performed by automated high performance liquid chromatography (HPLC) according to Schrijver *et al.*[26] with some modifications. Total homocysteine concentrations were measured by an HPLC method with reverse phase separation and fluorescent detection (Gilson 232-401 sample processor, Spectra Physics 8800 solvent delivery system and Spectra Physics LC 304 fluorometer). F_{1+2} and TAT were determined by ELISA using commercial kits (Enzyngnost®F1+2 micro and Enzyngnost®TAT micro, Dade-Behring, Marburg, Germany). D-dimer was also determined by ELISA (Fibrinostika®FbDP, Organon Teknika, Boxtel, the Netherlands).

Statistical Analysis

Differences in study characteristics between groups were tested by analysis of variance (ANOVA) for continuous variables and Pearson's χ^2 test for categorical variables.

Correlation analyses with baseline values of all volunteers combined were performed 1) to check whether the three clotting activation markers were intercorrelated, 2) to study whether homocysteine concentrations were correlated with the concentrations of the clotting activation markers. Correlation was assessed by calculating Pearson's correlation coefficients. In addition, the correlation analysis between homocysteine and clotting activation markers was conducted with adjustment for age and sex.

The difference in baseline concentrations of homocysteine and clotting activation markers between the high homocysteine group and the normal homocysteine group was tested by student *t* test. The change in concentrations of homocysteine and clotting activation markers in response to B-vitamin treatment was

expressed as a percentage relative to the baseline concentration. To increase power, data of high and normal homocysteine groups were combined. The difference in response between the two treatment groups was tested with a student *t* test. Baseline values, post-intervention values and responses to treatment of homocysteine and clotting activation markers were log-transformed to obtain better normally distributed data. Data-analyses were performed with the Statistical Application Software for PC, version 6.12 (SAS institute Inc., Cary, North Carolina, USA).

Results

Baseline Characteristics

Baseline characteristics of all groups are listed in table 5.1. Mean creatinine concentration was significantly lower in the normal homocysteine placebo group compared to the high homocysteine placebo group. Mean folate concentrations were significantly higher in the normal homocysteine groups compared to the high homocysteine B-vitamin group. Although there seemed to be slight differences between groups in the distribution of determinants of homocysteine and risk factors for cardiovascular disease, none of these differences were statistically significant.

Correlations at Baseline

Clotting activation markers at baseline were intercorrelated; F_{1+2} versus TAT r=0.32 (*P*=0.0004), F_{1+2} versus D-dimer r=0.28 (*P*=0.002) and TAT versus D-dimer r=0.39 (*P*=0.0001).

Figure 5.1 shows scatter plots of homocysteine versus clotting activation markers at baseline for all groups combined. In addition, correlation and regression coefficients of the variables are presented. Homocysteine was positively correlated with all three clotting activation markers. The correlation with D-dimer was the strongest. For F_{1+2} and TAT the correlation became stronger after adjustment for age and sex. Adjustment for age and sex did not affect the correlation between homocysteine and D-dimer.

Treatment Effect

Table 5.2 shows concentrations of homocysteine and clotting activation factors at baseline and at the end of 8 weeks of intervention. When the participants entered the intervention study, some subjects with elevated homocysteine levels in the case-control study had normal value at the start of the intervention study and vice versa. However, baseline mean homocysteine concentrations was still significantly higher in the high homocysteine group compared with the normal homocysteine group (*P*=0.0001). There were no significant differences in mean concentrations of TAT (*P*=0.83) and D-dimer (*P*=0.12) between the high homocysteine group and normal homocysteine group. However, the baseline mean concentration of F_{1+2} was significantly higher in the high homocysteine compared with the normal homocysteine group (*P*=0.02).

Table 5.1 Baseline characteristics (mean ± SD or n)

	Homocysteine			
	> 16 µmol/L		≤ 16 µmol/L	
	Placebo (n=27)	**B-vitamins (n=23)**	**Placebo (n=35)**	**B-vitamins (n=33)**
Age (years)	54 ± 17	59 ± 13	53 ± 11	53 ± 14
Body mass index (kg/m^2)	25.6 ± 3.1	26.4 ± 4.4	27.7 ± 4.7	26.4 ± 3.9
Cholesterol (mmol/L)	5.8 ± 1.0	6.2 ± 1.0	6.2 ± 1.3	5.9 ± 1.1
Systolic blood pressure (mmHg)	136 ± 21	142 ± 30	136 ± 19	131 ± 16
Diastolic blood pressure (mmHg)	84 ± 11	88 ± 13	86 ± 13	83 ± 11
Creatinine (µmol/L)	86 ± 21	81 ± 17	73 ± 17*	75 ± 14
Sex (n males)	11	12	13	13
Smoking (n yes)	13	9	11	10
Alcohol (n yes)	19	17	24	24
Vitamin use (n yes)	2	1	4	6
MTHFR[#] 677C>T: CC/CT/TT (n)	12/13/2	9/9/5	21/11/3	19/11/3
Factor V Leiden 1691G>A:GG/GA (n)	25/2	22/1	31/4	28/3
Prothrombin 20210G>A: GG/GA (n)	26/1	23/0	35/0	28/3
Folic acid (nmol/L)	11.4 ± 4.0	10.5 ± 3.9	13.9 ± 4.3[†]	13.8 ± 4.9[†]
Vitamin B_{12} (pmol/L)	272 ± 124	213 ± 86	275 ± 105	280 ± 110
Vitamin B_6 (PLP) (nmolL)	49 ± 19	45 ± 11	42 ± 8	42 ± 9

[#]methylenetetrahydrofolate reductase
**P*<0.05 versus placebo group with homocysteine > 16 µmol/L
[†]*P*<0.05 versus B-vitamin group with homocysteine > 16 µmol/L

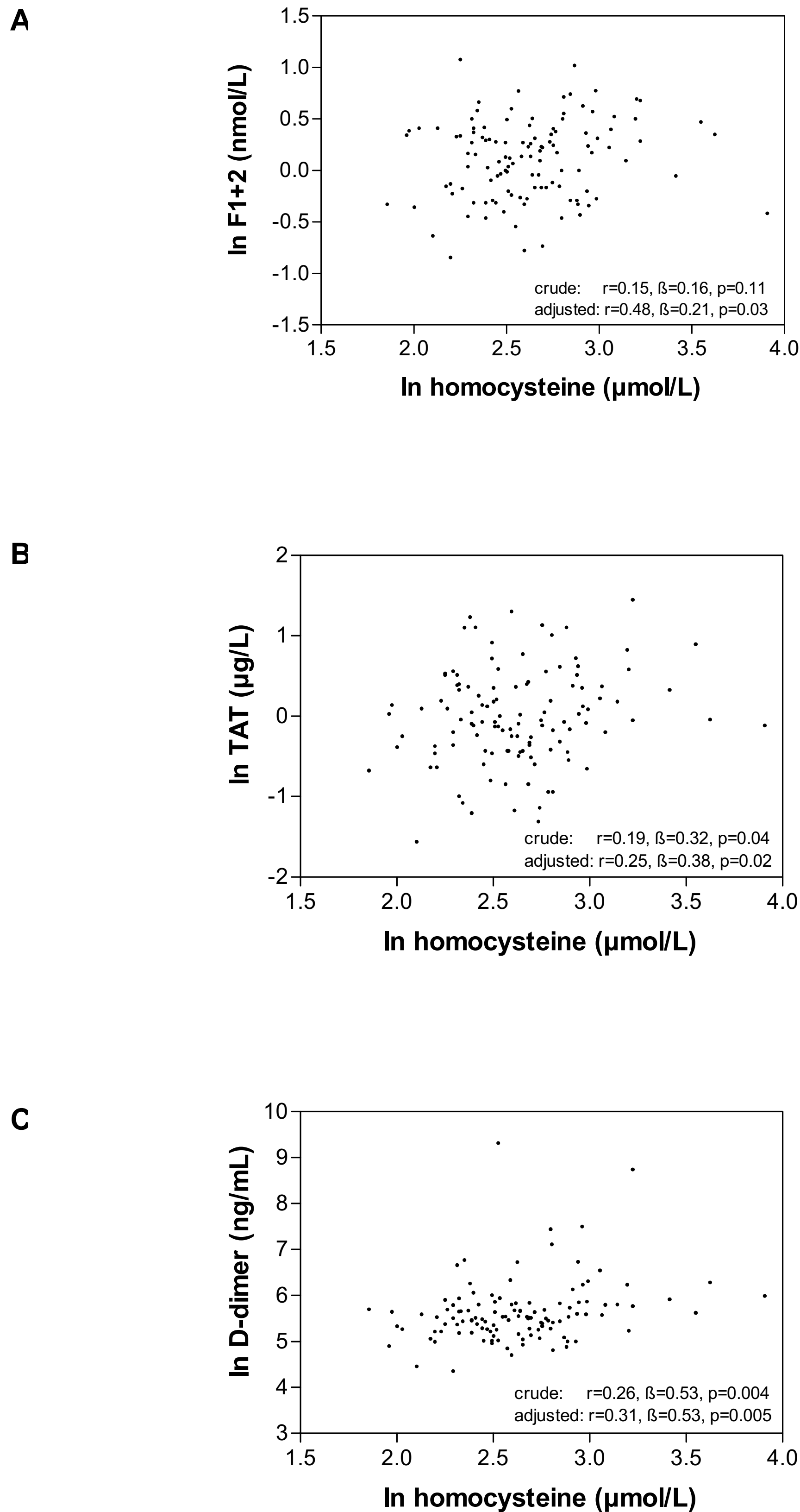

Figure 5.1 Scatter plots of logtransformed homocysteine concentrations versus logtransformed F_{1+2} (A), TAT (B), and D-dimer (C) concentrations. In addition, correlation and regression coefficients including p-values are presented; crude and adjusted for age and sex.

Table 5.2 Concentrations of homocysteine, F_{1+2}, TAT and D-dimer before and after 8 weeks of intervention*

	Homocysteine > 16 µmol/L		Homocysteine ≤ 16 µmol/L	
	Placebo (n=27)	B-vitamins (n=23)	Placebo (n=35)	B-vitamins (n=33)
Homocysteine (µmol/L)				
before intervention	17.9 (15.8-20.2)	17.3 (15.0-19.9)	11.5 (10.6-12.5)	11.7 (10.7-12.8)
after intervention	17.2 (15.5-19.1)	11.0 (9.8-12.2)	11.0 (10.2-11.8)	8.4 (7.9- 8.9)
F_{1+2} (nmol/L)				
before intervention	1.24 (1.06-1.46)	1.22 (1.06-1.40)	1.01 (0.88-1.16)	1.09 (0.96-1.23)
after intervention	1.22 (1.01-1.48)	1.17 (1.02-1.35)	1.04 (0.90-1.20)	1.12 (0.95-1.30)
TAT (µg/L)				
before intervention	1.04 (0.84-1.27)	0.91 (0.72-1.15)	0.87 (0.69-1.09)	1.17 (0.95-1.43)
after intervention	0.90 (0.67-1.19)	1.03 (0.85-1.25)	0.86 (0.64-1.16)	0.87 (0.70-1.08)
D-dimer (ng/mL)				
before intervention	315 (209-474)	322 (264-393)	222 (201-244)	301 (231-394)
after intervention	353 (229-543)	315 (236-420)	213 (190-239)	247 (195-313)

*geometric means and 95% CI

From this table it is clear that there was a marked decrease in homocysteine concentration in both the high homocysteine group and normal homocysteine group treated with B-vitamins. From this table, however, it is difficult to draw conclusions with regard to the effect of the intervention on clotting activation markers. Therefore, response to treatment of homocysteine and clotting activation markers was calculated as a percentage change relative to baseline values (figure 5.2). Since there was also a significant reduction in homocysteine in the normal homocysteine group, data of the high and normal homocysteine groups were combined to increase power. At the end of 8 weeks intervention homocysteine concentrations had decreased by 27.7% (P<0.0001) in the B-vitamin group compared with the placebo group. B-vitamin supplementation compared with placebo had no effect on F_{1+2} (-0.9%, P=0.86) concentrations. TAT concentrations seemed slightly reduced after treatment in the B-vitamin group compared with the placebo group (-5.1%, P=0.62), but this was due to one subject in the placebo group that showed a large increase (+2063%). When the analysis was repeated without this outlier, there was no effect of B-vitamin treatment on TAT (-0.4%, P=0.89). D-dimer concentration was 14.8% (P=0.10) reduced in the B-vitamin group compared with the placebo group. When

the analysis was repeated without one subject in the placebo group that showed a large increase in D-dimer concentration (+1409%), the reduction in the B-vitamin group compared with the placebo group was somewhat smaller (10.4%, P=0.08). Sixty-four percent of the subjects showed a reduction in D-dimer in the B-vitamin group, compared with 53% in the placebo group.

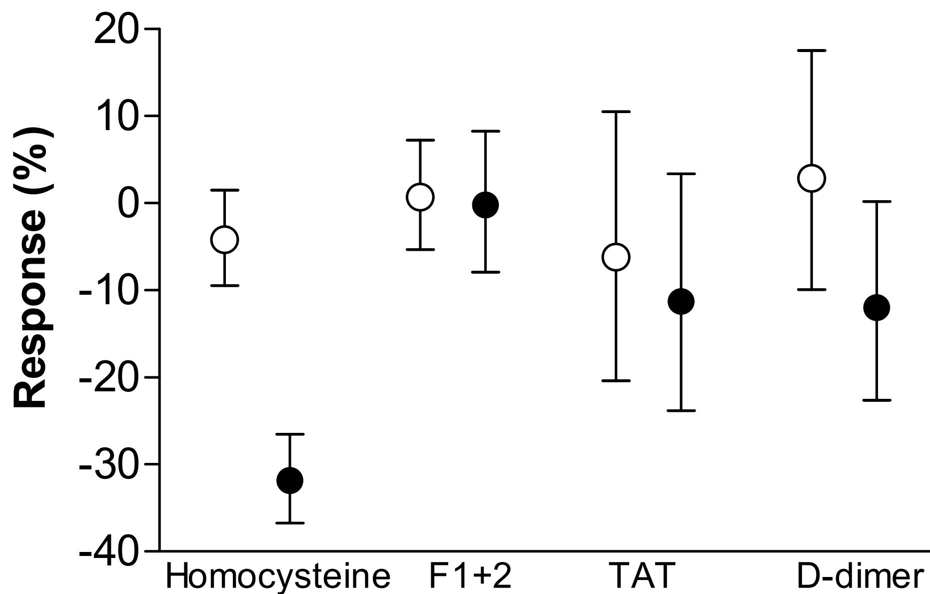

Figure 5.2 Response in homocysteine, F_{1+2}, TAT and D-dimer concentrations as percentage change relative to baseline values (geometric means and 95% confidence intervals):
○ Placebo (n=62) ● B-vitamins (n=56)

Discussion

This is the first randomized placebo controlled intervention study that investigated the effect of homocysteine lowering on clotting activation markers. In this study, homocysteine lowering caused by B-vitamin treatment had no effect on F_{1+2} and TAT concentrations. B-vitamin treatment reduced D-dimer concentrations, although this reduction was not statistically significant.

Our prior hypothesis was that homocysteine may damage the endothelium and consequently lead to a prethrombotic state. A prethrombotic state is defined as a condition characterized by an imbalance in hemostasis with a tendency to hypercoagulability (i.e. increased clotting activation), but without clinical signs of thrombosis or evidence of fibrin deposition.[22] We used F_{1+2}, TAT and D-dimer as markers of clotting activation.[21]

F_{1+2} and TAT are markers of thrombin generation.[17; 22; 27; 28] F_{1+2} is released when prothrombin is converted to thrombin.[17; 22] TAT is an enzyme-inhibitor complex, which reflects the inhibition of thrombin by antithrombin.[27; 28] Procoagulant reactions

producing fibrin stimulate the production of plasmin, which degrades fibrin to produce D-dimer.[29] In other words, D-dimer concentration is a marker of fibrin turnover.[17] The combination of these usually correlated markers is a good reflection of clotting activation.[17-20] Also in the present study, F_{1+2}, TAT and D-dimer concentrations were intercorrelated at baseline. The advantage of these markers is that they have a longer half-life in contrast to their precursor molecules, and therefore they are considered as sensitive prethrombotic markers.[18; 21] Furthermore, there is increasing evidence that these markers are positively associated with[16] or even predict risk of cardiovascular disease.[29-32]

Based on our prior hypothesis, we expected that homocysteine and clotting activation markers would show a positive correlation. In the present study, homocysteine was positively correlated with all three clotting activation markers at baseline.[33] There are only limited observational studies that investigated the relation between homocysteine and markers of clotting activation in humans. Cross-sectional studies performed in vascular patients have shown positive correlations with homocysteine for F_{1+2}[11; 13] and TAT.[16] Cross-sectional studies performed in healthy individuals have shown no correlations between homocysteine and $F_{1+2,}$[34; 35] but positive correlations for homocysteine and D-dimer.[34; 36] Our study shows that in subjects without clinical disease there is a positive relation with all three markers.

Since homocysteine was correlated with all three markers, and the markers were intercorrelated at baseline, we expected that homocysteine lowering would reduce concentrations of all three markers. B-vitamin supplementation significantly reduced homocysteine in both the high homocysteine and normal homocysteine groups. Therefore, it was not possible to answer the question whether the effect on clotting activation markers might be attributed to homocysteine lowering or B-vitamins itself, and we decided to combine the groups to study the effect of on homocysteine lowering by B-vitamin supplementation with more power. No effect of homocysteine lowering was found on F_{1+2} and TAT. There was a small reduction in D-dimer concentration, which may indicate a beneficial effect of homocysteine lowering on clotting activation. However, this reduction was not significant at the 0.05-level. Although the baseline mean D-dimer concentration was significantly higher in the high homocysteine B-vitamin group compared with the normal homocysteine placebo group, this difference cannot explain the observed reduction in D-dimer in the B-vitamin group, because we evaluated this effect by comparing the percentage change relative to baseline between both intervention groups.

The relatively small effect on D-dimer and absence of effect on F_{1+2} and TAT might be explained by some elements of the design. First of all the intervention period might have been too short or the sample size too small. However, Undas *et al.*[15] supplemented only 17 healthy subjects with fasting homocysteine levels > 16 μmol/L with a similar dose of B-vitamins for a similar intervention period and found a significant reduction of both F_{1+2} and TAT. However, the study of Undas *et al.* did not include a placebo group, which makes results more difficult to interpret. Other studies have shown that the plasma concentrations of these clotting activation markers in vascular patients are higher than in healthy subjects.[13; 16; 18; 31; 37-39] Both the study of Undas *et al.* and our study comprised healthy volunteers instead of vascular patients but the extend of other vascular risk factors could be different. It

might be that homocysteine affects primarily these markers in patients with subclinical cardiovascular disease or in subjects with a combination of hyperhomocysteinemia and other cardiovascular risk factors (e.g. smoking, hypertension).

In conclusion, the present study shows that homocysteine reduction by B-vitamin supplementation for 8 weeks in healthy volunteers had no effect on F_{1+2} and TAT, but slightly reduced D-dimer concentrations. It appears that in healthy subjects homocysteine reduction has a modest beneficial effect on clotting activation. In patients with (sub)clinical cardiovascular disease or subjects with various other risk factors for cardiovascular disease present, the effect might be stronger. However, randomized placebo controlled intervention studies in those groups are needed to confirm this hypothesis.

Acknowlegdements

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6

The effect of folic acid supplementation for 1 year on hemostasis markers in healthy elderly

Mariska Klerk, Jane Durga, Evert G. Schouten, Cornelis Kluft,
Frans J. Kok, and Petra Verhoef

Submitted

Abstract

Background Elevated homocysteine levels are associated with increased risk of cardiovascular disease (CVD). However, the mechanism by which high levels of homocysteine could lead to CVD is still unclear. It has been suggested that homocysteine might affect the endothelium, and consequently lead to impaired hemostasis.

Methods In a randomized placebo controlled trial with 276 healthy elderly volunteers, with homocysteine concentrations above 13 µmol/L at screening, we investigated the effect of homocysteine reduction by folic acid supplementation (0.8 mg/d) for 1 year on markers of several stages of hemostasis, including a marker of endothelial function (von Willebrand factor), markers of coagulation (tissue factor, factor VIIa, fragment 1+2), and markers of fibrinolysis (fibrin degradation products, tissue-type plasminogen activator). In addition, C-reactive protein (CRP), a marker of inflammation, was measured, because changes in hemostasis might be related to changes in inflammatory condition.

Results Despite a considerable reduction in homocysteine (≈25%) and a large increase in serum folate (≈400%) concentrations, there were no clear effects on hemostasis markers, nor CRP. Furthermore, subjects with different genotypes for the MTHFR 677C>T polymorphism did not differ in their response.

Conclusions Our findings indicate that it is unlikely that normal to slightly elevated homocysteine levels increase CVD risk through effects on hemostasis.

Introduction

Elevated homocysteine levels are associated with increased risk of cardiovascular disease (CVD). Elevation of plasma homocysteine levels can be caused by genetic alterations in enzymes or sub-optimal intake of vitamins involved in homocysteine metabolism, like folate, vitamin B_{12} and vitamin B_6. However, homocysteine levels can generally be effectively lowered by supplementation with folic acid. Whether homocysteine plays a causal role in the occurrence of CVD has been under debate. In a recent meta-analysis we have shown that homozygosity for the 677C>T mutation in the gene coding for methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in homocysteine remethylation, was associated with a 16% increase in risk of coronary heart disease (CHD; see also chapter 2).[1] This finding supports the hypothesis that impaired folate metabolism, resulting in higher homocysteine concentrations, is causally related to CHD.

The mechanism by which high levels of homocysteine could lead to vascular disease is still unclear. *In vitro* and animal studies have suggested that homocysteine might damage the endothelium, thereby inducing atherothrombosis by stimulation of procoagulant pathways and impairment of anti-coagulant and fibrinolytic pathways.[2] However, several cross-sectional studies that examined the relation between homocysteine and hemostasis markers have shown inconsistent results, with some finding a correlation between homocysteine and hemostasis markers, but other studies finding no correlation.[3-13] Also, intervention studies with healthy volunteers or vascular patients investigating effects of homocysteine lowering on hemostasis markers have shown mixed results.[14-20]

In this study we investigated the effect of homocysteine reduction by folic acid supplementation for 1 year on a marker of endothelial function (von Willebrand factor [vWF]), activation markers or factors of several stages of coagulation (tissue factor [TF], factor VIIa [FVIIa], prothrombin fragment 1+2 [F_{1+2}]) and fibrinolyis (fibrin degradation products [FbDP] and tissue-type plasminogen activator activity [tPA-activity]) and inflammation (C-reactive protein [CRP]) in healthy volunteers aged 50-70 years. In addition, we explored whether any effect was modified by MTHFR 677C>T polymorphism. We anticipated that TT subjects, starting out with about 25% higher homocysteine levels, might potentially show larger effects on hemostasis upon folic acid supplementation than the CC and CT subjects. We examined the effect of homocysteine reduction at various stages of the coagulation pathway (see figure 6.1). Our prior hypothesis was that homocysteine may affect the endothelium in a way that leads to enhanced coagulation. Adverse effects on the endothelium could be reflected by an increase of vWF, a marker of endothelial function.[21] When the endothelium and arterial wall are affected, blood coagulation may be stimulated additionally by the release of TF from the vessel wall. Homocysteine has been shown to increase transcription and activity of TF from macrophages in the vessel wall.[22-25] Consequently, this may lead to an increased state of activation of the extrinsic coagulation cascade resulting in increased levels of clotting activation markers down the coagulation cascade such as FVIIa and F_{1+2}. Furthermore, thrombin generation, causing increased fibrin formation, would result in reactive fibrinolysis, with possibly increasing tPA-activity and subsequently elevated FbDP

concentration. Based on this hypothesis, we expected that homocysteine reduction by folic acid supplementation would decrease the concentration or activity of vWF and coagulation markers. However, homocysteine might also affect fibrinolyis independent of coagulation and inhibit fibrinolytic activity.[26;27] In this case, homocysteine reduction would be expected to stimulate fibrinolysis, and thus increase tPA-activity and FbDP concentration. Besides these hemostasis markers, CRP - a marker of low-grade inflammation - was evaluated, because it has been proposed that hyperhomocysteinemia contributes to low-grade inflammation.[8;28] Several studies have suggested that inflammation may lead to coagulation activation,[29] and therefore changes in hemostasis in response to homocysteine lowering might be reflected by changes in CRP. To our knowledge this is the first study placebo controlled study on homocysteine reduction that covers all stages of hemostasis.

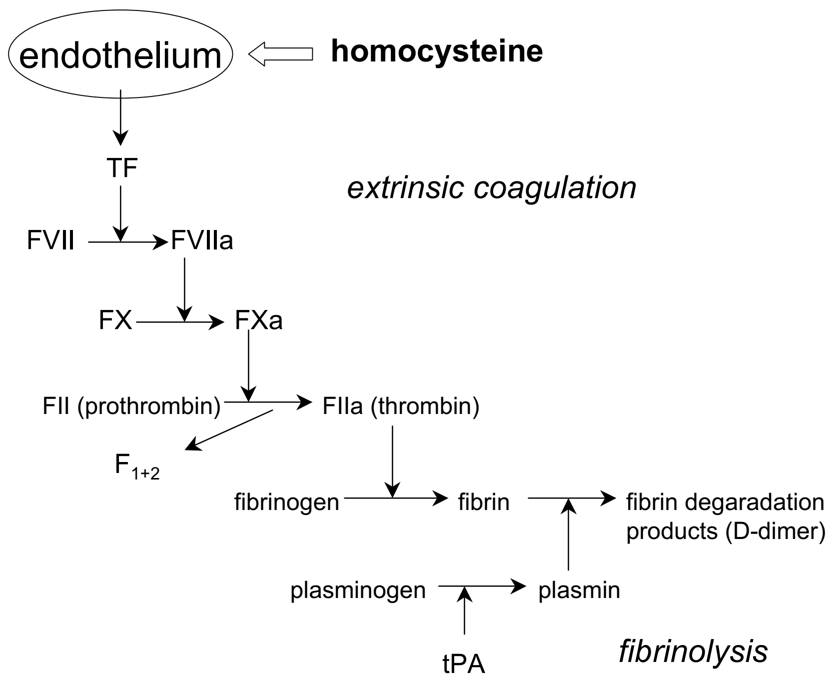

Figure 6.1 Simplified overview of coagulation and fibrinolysis

Methods

Subjects

This study is part of a larger, ongoing trial called FACIT (Folic Acid and progression of Carotid Intima-media Thickness). FACIT is a placebo controlled, double blind trial among 834 healthy volunteers examining the effect of folic acid supplementation for 3 years on the progression of carotid artery intima-media thickness, as measured by B-mode ultrasound.[30] For this sub-trial focusing on the effect of folic acid supplementation for 1 year on hemostasis markers we used data of the first 276 subjects. Subjects were recruited from local blood banks and municipal registries in Wageningen and surroundings. Subjects were eligible if they were between 50 and 70 years old, had no thyroid or renal disease, did not use drugs that affect homocysteine or progression of intima-media thickness (i.e. hormone replacement or lipid lowering therapy), and did not use vitamin supplements containing folic acid, vitamin B_{12} or vitamin B_6. Women had to be postmenopausal or in case of uterus hysterectomy > 55 years. Eligible subjects were then screened for plasma homocysteine and serum vitamin B_{12} concentrations. Plasma homocysteine values had to be higher than 13 µmol/L, but lower than 26 µmol/L. Serum vitamin B_{12} concentrations had to be higher than 200 pmol/L to reduce the chance of developing vitamin B_{12} deficiency during the trial which could be masked by folic acid supplementation (i.e. delayed symptoms of anemia).

Design

The study started with a run-in period of 6 weeks in which all subjects took placebo capsules. After the run-in period, subjects with > 80% compliance were randomized to treatment with folic acid (0.8 mg) or placebo for 3 years, using permuted blocks with block sizes 4 and 6. There were 139 participants in the placebo group and 137 participants in the folic acid group. In order to avoid mix-up of supplements, randomization was done in such a way that members of the same household received the same treatment. The capsules used for folic acid or placebo supplements looked identical. During the trial, compliance (defined as > 80% of capsules taken) was judged by an assistant based on 12-week periods for which subjects reported on a 'missed pill calendar' and returned the used strips. At baseline and after one year, blood was collected after an overnight fast. The study was approved by the medical ethics committee of Wageningen University, and written informed consent was obtained from all participants.

Blood collection and analysis

After the subject has been seated for 15 minutes, fasting blood samples were drawn into evacuated tubes without moving the needle in the vein. Blood used for determination of serum vitamin B_{12}, folate and lipids was collected in serum separator tubes, which were placed in the dark and stored at room temperature for at least 30 minutes before centrifugation for 20 minutes at 2000g at 4°C. Blood used for determination of homocysteine was collected in EDTA-containing tubes. Blood used for determination of FVIIA, vWF, TF, F_{1+2} and CRP was collected in citrate-containing

tubes. Blood used for determination of tPA-activity was collected in Stabilyte tubes. After blood collection these vacutainers were turned 'head over head' 5 times and, where necessary, directly put on ice until centrifugation. The plasma was separated from blood cells within 30 minutes by centrifugation at 2000 x g for 20 minutes at the appropriate temperature. Samples were quickly frozen using dry-ice and stored at –80°C until analysis. Samples that had to be transferred to other laboratories were transported with sufficient dry-ice to keep the samples frozen.

Pre- and post-intervention samples of a participant, except for serum folate, were analyzed in the same run to minimize variability. Serum folate and vitamin B_{12} concentrations were measured with a commercial chemiluminescent immunoassay analyzer (Immulite 2000, Diagnostic Products Company, Los Angeles, USA). The intra-assay coefficients of variation of the serum folate and vitamin B_{12} assays were < 10%. Creatinine concentrations and lipid profile were determined by Hitachi 747. These analyses were all performed at the University Medical Center St Radboud, Nijmegen, The Netherlands. Total plasma homocysteine concentrations were measured by HPLC with fluorimetric detection (intra- and interassay coefficient of variation 2% and 7%, respectively) at the Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands.[31;32] Commercial ELISA kits were used to determine plasma concentrations of TF (Actichrome TF, Amercan Diagnostica, Greenwich, USA), Factor VIIa (StaClot VIIa-rTF, Stago, Asnières, France), F_{1+2} (Enzygnost, Dade-Behring, Marburg, Germany), and tPA-activity (Chromalize t-PA, Biopool, Umeå, Sweden). Home-made ELISA with polyclonal antobodies was used for determination of vWF and CRP (Dako, Glastrup, Denmark). Measurement of hemostasis factors was performed at TNO Prevention and Health, Gaubius Laboratory, Leiden, The Netherlands. All laboratory staff was blinded for the treatment allocation. Intra- and interassay coefficients of variation (CV) for hemostasis markers and CRP were < 10%, except for FVIIa (intra-assay CV < 15%) and for TF (inter-assay CV < 15%).

MTHFR 677C>T genotyping

Genomic DNA was isolated from whole blood. The MTHFR 677C>T genotype was determined by PCR of genomic DNA and restriction enzyme digestion with *Hin*FI, according to Frosst *et al.*[33] at Wageningen University.

Other measurements

At baseline, systolic and diastolic blood pressure was measured using a DINAMAP Compact PRO 100 (Critikon, Tampa, USA). The participant rested in the supine position for fifteen minutes before measurements were done. The second and third of three measurements were averaged and the mean was used as the blood pressure value. Weight was determined using an electronic weight scale to the nearest 0.1 kg. Height was determined by the nearest 0.1 cm. Body mass index (BMI) was expressed as kg/m^2.

Statistics

Baseline values of homocysteine, folate and hemostasis markers were log-transformed to obtain normally distributed data. Correlation analyses with baseline values of all volunteers combined were performed 1) to study whether homocysteine concentrations correlated with the concentrations or activity of the hemostasis markers, and 2) to study whether the hemostasis markers were interrelated. Correlation was assessed by calculating Pearson's correlation coefficients. Baseline differences in homocysteine, folate and hemostasis markers between treatment groups were tested by student *t* test. Treatment effects were expressed as percentage change relative to baseline values. The differences in treatment effect between the two intervention groups were tested with student *t* test. To explore potential differences in effects between MTHFR genotypes, treatment effects were calculated for each genotype separately and tested with analysis of variance (ANOVA). Data-analysis was performed with the Statistical Application Software for PC, version 6.12 (SAS institute Inc., Cary, North Carolina, USA).

Results

Baseline characteristics

Baseline characteristics of the two treatment groups are shown in table 6.1. The distribution of conventional cardiovascular risk factors, many of which are also determinants of homocysteine and some also of hemostasis factors, was similar in both groups. In both groups, subjects had normal vitamin B_{12} and B_6 status, and also total cholesterol levels and blood pressures were in the normal range. Coincidentally, the MTHFR 677TT genotype was more prevalent and the CT genotype less prevalent in the folic acid group compared with the placebo group.

Baseline correlations

Baseline correlations between homocysteine and hemostasis makers and mutual correlations between hemostasis markers are shown in table 6.2. Homocysteine was not correlated with any of the hemostasis markers. Mutual correlations between hemostasis varied considerable, ranging from no correlation to a relatively strong correlation. The strongest correlations were observed between F_{1+2} and FbDP, and between FbDP and CRP.

Table 6.1 Baseline characteristics (mean ± SD or %)

	Placebo (n=139)	Folic acid (n=137)
Age (years)	60.2 ± 5.2	59.5 ± 5.8
Sex (% M/F)	70/30	77/23
BMI (kg/m^2)	26.8 ± 3.9	26.8 ± 3.9
Total cholesterol (mmol/L)	5.6 ± 0.8	5.6 ± 1.0
HDL cholesterol (mmol/L)	1.2 ± 0.3	1.2 ± 0.3
LDL cholesterol (mmol/L)	3.8 ± 0.7	3.8 ± 0.9
Systolic blood pressure (mmHg)	131 ± 14	130 ± 17
Diastolic blood pressure (mmHg)	76 ± 8	77 ± 9
Creatinine (µmol/L)	92 ± 11	95 ± 13
Current smoking (%)	24	18
Vitamin B_{12} (pmol/L)	321 ± 94	314 ± 112
Vitamin B_6 (nmol/L)	35 ± 16	38 ± 21
MTHFR genotype (% CC/CT/TT)	34/57/10	37/42/19

Table 6.2 Correlations between homocysteine and hemostasis markers and mutual correlations between hemostasis markers at baseline*

	Hcy	TF	FVIIa	F_{1+2}	FbDP	tPA-activity	vWF	CRP
Hcy	1							
TF	0.06	1						
FVIIa	-0.00	-0.09	1					
F_{1+2}	-0.04	-0.05	0.23†	1				
FbDP	-0.09	0.18*	0.14†	0.43†	1			
tPA-activity	0.09	0.10	-0.19†	-0.02	-0.01	1		
vWF	0.02	0.11	-0.08	0.01	0.28†	-0.09	1	
CRP	-0.11	0.02	0.13†	0.16†	0.36†	-0.27†	0.12†	1

Hcy = homocysteine; *based on logtransformed variables
$^{\dagger}P < 0.05$

Table 6.3 Baseline concentrations of folate, homocysteine and hemostasis markers, percentages change relative to baseline per treatment group, and differences in response between groups*

	Placebo	**Folic Acid**	**Difference between groups (%) (folic acid – placebo)**
Folate			
baseline (nmol/L)	12.8 (12.0 to 13.5)	12.3 (11.6 to 13.1)	
change (%)	-10 (-16 to -5)	383 (339 to 431)	393 (349 to 442)†
Homocysteine			
baseline (µmol/L)	12.6 (12.2 to 13.1)	13.2 (12.8 to 13.6)	
change (%)	-1 (-4 to 1)	-27 (-29 to -25)	-25 (-28 to -22)†
Tissue Factor			
baseline (pg/mL)	136 (123 to 151)	118 (105 to 133)	
change (%)	-6 (-13 to 3)	5 (-4 to 14)	10 (-1 to 23)
FVIIa			
baseline (mU/mL)	64 (58 to 70)	66 (61 to 72)	
change (%)	7 (1 to 13)	6 (0 to 11)	-1 (-9 to 7)
F_{1+2}			
baseline (nmol/L)	0.70 (0.67 to 0.75)	0.69 (0.66 to 0.73)	
change (%)	15 (11 to 20)	17 (13 to 22)	2 (-4 to 9)
FbDP			
baseline (ng/mL)	195 (179 to 212)	187 (170 to 206)	
change (%)	3 (-4 to 10)	3 (-4 to 10)	0 (-10 to 10)
tPA-activity			
baseline (IU/mL)	0.50 (0.45 to 0.55)	0.46 (0.41 to 0.51)	
change (%)	12 (3 to 23)	12 (1 to 24)	0 (-15 to 16)
vWF			
baseline (%)	116 (105 to 128)	116 (105 to 128)	
change (%)	3 (-4 to 11)	-5 (-13 to 3)	-8 (-19 to 3)
CRP			
baseline (mg/L)	1.07 (0.90 to 1.28)	1.18 (1.00 to 1.38)	
change (%)	5 (-8 to 21)	-3 (-16 to 12)	-8 (-27 to 13)

*geometric means (95% CI)
†$P < 0.001$

Treatment effect

In table 6.3, concentrations of folate, homocysteine and hemostasis markers at baseline, and the percentage changes relative to baseline within the two groups are shown. In addition, the difference in response between the folic acid and placebo group is shown. At baseline, serum folate was in the normal range in both treatment groups. Baseline values of folate and hemostasis factors were similar for the groups. However, baseline homocysteine concentration was significantly higher in the folic acid group. After 1 year supplementation, the folate concentration was significantly increased by 393% (≈ 50 nmol/L) and homocysteine significantly reduced by 25% (≈3 µmol/L) in the folic acid group compared with the placebo group. Concentrations of FVIIa, F_{1+2} and FbDP, as well as tPA-activity, increased slightly in both treatment groups. There were no clear differences in response to treatment between the two groups. TF concentration was slightly decreased in the placebo group and increased in the folic acid group. vWF and CRP were slightly increased in the placebo group, and decreased in the folic acid group. However, the differences between groups were not statistically significant.

We also investigated whether any response was modified by the MTHFR 677C>T genotype. Table 6.4 shows percentage changes relative to baseline within the two treatment groups per genotype, and the difference in these changes between the two treatment groups. At baseline, a trend of lower folate and higher homocysteine concentrations with increasing number of T alleles was observed, but baseline values of hemostasis factors were not different between genotypes (data not shown). In the folic acid group, the relative reduction in homocysteine was significantly larger with increasing number of T alleles. However, no trend over genotypes was observed for the response in folate concentration. There were some minor differences in response to folic acid, corrected for the placebo effect, between genotypes for TF, F_{1+2}, FbDP, vWF, and CRP. However, no clear pattern could be observed, indicating that the response of these markers to folic acid supplementation is not modified by the MTHFR 677C>T genotype. For FVIIa a reduction was observed in CC subjects, whereas an increase was observed in the CT and an even larger increase in TT subjects. tPA-activity showed a large, although non-significant, reduction in TT subjects, whereas no effect was observed in CT subjects, and a slight increase in CC subjects. CRP showed an increase in CC subjects, whereas a reduction was shown in CT and an even larger reduction in TT. Since 95% confidence intervals are wide and overlapping, these might be chance findings.

Table 6.4 Percentage change relative to baseline for folate, homocysteine and hemostasis markers within MTHFR 677C>T genotypes, and differences in response between groups*

	placebo	**folic acid**	**difference between groups (folic acid – placebo)**
Folate			
CC	-9 (-20 to 2)	354 (292 to 424)	363 (301 to 435)
CT	-9 (-15 to -1)	442 (364 to 534)	451 (372 to 543)
TT	-21 (-35 to -4)	348 (250 to 474)	369 (270 to 497)
Homocysteine			
CC	-1 (-5 to 2)	-22 (-25 to -20)	-21 (-25 to -17)
CT	-3 (-5 to 0)	-27 (-30 to -24)	-24 (-29 to -20)
TT	6 (-6 to 21)	-35 (-39 to -30)	-41 (-54 to -26)
Tissue Factor			
CC	-5 (-20 to12)	7 (- 8 to 25)	13 (-8 to 37)
CT	-4 (-13 to 7)	5 (- 6 to 16)	8 (-6 to 24)
TT	-16 (-33 to 6)	0 (-23 to 32)	16 (-13 to 54)
Factor VIIa			
CC	11 (3 to 20)	0 (-7 to 8)	-11 (-22 to 1)
CT	4 (..-4 to 13)	7 (-2 to 16)	3 (-10 to 16)
TT	8 (-11 to 31)	18 (3 to 34)	10 (-14 to 38)
F_{1+2}			
CC	14 (5 to 23)	16 (9 to 22)	2 (-9 to 14)
CT	16 (11 to 22)	17 (9 to 25)	1 (-9 to 11)
TT	15 (0 to 29)	22 (10 to 36)	9 (-10 to 29)
FBDP			
CC	3 (- 7 to 15)	4 (- 9 to 18)	0 (-16 to 19)
CT	2 (- 7 to 11)	-1 (-11 to 9)	-3 (-17 to 11)
TT	5 (-15 to 30)	9 (- 8 to 30)	4 (-22 to 37)
tPA-activity			
CC	10 (-4 to 27)	20 (-2 to 46)	9 (-17 to 41)
CT	9 (-4 to 24)	8 (-5 to 24)	-1 (-20 to 21)
TT	36 (-3 to 91)	2 (-18 to 26)	-35 (-78 to 26)
vWF			
CC	4 (-8 to 18)	3 (-11 to 18)	-1 (-20 to 19)
CT	2 (-7 to 11)	-13 (-24 to -1)	-15 (-29 to 0)
TT	8 (-15 to 38)	-2 (-18 to 16)	-11(-39 to 24)
CRP			
CC	12 (-8 to 36)	-7 (-25 to 14)	-19 (-46 to 13)
CT	1 (-17 to 24)	0 (-22 to 29)	-1 (-30 to 35)
TT	5 (-40 to 84)	-3 (-33 to 41)	-8 (-62 to 83)

*geometric means (95% CI)

Discussion

In this randomized placebo controlled trial with healthy elderly volunteers, folic acid supplementation for 1 year did not have a clear effect on hemostasis markers, despite a considerable reduction in homocysteine (≈25%) and a large increase in serum folate (≈400%) concentrations. Furthermore, the effect of folic acid did not clearly differ between the three MTHFR 677C>T genotypes. Our findings indicate that it is unlikely that moderately elevated homocysteine levels affect hemostasis in apparently healthy elderly. However, it should be noted that we would never have been able to exclude the possibility that folic acid supplementation would directly affect hemostasis, independent of homocysteine reduction.

Besides the absence of a clear effect of homocysteine reduction on hemostasis, homocysteine concentration was not correlated with any of the hemostasis markers at baseline. These findings further support our conclusion that homocysteine levels in the normal range do not affect hemostasis in apparently healthy volunteers. In contrast to our findings, however, several other studies in healthy populations have shown moderate correlations (r=0.20-0.30) between homocysteine and FVIIa (antigen and activity), F_{1+2}, D-dimer (≈FbDP), tPA (antigen and activity), vWF and CRP.[4;9-11;13;18] In addition, we assessed baseline correlations between hemostasis factors to examine whether these markers were dependent or independent. The results ranged from no correlation to a relatively strong correlation, and were in line with other studies.[34-36] These correlations were calculated to check whether a change in one marker in response to folic acid supplementation was in line with a change in another marker. However, as we did not observe any clear changes in the markers after folic acid supplementation, these correlations are of minor importance now.

This is the largest intervention trial on homocysteine and hemostasis performed so far, and to our knowledge the first study that examined the effect of homocysteine reduction at various stages of the coagulation pathway, together with markers of endothelial function and inflammation. There is considerable evidence for most of these markers that they are positively associated with[37;38] or even predict risk of CVD.[8;39-45] Our results cannot be explained by poor quality of plasma samples used for determination of hemostasis markers, since we followed a strict protocol for sampling and handling of the blood. Furthermore, the coefficients of variation of hemostasis markers were all below 15%.

We assessed the effect of homocysteine reduction by folic acid supplementation on hemostasis markers. Several other studies have investigated the effect of acute hyperhomocysteinemia induced by methionine loading on hemostasis factors. In these studies, methionine loading significantly increased levels of vWF, F_{1+2}, D-dimer, tPA and PAI.[4;46;47] Homocysteine levels are usually 3-4 fold higher after methionine loading compared to fasting homocysteine concentrations, so these results might indicate that homocysteine affects hemostasis only at high homocysteine levels. However, it could be questioned whether these acute effects can be compared with chronically elevated fasting homocysteine levels. Some studies in homocystinuria patients suggest that chronically high homocysteine levels are associated with increased hemostatic dysbalance.[48]

The average homocysteine concentrations of our participants might have been just too low to find any effects. At screening, we selected elderly people with a homocysteine concentration > 13 µmol/L. However at baseline, only 46% of the subjects still had homocysteine values > 13 µmol/l, probably due to regression to the mean. In a non-placebo controlled trial, Undas *et al.*[49] supplemented 17 healthy subjects with fasting homocysteine levels > 16 µmol/L with a high dose of B-vitamins for 8 weeks and found a significant reduction of F_{1+2} and thrombin-antithrombin (TAT). However, the results of our study did not change when we confined the analysis to the 32 subjects with homocysteine > 16 µmol/L (data not shown). In addition, we observed no difference in effect on hemostasis between MTHFR 677C>T genotypes, although subjects with the TT genotype had higher baseline homocysteine concentration and showed a larger relative reduction in homocysteine concentration. These results are in line with other studies[17;20] in which no difference in effect on vWF between subgroups of MTHFR 677C>T genotypes was observed after folic acid supplementation.

In our healthy elderly population, baseline levels of hemostasis factors were relatively low compared with vascular patients and possibly could not be reduced any further.[3;35;38;42;50-52] Even in the highest tertile of baseline hemostasis no effect of folic acid supplementation could be observed (data not shown). Two other placebo-controlled trials studied the effect of B-vitamin supplementation on hemostasis in healthy volunteers and also did not find a clear effect.[17;18] In addition, five studies investigated the effect of high dose folic acid supplementation on hemostasis for a period of three months to one year in vascular patients. One of these studies showed a significant reduction in vWF.[14] Two other studies did not find an effect on the same markers as we measured in our study,[15;16] while two studies observed significant effects on some other hemostasis markers.[19;20] However, it should be noted that most of these studies did not include a placebo group and sample sizes were generally small.

In conclusion, homocysteine reduction by folic acid supplementation for 1 year had no effect on markers of hemostasis in this trial with elderly healthy volunteers. It might be possible that homocysteine affects hemostasis only in patients with (sub)clinical cardiovascular disease; i.e the endothelium might be more sensitive to detrimental effects of homocysteine on hemostasis when it is already affected by atherosclerosis. Specifically designed intervention trials among subjects with documented atherosclerosis are needed to evaluate this hypothesis. It is also possible that homocysteine exerts its adverse effects via completely different pathogenic mechanisms like promotion of atherogenesis, impairing of nitric oxide availability or LDL oxidation.

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7

General discussion

Introduction

The main objectives of this thesis were I) to contribute evidence for causality of homocysteine in the occurrence and pathogenesis of coronary heart disease (CHD) by investigating the role of polymorphic genes, and II) to explore the role of hemostasis as a potential mechanism. Within these two main objectives we formulated several specific research questions. An overview of these research questions, main findings and conclusions are presented in table 7.1. In this chapter, we will further discuss these main findings, address several methodological aspects, put our findings in perspective by comparing them with other studies, and draw conclusions.

The structure of the chapter will be as follows:
I Polymorphic genes: investigating causality
- The MTHFR 677C>T polymorphism and CHD risk (chapter 2)
- The MS 2756C>G polymorphism and CHD risk (chapter 3)
- Folate requirements among individuals with the MTHFR 677TT genotype (chapter 4)

II Hemostasis: insight into mechanism (chapter 5 and 6)
III Suggested alternative pathogenic mechanisms
IV Overall conclusions
V Suggestions for future research
VI Implications for public health

I Polymorphic genes: investigating causality

The association between homocysteine and CHD has been well established, but whether homocysteine is a causal risk factor is still under debate. The advantage of studying polymorphisms is that the metabolic effects of a particular genotype start early in life, and therefore the level of the risk factor – in this case homocysteine – cannot be influenced by the presence of the disease. Therefore, investigating the association between a genetic polymorphism known to elevate homocysteine and risk of CHD can potentially reveal whether the association between homocysteine and CHD is causal. Furthermore, due to so-called Mendelian randomization bias and confounding are theoretically absent, even in case-control studies.[1]

Several polymorphisms of genes coding for enzymes involved in homocysteine metabolism (MS, MTHFR and CBS, see figure 1.1 in chapter 1) have been detected. For most of these polymorphisms presence or absence of an association with homocysteine or CHD has not consistently been shown yet.[2] In this thesis we focused the MTHFR 677C>T and the MS 2756A>G polymorphism, two polymorphisms in genes coding for enzymes involved in homocysteine remethylation, since these are the most frequently studied polymorphisms and therefore enable pooling of data.

Table 7.1 Overview of main findings and conclusions

Ch.	Research question	Study	Results	Conclusion
2	Is the MTHFR 677TT genotype associated with increased CHD risk?	pooled meta-analysis of 40 case-control studies (11162 cases and 12758 controls)	• The OR of CHD (TT vs. CC): - Overall: 1.16 (95%CI 1.05-1.28)* - Europe: 1.14 (95%CI 1.01-1.28) - N. America: 0.87 (95%CI 0.73-1.05) • Heterogeneity largely explained by interaction with folate status	• MTHFR 677TT genotype associated with 16% increased CHD risk • Impaired folate metabolism leading to increased homocysteine plays a causal role in CHD.
3	Is the MS 2756A>G polymorphism associated with homocysteine and CHD risk?	Case-control study with 123 cases and 540 controls and meta-analysis of 5 studies	*Case-control study:* • No association with homocysteine • The OR of CHD (vs. AA genotype): - AG: 0.7 (95%CI 0.4-1.2) - GG: 4.0 (95%CI 1.4-11.6) *Meta-analysis:* no association with CHD risk	• Still unclear whether MS 2756A>G polymorphism is functional • GG genotype associated with increased CHD risk in our study, but not in meta-analysis.
4	Do individuals with the MTHFR 677TT genotype have higher folate requirement for regulation of homocysteine?	Dose-finding study with 307 healthy volunteers	• Relative reduction in homocysteine: - larger with higher folic acid dose for all genotypes, but leveled off - larger for CT and TT than CC at low doses, but no difference at high doses • TT had higher post-intervention levels with all doses	Individuals with the TT genotype: • did not need more folate to achieve a maximal reduction in homocysteine • might need more folate to achieve a certain target level of homocysteine
5	Does homocysteine lowering by B-vitamin supplementation have a beneficial effect on hemostasis in healthy volunteers?	Placebo controlled intervention trial of 8 weeks with high-dose B-vitamin supplements in 118 volunteers	• Δ homocysteine -28% (p<0.0001) • Δ D-dimer -10% (p=0.08) • no effect on F_{1+2} and TAT	High-dose B-vitamin supplementation had modest beneficial effect on clotting activation
6		Placebo controlled intervention trial of 1 year with 0.8 mg folic acid in 276 volunteers	• Δ homocysteine -25% (p<0.0001) • no effect on hemostasis and CRP • no differences in effects between MTHFR 677C>T genotypes	Folic acid supplementation had no effect on hemostasis and CRP

CHD = coronary heart disease; MTHFR = methylene tetrahydrofolate reductase; MS = methionine synthase; F1+2 = fragment 1+2; TAT = thrombin antithrombin; CRP = C-reactive protein
*N.B. Overall OR also includes 3 Japanese, 2 Australian, 2 Turkish and 1 Israelian study

The MTHFR 677C>T polymorphism and CHD risk

Main findings

The results of our pooled meta-analysis on the MTHFR 677C>T polymorphism and CHD risk are summarized in table 7.1. The study demonstrated that individuals with the MTHFR 677TT genotype have a significantly 16% higher risk of CHD compared with the CC genotype. The TT genotype was associated with an increased in CHD risk in European populations, but not in North American populations. We suggested that heterogeneity between European and North American studies could largely be explained by differences in folate intake between the two continents. This suggestion was substantiated by the fact that both the difference in homocysteine and folate concentration between the TT and CC genotype were smaller in North American compared with European populations. Furthermore, we were able to show an interaction between the MTHFR 677C>T polymorphism and folate status; the TT genotype was associated with increased CHD risk only when folate status was low.

Others have suggested that the association might be modified by CHD risk factors.[3-6] We explored effect modification by CVD risk factors among the studies for which these data were available (see appendix). These analyses showed that heterogeneity in the association between the MTHFR 677C>T polymorphism and CHD risk between European and North American studies could not be explained by interaction between the MTHFR 677C>T polymorphism and CHD risk factors.

Methodological aspects

Bias - As stated before, studies investigating associations between a genetic polymorphism and disease risk are less prone to traditional biases often encountered in case-control studies. To minimize selection bias for the case-control studies, we checked whether control subjects were derived from the source population of the cases, before a study was included in the pooled meta-analysis.

Although confounding is not expected in analyses of an association between a polymorphism and disease risk (i.e. due to Mendelian randomization), the slightly uneven distribution of CHD risk factors over genotypes we observed, could potentially have confounded the association between the MTHFR 677C>T polymorphism and CHD risk. However, when the analyses were adjusted for these CHD risk factors, the OR of CHD only slightly changed.

Exposure measurement - The quality of genotyping is essential in genetic epidemiology as errors in determination of genotypes will result in misclassification. All studies that were included in our pooled meta-analysis used the same standardized method to determine MTHFR 677C>T genotype,[7] with two exceptions where the method had been validated with the standard method.[8;9] It is not clear whether the researchers of the individual studies performed any type of quality control on the genotyping. In all but three studies, the genotype distribution was in Hardy-Weinberg equilibrium, which means that the observed distribution will remain constant from one generation to the next. The Hardy-Weinberg equilibrium is often regarded as a measure of quality for genotyping, but whether this is a valid approach is questionable. Human populations never truly meet the conditions for Hardy-Weinberg equilibrium, since there is always some selection, migration, and non-

random mating. In addition, small population sizes may lead to genetic drift.[10] Thus, if a population is in Hardy-Weinberg equilibrium this does not per se guarantee the absence of misclassification.

Sample size - Our meta-analysis illustrates the need to study very large numbers of cases and controls to provide conclusive evidence for an association between a genetic polymorphism and disease, when the impact of the polymorphism on disease risk is small. Although we realized the power would be too small to investigate interaction properly, we wanted to get an indication of possible effect modification of the association between the MTHFR 677C>T polymorphism and CHD risk by folate status and CHD risk factors. To be able to investigate interaction with sufficient statistical power, the sample size should have been multiplied by four,[11] i.e. numbers which were simply not available.

Endpoint - An important difference between the prospective studies and retrospective case-control studies included in our meta-analysis was that in the prospective studies also fatal cases were included, whereas the retrospective studies included only non-fatal cases. Thus, a higher case-fatality of one of the genotypes, could lead to a different association between prospective and retrospective studies. However, several studies have shown that the MTHFR 677C>T polymorphism is not associated with mortality[12-14] nor longevity.[15;16] The difference in association between prospective and retrospective studies observed in this meta-analysis was more likely to reflect heterogeneity between continents, since three large studies out of five prospective studies were North American studies.

We included both studies on coronary artery disease (CAD) and myocardial infarction (MI). We realize there might be a difference in outcome between MI and CAD, as thrombosis is a precipitating event primarily in MI. However, we were unable to address this in our pooled analysis, since information about specific endpoints was not available for all studies.

Other studies

Our summary estimate is in line with the result of a previous meta-analysis performed by Brattstrom *et al*.[15] with about 4700 cases and 5200 controls. They observed an OR of CHD of 1.11 for individuals with the TT genotype compared with the CC genotype. However, the 95% confidence interval included 1, and the authors thus concluded that the polymorphism was not associated with increased CHD risk. Nevertheless, their confidence interval included values that fitted perfectly with the risk expected beforehand, based on the difference in homocysteine concentration between TT and CC subjects.

Since individuals with the TT genotype have on average 25% higher homocysteine levels than individuals with the CC genotype, our results are in concordance with a recent pooled meta-analysis of prospective studies on homocysteine and risk of ischaemic heart disease (IHD) in which a 25% lower fasting homocysteine concentration was associated with an 11% (95% CI: 4 to 17%) lower IHD risk.[17] Furthermore, our findings are supported by the results of the majority of the studies investigating the relation between the MTHFR 677C>T polymorphism and carotid intima-media thickness (IMT), an early marker of

atherosclerosis, that have reported that individuals with a MTHFR 677T allele have larger IMT.[18]

Many studies have shown that the MTHFR 677C>T polymorphism is associated with homocysteine, mainly when folate status is marginal, and we observed that this also applies to the association with CHD. Recently, several studies showed that also riboflavin (vitamin B_2) status may modify the association between the MTHFR 677TT genotype and homocysteine.[19-21] Riboflavin is the precursor of flavin adenine dinucleotide (FAD), which is the co-factor of MTHFR. In the United States products made from refined flour have been enriched with riboflavin since the 1940s. It is likely that effect modification by dietary intake of riboflavin, in addition to folate intake may account for some of the heterogeneity between European and North American populations.[21;22] Others suggested that observed heterogeneity might also be due to linkage dis-equilibrium with other polymorphisms, for example the MTHFR 1298A>C polymorphism.[23-25]

However, effect modification cannot explain why the association between the MTHFR 677TT genotype and CHD risk was even inverse in North American populations, although the association was not statistically significant. *In vitro* studies have suggested that the TT genotype might have beneficial effects, e.g. increased DNA repair, which may counterbalance the adverse effects of the genotype via homocysteine elevation.[26-28]

Conclusions

In our pooled analysis, the MTHFR 677TT genotype was associated with a 16% increase in risk. The TT genotype was significantly associated with a 14% increase in CHD risk in European populations, but not in North American populations, which might largely be explained by interaction between the MTHFR 677C>T polymorphism and folate intake. Another potential effect modifier might be vitamin B_2. CHD risk factors do not seem to modify the association between the MTHFR 677C>T polymorphism and CHD risk. This result provides support for the hypothesis that impaired folate metabolism leading to high homocysteine concentrations is causally related to CHD risk.

The MS2756A>G polymorphism and CHD risk

Main findings

In our case-control study with 123 CHD cases and 540 healthy controls, the MS2756A>G polymorphism was not associated with fasting or post-methionine load homocysteine concentrations. However, the GG genotype showed a 4-fold increase in CHD risk, whereas the risk of the AG genotype was not elevated (table 7.1). The overall result of our subsequent meta-analysis indicated that neither the GG nor the AG genotype is associated with CHD risk. However, the overall odds ratio for the GG genotype (OR = 1.0) was strongly influenced by the quite discrepant increased risk that was found in our own study. The majority of the other available case-control studies suggested that the MS 2756GG genotype has a protective rather than an adverse effect on CHD.[29-32]

Methodological aspects

Selection bias - To increase the power of our study we a priori added a group of healthy controls from The Hague to a case-control study conducted in Rotterdam. We considered this a valid approach, as Rotterdam and The Hague are both large cities, close together in the Western part of Holland, and the populations were very similar with respect to CHD risk profile. However, the genotype distributions in controls from Rotterdam and The Hague appeared to be different, although both were in Hardy-Weinberg equilibrium. When the controls from The Hague were omitted from the analyses the association between the MS 2756GG genotype and CHD was less strong, but still positive as opposed to most other studies.
Exposure measurement - Due to miscommunication, 153 DNA samples were genotyped twice, by two different persons. When we compared the duplicates, seven of these samples (=4.6%) did not match. The discrepancies only occurred for the AA and AG genotype. These samples were analyzed for a third time and these last results were used in the analyses. Similar misclassification in the samples that were not analyzed in duplicate cannot be ruled out. However, this could not explain the large OR we found in contrast to other studies. One would expect that misclassification would have attenuated the risk rather than increasing it.
Sample size - We realize that our case-control study, but also our meta-analysis was too small to provide conclusive evidence.

Other studies

It is still not clear whether the MS 2756A>G mutation has functional consequences for enzyme activity. Studies with the bacterial enzyme, suggest the mutation lies in a region connecting the cobalamin (= vitamin B_{12}) binding domain to the activation domain. The mutated bacterial enzyme displayed a slight decrease in activity.[2] However, researchers were unable to express human MS at sufficient levels in active form to evaluate the biochemical effects of this polymorphism.[33]

Just like our study, several other studies did not show an association between the MS 2756A>G polymorphism and homocysteine concentrations.[29;34-38] Some studies, however, found that both fasting and post-methionine load homocysteine concentration decreased with increasing number of G alleles.[30;31;33;39;40]

In addition to the case-control studies that were included in the meta-analysis, three other studies investigated the association between the MS 2756A>G polymorphism and CVD. Hyndman *et al*.[35] showed that heterozygosity for MS 2756A>G polymorphism reduced risk for recurrent cardiovascular events. In the study of Wang *et al*.,[39] no association was found between the MS 2756 A>G polymorphism and severity of coronary artery disease. Recently, a study on MS 2756A>G polymorphism and thrombotic events was published. In this study, heterozygotes had lower homocysteine levels, higher levels of B-vitamins and reduced risk for thrombotic events.[41]

Conclusions

Studies investigating the association between the MS 2756A>G polymorphism and homocysteine have shown inconsistent results. Some studies showed that the GG

genotype is associated with lower homocysteine levels. This is in line with the lower CHD risk in GG subjects, as observed in the majority of the other available case-control studies. Our meta-analysis indicated that the polymorphism is not associated with CHD risk, but the result was mainly driven by the discrepant strong positive association found in our own case-control study. Before putting effort in additional epidemiological studies, it needs to be established whether this polymorphism has functional consequences for enzyme activity.

Folate requirements among individuals with the MTHFR 677TT genotype

Many studies have shown that the MTHFR 677C>T polymorphism is associated with high homocysteine levels only when folate status is low,[15] and therefore it was hypothesized that subjects with the MTHFR TT genotype might have higher folate requirements for regulation of plasma homocysteine concentrations.[42]

Main findings

In this study with 307 healthy elderly volunteers, we evaluated whether there was a difference in dose-response relation of plasma homocysteine to folic acid between MTHFR 677C>T genotypes. The main findings of the study are summarized in table 7.1. For all MTHFR 677C>T genotypes, the reduction in plasma homocysteine concentrations relative to baseline concentrations increased with increasing dose of folic acid. At the dose of 600-800 µg/day there was little additional lowering compared with 200-400 µg/day, and the relative reduction in homocysteine reached about 25% for all genotypes. However, even in the dose-group of 600-800 µg/day, homocysteine concentration after supplementation was still about 10% higher in subjects with the TT genotype compared with CC and CT genotype.

Methodological aspects

Design - Daily supplementation with 500 µg to 5 mg produces similar homocysteine –lowering effects.[43] Therefore the doses included in our study were sufficiently high to achieve maximal homocysteine reduction, which was supported by the finding that the relative homocysteine reduction leveled off at the dose of 600-800 µg/day. Furthermore the range of doses that were used are interesting from a public health point of view because they can be achieved by increasing dietary intake of folate or moderate food fortification.
Outcome - We focused on the maximal relative reduction in homocysteine, but one could also be interested in lowering homocysteine below a certain target level, for example 10 µmol/L.[44] However, there is no consensus yet with regard to target values for ‘healthy’ homocysteine levels.
Measurements - The quality of the MTHFR 677C>T genotyping was evaluated by analyzing 10% of the samples in duplicate, and randomly adding blind duplicate samples as well. Furthermore, our laboratory takes part in a national proficiency test. In all these tests the laboratory scored 100% accuracy.
Power - The study was not designed to investigate differences in dose-response relation between MTHFR 677C>T genotypes. Therefore the number of subjects with the TT genotype in each dose-group was small (maximal 7 per dose-group). To

increase the number of TT subjects per treatment group we combined the data of two subsequent dose-groups, but even then the numbers per group were small. Nevertheless, this is the first study that explored the difference in dose-response relation between MTHFR 677C>T genotypes, so for now it is the best information available.

Other studies

Results of other trials investigating the effect of folic acid supplementation within MTHFR 677C>T genotypes are shown in table 7.2. These trials confirm that TT subjects compared with CC and TT subjects show a larger relative reduction in homocysteine when supplemented with 400-500 µg folic acid per day,[45-47] but that there is no difference between genotypes with doses above 800 µg per day.[48] One study observed a larger relative reduction in homocysteine concentrations in TT subjects with folic acid doses of 1 or 2 mg per day.[49] However, the intervention period was only 3 weeks, and these differences between genotypes might have disappeared with a longer intervention period. Other studies have shown inconsistent results with regard to final homocysteine concentrations within the three MTHFR 677C>T genotypes. Even the studies that used similar doses of folic acid showed opposite results. From these trials it could not be concluded whether individuals with the TT genotype would need more folate for regulation of homocysteine since they did not investigate a dose-response relation.

Our trial had a longer intervention period than the majority of the trials mentioned above. However, we do not know what the effect would have been if we had supplemented for even a longer period. In another trial (chapter 6) we observed that subjects with the TT genotype showed a larger relative reduction in homocysteine after supplementation with 800 µg folic acid per day for one year. In addition, the final homocysteine was lower in TT subjects compared with CC and CT subjects. This could indicate that 800 µg folic acid per day is sufficiently high for TT subjects to reach homocysteine concentrations similar to those of CC and CT subjects when supplemented for a longer period than 12 weeks. However, we do not know whether similar final homocysteine concentrations could be achieved if TT subjects would be supplemented for a longer period with a lower dose of folic acid.

Conclusions

Our findings suggest that individuals with the MTHFR 677TT genotype do not need more folic acid than individuals with the CC and CT genotype to achieve at least the same maximal relative homocysteine reduction (e.g. velocity of homocysteine reduction). The required dose of folic acid appears to be no more than 800 µg per day. However, TT subjects might need to be supplemented for longer than 12 weeks to reach final homocysteine concentrations similar to CC and CT subjects.

Table 7.2 Trials investigating the effect of folic acid supplementation on homocysteine within MTHFR 677C>T genotypes

Study	Subjects	Duration	Treatment	Homocysteine results			
Fohr[45]	160 healthy	8 weeks	400 µg/d folic		TT	CT	CC
	women		acid or	baseline	7.3	8.1	8.5
	(age: 19-39)		placebo	follow up	6.9	6.9	7.4
				change	-0.5	-1.2	-1.1
				change %	-7	-14	-13
				placebo showed 18% rise in homocysteine in TT, no effect in CC and TT			
Ashfield-Watt[46]	126 healthy	3 x 4 months	400 µg/d folic		TT	CT	CC
	subjects	(cross-over)	acid or 200	baseline	12.5	9.3	8.8
	(35CC/ 35CT/		µg/d extra	*supplement*			
	36TT)		dietary folate	follow up	9.5	8.1	8.1
	(age: 39 ± 12)		or placebo	change	-3.0	-1.2	-0.8
				change %	-24	-13	- 9
				diet			
				follow up	10.2	8.2	7.9
				change	-2.3	-1.1	-0.9
				change %	-18	-12	-10
Nelen[47]	49 women	2 months	500 µg/d folic		TT	CT	CC
	with recurrent		acid	baseline	14.9	12.8	13.1
	miscarriages			follow-up	8.8	9.5	9.7
	(age: 22-41)			change	-6.9	-2.4	-3.3
				change %	-46	-19	-25
Woodside[48]	132 healthy	8 weeks	1 mg/d folic	in groups with B-vitamins			
	men with		acid + 7.2		TT	CT	CC
	homocysteine		mg/d B_6 +	baseline	11.2	10.3	9.1
	> 8.34 µmol/L		0.02 mg/d B_{12}	follow up	8.2	6.9	6.6
	(age: 30-49)		or placebo	change	-3.0	-3.4	-2.5
				change %	-26	-33	-27
Malinow[49]	143 non-	3 weeks	1 or 2 mg/d		TT	CT	CC
	vitamin users		folic acid	baseline	11.8	10.0	9.9
	discharged			follow up	9.1	8.5	9.0
	with diagnosis			change	-2.7	-1.5	-0.9
	of IHD			change%	-21	-13	-7
	(age: 45-85)						
Klerk[(chapter 6)]	276 healthy	1 year	800 µg/d folic		TT	CT	CC
	subjects		acid or	baseline	13.9	13.1	13.0
	(age: 50-70)		placebo	follow up	9.1	9.6	10.1
				change	-4.8	-3.5	-2.9
				change %*	-41	-24	-21
				** treatment vs. placebo*			

IHD = ischaemic heart disease

II Hemostasis: insight into mechanism

There is evidence that elevated homocysteine levels lead to impairment of endothelial function, which could lead to both atherosclerosis and thrombosis. Several findings, however, suggested that homocysteine promotes thrombogenesis rather than atherogenesis.[50] Therefore, we investigated the effect of homocysteine lowering by B-vitamin supplementation on hemostasis markers in healthy volunteers.

In addition, the inflammation marker C-reactive protein (CRP) was evaluated, because it has been proposed that hyperhomocysteinemia might contribute to low-grade inflammation.[51; 52] Furthermore, studies have suggested that inflammation may lead to increased propensity for coagulation[53;54] and therefore we hypothesized that changes in hemostasis in response to homocysteine lowering might be paralleled by changes in CRP.

Main findings

The main findings of the two trials we performed are summarized in table 7.1. In both trials a reduction in homocysteine concentration of about 25% was observed. In the first trial (chapter 5), a non-significant reduction in concentration of fibrin degradation products (FbDP, in chapter 5 referred to as D-dimer) concentration was observed, indicating a modest reduction in clotting activation. In our second intervention trial (chapter 6), folic acid supplementation for 1 year had no effect on any of the hemostasis markers, nor CRP levels. Furthermore, no differences in effect between MTHFR 677C>T genotypes were observed.

Methodological issues

Design - We hypothesized that homocysteine reduction would have a beneficial effect on hemostasis. However, it is also possible that B-vitamin supplementation would directly affect hemostasis, independent of homocysteine reduction. If the latter were the case this could indicate that the dose of folic acid in the second study (chapter 6) was too low to find an effect on hemostasis. However, this is unlikely, since we observed a considerable increase in serum folate levels (≈400%). An alternative explanation could be that the effect on D-dimer in the first study (chapter 5) was caused by the additional supplementation vitamin B_{12} or B_6. However, since the effect on D-dimer was not significant, it might also be due to chance.
Subjects - It might be possible that homocysteine affects hemostasis only above a certain level. In both studies, however, no clear difference in effect on hemostasis markers was observed between hyperhomocysteinemic (homocysteine > 16 µmol/L) and normohomocysteinemic subjects (data not shown). In addition, we observed no difference in effect on hemostasis between MTHFR 677C>T genotypes (chapter 6), although subjects with the TT genotype had higher baseline homocysteine concentrations and additionally showed a larger relative reduction in homocysteine. Another explanation could be that these healthy volunteers had relatively low levels of hemostasis markers compared with vascular patients, which could not be reduced any further.
Outcome - In the first trial (chapter 5) we chose to focus on the effect of clotting activation (F_{1+2}, TAT and D-dimer), since these usually intercorrelated markers are

considered as sensitive prethrombotic markers. In the second trial (chapter 6) we tried to cover all stages of hemostasis, including with markers of endothelial function and inflammation. For most of these markers there is considerable evidence that they are positively associated with or even predict risk of CVD.
Measurements - Hemostasis is a carefully regulated process. When the vascular wall is damaged, the coagulation process is immediately triggered. Therefore, collection of blood to determine hemostasis factors should be performed a-traumatically. To minimize damage to the vascular wall, the needle should be immobilized. Also the handling of the blood after collection needs extra attention. In both our studies we used strict collection and handling procedures, which were provided by the TNO Prevention & Health institute. In this way, we did the utmost to prevent that our results could have been influenced by inappropriate sample treatment. Intra- and interassay coefficients of variation (CV) for markers measured in the second study (chapter 6) were < 10%, except for FVIIa (intra-assay CV < 15%) and for TF (inter-assay CV < 15%). Unfortunately, coefficients of variation were not available for the markers measured in the first study (chapter 5).

Other studies

In table 7.3, an overview of trials investigating the effect of homocysteine lowering by B-vitamin supplementation on hemostasis factors is presented. Only three other trials investigated the effect of B-vitamin supplementation in healthy volunteers. In two of these studies a significant beneficial effect on several markers of hemostasis was observed.[55;56] However, these studies did not include a placebo group, so a period effect cannot be excluded. Recently, in a cross-over trial, folic acid supplementation had no effect on vWF, overall nor stratified by MTHFR 677C>T genotype.[57]

Five studies investigated the effect of high dose B-vitamin supplementation on hemostasis markers for a period of three months to 1 year in vascular patients. Three non-placebo controlled trials observed beneficial effects of B-vitamin supplementation on several hemostasis markers,[56;58;59] whereas a fourth did not.[60] In the only placebo-controlled trial, Thambyrajah *et al.*[61] did not find any effect on vWF.

In summary, the experimental evidence with regard to the effect of homocysteine lowering by B-vitamin supplementation on hemostasis in humans is limited and inconsistent. Furthermore it should be noted that most of these trials used pharmacological doses of B-vitamins, and the majority of the available studies was poorly designed.

Conclusions

B-vitamin or folate supplementation does not seem to have a beneficial effect on hemostasis in healthy volunteers. In addition, the effect appears not to be modified by the MTHFR 677C>T polymorphism. More well-designed intervention studies are needed to evaluate whether hemostasis is affected in individuals with higher homocysteine (or low folate) concentrations, or in patients with vascular disease.

Table 7.3 Trials on homocysteine lowering by B-vitamin supplementation

Study	Subjects	Treatment	Duration	Markers	Results
Undas[55]	17 healthy subjects with homocysteine > 16 μM	5 mg/d folic acid, 300 mg/d B_6, and 1000 μg/week B_{12}	8 weeks	fibrinogen F_{1+2} TAT antithrombin protein C protein S factor VII	Significant decrease in F_{1+2} and TAT, but no effect on other markers
Mayer[56]	26 healthy elderly subjects with homocysteine > 20 µM	5 or 10 mg/d folic acid	3 months	fibrinogen plasminogen antithrombin	Significant decrease in fibrinogen Significant increase in plasminogen Nonsig. increase in anti-thrombin
Pullin[57]	126 healthy subjects (42 MTHFR 677 CC, 42 CT and 42 TT)	0.4 mg/d folic acid, extra dietary folate or placebo	Cross-over trial with 3 * 4 months	vWF	No effect on vWF
van den Berg[58]	18 patients with CVD	5 mg/d folic acid + 250 mg/d vitamin B_6	1 year	vWF tPA FbDP TM endothelin P-selectin	Significant decrease in vWF, TM and endothelin No effect on tPA Nonsign. increase in FbDP Nonsign. decrease in P-selectin
Mayer[59]	57 with homocysteine > 20 µmol/L at high risk of CVD or with manifest CVD	placebo, followed by 10 mg/d folic acid	1 month + 2 months	fibrinogen plasminogen antithrombin vWF	Significant decrease in fibrinogen and significant increase in plasminogen, antithrombin and vWF
Constans[60]	18 patients with arterial or venous disease	5 mg/d folic acid + 250 mg/d vitamin B_6	3 months	VWF TM	No effect on fasting vWF, but significant reduction in post-methionine rise Significant decrease in baseline TM
Thambyrajah[61]	90 patients with CAD	5 mg/d folic acid or placebo	3 months	VWF	No effect on vWF
Mayer[56]	33 patients with peripheral vascular disease	5 or 10 mg/d folic acid	3 months	fibrinogen plasminogen antithrombin	Significant decrease in fibrinogen and significant increase in plasminogen and anti-thrombin

CVD = cardiovascular disease; CAD = coronary artery disease; F_{1+2} = fragments 1+2; TAT = thrombin-antithrombin; vWF = von Willebrand factor; tPA= tissue lasminogen activator; FbDP= fibrin degradation products; TM = thrombomodulin;

III Suggested alternative pathogenic mechanisms

Many pathogenic mechanisms for homocysteine have been suggested, like inhibition of nitrogen oxide (NO) synthesis and/or availability, smooth muscle cell proliferation, oxidative modification of low density lipoproteins (LDL), and platelet activation.[62-64] These are all mechanisms which are thought to be caused by oxidative stress due to auto-oxidation of homocysteine.

Homocysteine has also been associated with increased levels of asymmetric dimethylarginine (ADMA), an endogenous NO synthase inhibitor, causing impairment of endothelial function. *In vitro* studies suggested that N-methylation of arginine to ADMA may occur concomitantly with the demethylation of methionine to homocysteine. However, dietary supplementation with B-vitamins did not decrease ADMA levels, although homocysteine levels were normalized.[65]

An alternative to the oxidative stress hypothesis is the molecular target hypothesis. Homocysteine itself may interact with specific compounds and modulate their activity.[66] Homocysteine has been shown to decrease activity of gluthathion peroxidase and superoxide dismutase. Homocysteine could react with NO to form nitrosothiols.[66] Elevated homocysteine has also been associated with enhanced synthesis of homocysteine thiolactone, an intramolecular thioester of homocysteine. Homocysteine thiolactone is chemically active and may be capable of inactivating specific proteins.[67] However, there is no evidence from epidemiological studies that homocysteine thiolactone is associated with atherothrombotic disease.[66]

Homocysteine is produced during methionine metabolism via the adenosylated compounds S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy). Elevations of homocysteine levels may lead to an increase in AdoHcy. Recently, it has been shown that AdoHcy levels were higher in patients with arterial occlusive disease compared with healthy controls. Increases in AdoHcy concentration relative to AdoMet may cause hypomethylation by inhibiting methyltransferases, which may lead to reduced protein repair. Therefore, it has been suggested that elevated AdoHcy levels rather than elevated homocysteine levels is the true risk factor for CVD.[68] However, this is still a hypothesis and the exact mechanism behind it is not clear.

Several experiments have shown that folate possesses anti-oxidant potential, which may reverse the impairment of endothelial function caused by reduced NO availability. Tetrahydrobiopterin (BH4) is a co-factor of the NO-synthesizing enzyme eNOS. *In vitro* studies have shown that 5-methyltetrahydrofolate increases the BH4-dependent synthesis of NO. Consequently, low folate levels might be associated with impaired BH4-dependent NO synthesis. Weighing all the evidence, this seems the most promising pathogenic mechanism, because it is supported by the results of several studies in humans in which folate had a beneficial effect on endothelial function, independent of changes in plasma homocysteine levels.[69]

IV Overall conclusions

The result of our pooled analysis on MTHFR 677C>T polymorphism and CHD risk provides support for the hypothesis that impaired folate metabolism leading to high homocysteine concentrations is causally related to CHD risk. The hypothesis was not confirmed by studies on the MS 2756A>G polymorphism. It is not clear whether the 2756A>G mutation has functional consequences for enzyme activity. Furthermore, available observational data so far did not consistently show a relation between the MS 2756A>G polymorphism and homocysteine concentrations, nor between this polymorphism and CHD risk.

These studies showed that large numbers of subjects are required to study the association between a genetic polymorphism and disease risk, when the impact of the genetic factor is relatively small. This would imply that investigation of genetic associations in observational studies is only worthwhile for common polymorphisms (e.g. prevalence of ‘mutant genotype’ of at least 10-20%) with a strong functional impact. At this moment no other polymorphism of an enzyme involved in homocysteine metabolism that meets these criteria is known.

The results of our dose-finding study suggest that individuals with the MTHFR 677TT genotype do not need more folic acid than individuals with the CC or CT genotype to achieve the same maximal relative homocysteine reduction (i.e. velocity of homocysteine reduction). The required dose of folic acid appears to be no more than 800 µg per day. However, TT subjects might need to be supplemented for longer than 12 weeks to reach final homocysteine concentrations similar to CC and CT subjects.

Our pooled meta-analysis study could not reveal whether it is elevated homocysteine, low folate, or impaired methylation that is the true cause of CHD. Many observational studies have shown that individuals with a low folate status or intake have a higher risk of CHD and it is possible that these associations may be independent of homocysteine. Homocysteine might be just a marker of low folate status. In addition, several studies have shown a beneficial effect of folate on endothelial function independent of changes in plasma homocysteine levels.[69]

Homocysteine does not seem to affect hemostasis in healthy volunteers. More randomized placebo-controlled intervention studies are needed to evaluate whether hemostasis is affected in individuals with very high homocysteine (or low folate concentrations), or in patients with advanced vascular disease. Despite previous doubt on an atherogenic effect of homocysteine (see chapter 1), recent findings do support the hypothesis that elevated homocysteine (or low folate) concentration has an atherogenic effect. In a placebo-controlled trial, treatment with a combination of folic acid, vitamin B_{12}, and vitamin B_6 for six months significantly reduced homocysteine levels and decreased the rate of restenosis, the need for revascularization, and the incidence of major adverse events in patients who had undergone coronary angioplasty.[70;71] In addition, B-vitamin therapy has been shown to decrease risk of atherosclerotic coronary events among healthy patients, and to inhibit the progression of carotid plaques. Results of ongoing trials investigating the effect of B-vitamin supplementation on early markers of atherosclerosis, like intima-media thickness,[72] are needed to confirm this hypothesis.

V Suggestions for future research

To disentangle whether homocysteine or folate is the true underlying cause of CHD, trials should explore the effects of homocysteine lowering substances other than folate on intermediate CHD markers as flow mediated dilatation or intima-media thickness, or clinical cardiovascular endpoints. A promising example of such a homocysteine lowering agent is betaine, which is already used in the clinical practice in renal patients in whom homocysteine levels do not respond to folic acid supplementation.[73] In addition, betaine has been shown to reduce homocysteine concentrations to the same extent as folic acid, also in healthy volunteers.[74]

Furthermore, it is very important to unravel the pathogenic mechanism. Most of the research is focused on homocysteine. However, it might be equally important to focus on adverse effects of low folate. A promising hypothesis is that low folate levels might be associated with impaired NO synthesis.[69] This hypothesis should be further explored in studies with humans.

The results of our dose-finding trial investigating the difference in homocysteine response between MTHFR 677C>T genotypes need verification by a similar, but larger trial with more equal numbers of subjects with CC, CT and TT genotype. Since individuals with the TT genotype have on average higher baseline homocysteine values, it seems logical that they generally show a larger reduction in homocysteine compared with individuals with CC and CT genotype (i.e regression to the mean). Therefore, it would be informative in a next trial to be able to stratify the groups for high and low homocysteine levels (> or ≤ 15 µmol/L).

Our results suggest that increasing folate intake is likely to have a beneficial effect on CHD risk, especially in countries where food fortification with folic acid has not been applied yet. Therefore, it would be useful to continue research on factors influencing bioavailability of folate from food, and on ways of improving bioavailability.

VI Implications for public health

Our pooled meta-analysis provides indirect evidence for a benefit of increasing population mean levels of folate. Several large trials are currently underway to assess if homocysteine lowering by supplementation with folic acid and other B-vitamins can reduce the risk of CHD, and may confirm the need of increasing the population mean intake of folate to reduce risk of CHD.

Dose-finding studies have shown that the optimal dose of folic acid for adequate homocysteine reduction is 400-800 µg per day on top of the usual dietary folate intake.[44;75;76] These doses are higher than the current dietary recommendation in, for example, The Netherlands. It should be noted, however, that it is still not clear whether elevated homocysteine, low folate levels or another factor related to homocysteine metabolism is the true cause of CHD. Therefore the adequate dose of folic acid to prevent CHD might be higher or lower than 400-800 µg per day.

Current dietary recommendations have not considered targeted dietary requirements, e.g. the MTHFR 677TT genotype. At the moment it seems that 800 µg folic acid on top of their usual dietary folate intake genotype is sufficient for

individuals with the TT to lower their homocysteine concentrations. Provided the folate status is adequate, there is little clinical value of screening for MTHFR 677C>T genotype in the general population for the prediction of CHD risk.

An increase in folate intake of 400-800 µg could never be achieved by increasing natural dietary intake (e.g. a diet rich in fruit and vegetables) alone, but substantial benefits could be achieved by improving bioavailability of folate from foods. However, additional food fortification or supplementation with folic acid might be needed. In general, folic acid supplementation or fortification up to 1 mg per day is considered safe. However, there is some concern that it may mask sub-clinical vitamin B_{12} deficiency.[77] Although the potential benefits of folate with regard to CHD prevention, will outweigh this potential problem, it could be prevented by including vitamin B_{12} in the supplements or adding it to fortified foods. Besides prevention of CHD, increasing folate intake might have more beneficial health effects, since low folate status or intake is also associated with increased risk of colon cancer,[78] pregnancy complications,[79] and dementia.[80;81]

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Summary

Introduction

Cardiovascular disease (CVD) is the major cause of morbidity and mortality in the Netherlands and other Western countries. Homocysteine is a sulfur-containing amino acid formed during catabolism of the essential amino acid methionine. Defects in genes coding for enzymes or sub-optimal intake of B-vitamins (e.g. folate) involved in homocysteine metabolism can lead to cellular accumulation of homocysteine and consequently elevated plasma levels. Numerous epidemiological studies have shown a positive association between elevated homocysteine levels and CVD risk. However, whether elevated homocysteine is truly a cause of CVD is still under debate. Furthermore, the pathogenic mechanism through which homocysteine would increase CVD risk is still unclear. One of the proposed mechanisms is that homocysteine would impair hemostasis. The studies described in this thesis explored the evidence for causality of homocysteine in the occurrence and pathogenesis of coronary heart disease (CHD) by investigating the role of polymorphic genes (chapters 2, 3, and 4). In addition, we investigated the role of hemostasis as potential mechanism (chapters 5 and 6).

Polymorphic genes: investigating causality

The advantage of studying polymorphisms is that the metabolic effects of a particular genotype start early in life, and therefore the level of the risk factor - in this case homocysteine - cannot be influenced by the presence of the disease. Therefore, Investigating the association between a genetic polymorphism known to elevate homocysteine and risk of CHD can potentially reveal whether the association between homocysteine and CHD is causal.

In **chapter 2**, we assessed the relationship between the MTHFR 677C>T polymorphism and CHD in a meta-analysis of 40 observational studies, involving 11,162 cases and 12,758 controls. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the formation of 5-methyltetrahydrofolate, which serves as the methyl donor in remethylation of homocysteine to methionine. People homozygous for the for the 677C>T mutation (TT genotype) in the gene coding for MTHFR generally have higher homocysteine levels, especially when their folate status is marginal. However, the relevance of this polymorphism to CHD risk was still uncertain. Our meta-analysis showed that individuals with the TT genotype had a 16% higher CHD risk compared with the CC genotype. The TT genotype was associated with increased CHD risk only when folate status was low. This interaction with folate may explain why the TT genotype was associated with increased CHD risk in Europe, but not in North America, where folate intake is higher because of widespread use of vitamin supplements and folate fortification. In summary, these results provide support for the hypothesis that impaired folate metabolism, leading to higher homocysteine concentrations, is causally related to increased CHD risk.

In **chapter 3**, we studied whether the MS 2756A>G polymorphism was associated with plasma homocysteine, folate and vitamin B_{12} concentrations, and CHD risk using data of a Dutch case-control study comprising 123 cases with CHD and 540 controls. Methionine synthase (MS) is an enzyme that catalyzes remethylation of homocysteine to methionine. In our study, the MS 2756A>G polymorphism was not associated with homocysteine concentrations. Nevertheless, the GG genotype was associated with a 4-times higher risk of CHD compared with the AA genotype. In contrast with our finding, the majority of the other available case-control studies suggested that the MS 2756GG genotype has a protective rather than an adverse effect on CHD. Before putting effort in additional epidemiological studies, it needs to be established first whether this polymorphism has functional consequences for enzyme activity.

In **chapter 4**, we evaluated whether individuals with the MTHFR 677TT genotype have higher folate requirement for regulation of plasma homocysteine concentrations. We used data of a dose-finding study in which 307 healthy subjects aged 50-70 years were randomized to placebo, 50, 100, 200, 400, 600 or 800 µg folic acid daily for twelve weeks. To increase the number of TT subjects per treatment group, we combined data of consecutive dose-groups, i.e. 50 and 100, 200 and 400, and 600 and 800 µg/day. For all MTHFR 677C>T genotypes, the reduction in plasma homocysteine concentrations relative to baseline concentrations increased with increasing dose of folic acid. However, at the dose of 600-800 µg/day there was little additional lowering compared with 200-400 µg/day, and the relative reduction in homocysteine reached about 25% for all genotypes. However, even in the dose-group of 600-800 µg/day, homocysteine concentration after supplementation was still about 10% higher in subjects with the TT genotype compared with CC and CT genotype. Our findings suggest that individuals with the MTHFR 677TT genotype do not need more folic acid than individuals with the CC and CT genotype to achieve at least the same maximal relative homocysteine reduction (e.g. velocity of homocysteine reduction). The required dose of folic acid appears to be no more than 800 µg per day. However, TT subjects might need to be supplemented for longer than 12 weeks to reach final homocysteine concentrations similar to CC and CT subjects.

Hemostasis: insight into mechanism

In chapter 5 and 6, we investigated the effect of homocysteine lowering by B-vitamin supplementation on hemostasis markers in healthy volunteers.

Chapter 5 focused on three markers of clotting activation: prothrombin fragments 1 and 2 (F_{1+2}), thrombin-antithrombin complex (TAT), and fibrin degradation products (D-dimer). Healthy volunteers (n=118) were randomized to placebo or high-dose B-vitamin supplements (5 mg folic acid, 0.4 mg hydroxycobalamin, and 50 mg pyridoxine) daily for 8 weeks. Homocysteine concentrations were 28% reduced in the B-vitamin group compared with the placebo group. D-dimer concentrations were reduced non-significantly by 10%, but no effect on F_{1+2} and TAT concentrations was observed. Thus, these data suggested that in

healthy subjects homocysteine reduction by B-vitamin supplementation had only a modest beneficial effect on clotting activation.

In **chapter 6**, results were shown of a randomized placebo controlled trial with 276 healthy elderly. We investigated the effect of 800 µg per day folic acid supplementation for 1 year on markers of several stages of hemostasis, including markers of endothelial function (von Willebrand factor), markers of coagulation (tissue factor, Factor VIIa, fragment 1+2), and markers of fibrinolysis (fibrin degradation products, tissue-type plasminogen activator). In addition, C-reactive protein (CRP), a marker of inflammation, was measured, because effects on hemostasis might be mediated by inflammation. Although folic acid supplementation caused a considerable reduction in homocysteine (≈25%) and large increase in serum folate (≈400%) concentrations, no clear effect on hemostasis markers nor CRP was observed. Furthermore, the responses in hemostasis markers were not different between the three MTHFR 677C>T genotypes.

Taking the findings of both studies together, it seems unlikely that slightly elevated homocysteine levels increase CHD risk through effects on hemostasis in healthy populations. However, it might be possible that homocysteine affects hemostasis in patients with advanced cardiovascular disease.

Conclusions

In **chapter 7**, we have put our studies in perspective by addressing several methodological aspects, and by comparing them with other studies. The result of our pooled meta-analysis on MTHFR 677C>T polymorphism and CHD risk provides support for the hypothesis that impaired folate metabolism leading to high homocysteine concentrations is causally related to CHD risk. It should be noted, however, that it is still not clear whether elevated homocysteine per se, or rather low folate or another factor related to homocysteine metabolism is the true cause of CHD. In contrast to our study, most other studies did not show an association between the MS 2756A>G polymorphism and CHD risk. In addition, it is still not clear whether this mutation has functional consequences for enzyme activity. We further showed that individuals with the MTHFR 677TT genotype do not need more folic acid than individuals with the CC and CT genotype to achieve at least the same maximal relative homocysteine reduction (e.g. velocity of homocysteine reduction). The required dose of folic acid appears to be no more than 800 µg per day. However, individuals with the TT genotype might need to be supplemented for longer than 12 weeks to reach final homocysteine concentrations similar to CC and CT subjects. Although homocysteine reduction by folic acid supplementation does not seem to affect hemostasis effect in healthy volunteers, the results of our pooled meta-analysis suggest that increasing population mean levels of folate would reduce the incidence of CHD. The average optimal dose of folate to regulate homocysteine (at least 400 µg) could never be achieved by increasing natural dietary intake (e.g. a diet rich in fruit and vegetables) alone, but substantial benefits could be achieved by improving bioavailability of folate from foods. However, additional food fortification or supplementation with folic acid might be needed.

Samenvatting

Inleiding

Hart- en vaatziekten zijn de meest voorkomende oorzaken van ziekte en sterfte in Nederland en andere Westerse landen. Homocysteïne is een zwavelbevattend aminozuur dat in het lichaam gevormd wordt uit het aminozuur methionine dat men via de voeding binnenkrijgt. Verminderde enzymwerking of een tekort aan B-vitamines (foliumzuur, vitamine B_{12} en vitamine B_6) die betrokken zijn bij de afbraak van homocysteïne kunnen leiden tot een ophoping van homocysteïne in de cel en vervolgens tot verhoogde homocysteïnespiegels in het bloed. Vele studies hebben aangetoond dat een verhoogde homocysteïnespiegel samenhangt met een verhoogd risico op hart- en vaatziekten. Het staat echter nog ter discussie of een verhoogd homocysteïnegehalte hart- en vaatziekten kan veroorzaken of dat het eerder een gevolg is van de ziekte. Dit komt mede doordat nog niet duidelijk is via welk mechanisme homocysteïne hart- en vaatziekten zou kunnen veroorzaken. Er zijn diverse mechanismen gesuggereerd en één daarvan is dat een verhoogd homocysteïnegehalte zou kunnen leiden tot een verhoogde neiging tot bloedstolling. In dit proefschrift wilden we met behulp van studies bij mensen met een genetische aanleg voor verhoogde homocysteïnespiegels aantonen dat homocysteïne een oorzakelijke rol speelt bij het ontstaan van hart- en vaatziekten. Verder hebben we onderzocht of het schadelijke effect van homocysteïne via een verhoogde neiging tot bloedstolling zou kunnen lopen.

Genetische aanleg voor hoog homocysteïne

Het voordeel van onderzoek bij mensen met een genetische aanleg voor verhoogde homocysteïnespiegels is dat het effect van deze genetische aanleg al begint bij de geboorte en dus niet door de aanwezigheid van ziekte kan zijn veroorzaakt. Daarom is dit soort onderzoek uitermate geschikt om te bestuderen of verhoogde homocysteïnespiegels een oorzakelijke rol spelen bij het ontstaan van hart- en vaatziekten.

In **hoofdstuk 2** hebben we de relatie tussen een bepaalde genetische variant in de homocysteïne stofwisseling en coronaire hartziekten (ziekte van de kransslagaders van het hart) onderzocht door data van 40 studies (in totaal 11.162 patiënten met coronaire hartziekten en 12.758 gezonde controles) samen te voegen in een zogenaamde meta-analyse. Methyleentetrahydrofolaat reductase (MTHFR) is een enzym dat helpt bij de omzetting van homocysteïne naar methionine. Bij deze omzetting is ook het B-vitamine foliumzuur betrokken. Bij mensen met een bepaalde variant in het gen dat de productie van het MTHFR enzym regelt, werkt het enzym slechter. Daarom hebben deze mensen over het algemeen hogere homocysteïnespiegels in het bloed, met name als het gehalte aan foliumzuur in hun bloed laag is. Tot nu toe was echter niet duidelijk of deze genetische variant van MTHFR ook een rol speelt bij het ontstaan van coronaire hartziekten. Onze studie

heeft aangetoond dat mensen met deze genetische variant van MTHFR een 16% hoger risico hadden op het krijgen van coronaire hartziekten dan mensen met normaal werkend MTHFR enzym. Dit was echter alleen het geval bij mensen met een laag gehalte aan foliumzuur in hun bloed. Dit verklaart waarschijnlijk grotendeels waarom de variant wel samenhing met een hoger risico op coronaire hartziekten in Europese studies, maar niet in Noord-Amerikaanse studies, waar de inname van foliumzuur over het algemeen hoger is door het gebruik van multivitamines en als gevolg van voedselverrijking met foliumzuur. Samengevat ondersteunen deze resultaten de hypothese dat verhoogde homocysteïnespiegels in het bloed een oorzakelijke rol spelen bij het ontstaan van coronaire hartziekten.

In **hoofdstuk 3** hebben we gekeken naar een genetische variant van een ander enzym dat betrokken is bij de omzetting van homocysteïne naar methionine, het enzym methionine synthase (MS). We gebruikten hiervoor gegevens van 123 patiënten met coronaire hartziekten en 540 gezonde personen. In onze studie was er geen duidelijk verschil in homocysteïnegehalte tussen mensen met de variant van het MS enzym en mensen met een normaal MS enzym. Mensen met de MS variant hadden een sterk verhoogd risico op het krijgen van coronaire hartziekten vergeleken met mensen met het normale enzym. Een aantal andere studies heeft echter laten zien dat mensen met de MS variant juist een lager risico op coronaire hartziekten hebben dan mensen met het normale enzym. Deze studie kon dus geen uitsluitsel geven over een mogelijk oorzakelijk verband tussen homocysteine en CHD. Voordat er nog meer onderzoek gedaan wordt naar de relatie tussen the MS variant en coronaire hartziekten, zou eerst uitgezocht moeten worden of de genetische variant van het MS enzym slechter of misschien juist beter zou werken.

In **hoofdstuk 4** hebben we geëvalueerd of mensen met de MTHFR variant meer foliumzuur nodig zouden hebben om hun homocysteïnegehalte te reguleren. We hebben hiervoor de data gebruikt van een studie met 307 gezonde vrijwilligers die gedurende 12 weken supplementen hebben gebruikt met daarin 0, 50, 100, 200, 400, 600 of 800 µg (1000 µg = 1 mg) foliumzuur per dag. Omdat het aantal mensen met de MTHFR variant in elke dosisgroep erg klein was hebben we in de analyses de resultaten van twee opeenvolgende dosisgroepen samengevoegd, ofwel 50 en 100, 200 en 400, en 600 en 800 µg/dag. Uit de resultaten bleek dat voor zowel mensen met de MTHFR variant als mensen met het normale MTHFR de procentuele daling in homocysteïnespiegels toenam met toenemende dosis foliumzuur. Het verschil in effect tussen 200-400 groep en de 600-800 groep was niet zo heel groot. Voor zowel mensen met het normale MTHFR als mensen met de variant was de maximale procentuele daling ongeveer 25%. Echter, zelfs in de 600-800 groep hadden mensen met de MTHFR variant na 12 weken nog steeds ca. 10% hogere homocysteïnespiegels dan mensen met het normale MTHFR enzym. Onze resultaten suggereren dat mensen met de MTHFR variant niet meer foliumzuur nodig hebben dan mensen met het normale MTHFR enzym voor eenzelfde procentuele daling in het homocysteïnegehalte. Het is nog onduidelijk of zij bij langdurige suppletie dezelfde lage homocysteïnespiegels bereiken als mensen met het normale MTHFR enzym.

Homocysteine en bloedstolling

In hoofdstuk 5 en 6 hebben we bij gezonde mensen het effect onderzocht van homocysteïneverlaging met vitamine B supplementen op stollingsfactoren in het bloed.

In **hoofdstuk 5** hebben we gekeken naar drie factoren die de activatie van het stollingsproces weerspiegelen. De studie duurde 8 weken en er namen 118 gezonde vrijwilligers aan deel, welke verdeeld waren over twee groepen. De ene groep kreeg dagelijks een vitaminesupplement met daarin foliumzuur, vitamine B_{12} en vitamine B_6; de andere groep kreeg een placebo. Ondanks het feit dat de B-vitamines een 28% daling van het homocysteïnegehalte veroorzaakten, werd op slechts één van de stollingsfactoren klein gunstig effect gevonden.

In **hoofdstuk 6** hebben we de resultaten laten zien van een studie met 276 gezonde ouderen. De deelnemers waren ook weer verdeeld over 2 groepen. De ene groep kreeg een jaar lang dagelijks een vitaminesupplement met daarin 800 µg foliumzuur, de andere groep kreeg een placebo. In deze studie hebben we het effect van het vitaminesupplement onderzocht op stoffen in het bloed die verschillende stadia van het stollingsproces weerspiegelen; dus zowel factoren die een rol spelen het ontstaan van het werkelijke stolsel, als factoren die betrokken zijn bij de afbraak van een stolsel. Daarnaast werd ook een factor meegenomen die het optreden van ontstekingen weerspiegelt, omdat gesuggereerd wordt dat het effect van homocysteïne op stolling via het bevorderen van ontsteking zou kunnen lopen. Hoewel de foliumzuursupplementen een duidelijke verlaging van het homocysteïnegehalte veroorzaakten, werd geen effect gevonden op de stollingsfactoren, noch op de ontstekingsfactor. Verder was er ook geen verschil in effect van het foliumzuursupplement tussen mensen met een normaal MTHFR enzym en mensen met de genetische variant.

De resultaten van deze 2 studies suggereren dat het niet waarschijnlijk is dat licht verhoogde homocysteïnespiegels bij gezonde mensen het risico op hart- en vaatziekten zouden verhogen via effecten op het stollingsproces. Het zou echter zo kunnen zijn dat homocysteïne wel een effect heeft op het stollingsproces bij mensen die al hart- en vaatziekten hebben.

Conclusies

In **hoofdstuk 7** hebben we de resultaten van ons onderzoek in een breder kader besproken door diverse methodologische aspecten te belichten en onze studies te vergelijken met andere studies. De resultaten van onze meta-analyse naar de relatie tussen de MTHFR variant en het risico op coronaire hartziekten ondersteunen de hypothese dat verhoogde homocysteïnespiegels in het bloed een oorzakelijke rol spelen bij het ontstaan van coronaire hartziekten. Wel moet opgemerkt worden dat het nog niet duidelijk is of het nu het verhoogde homocysteïnegehalte zelf is, of eerder een laag foliumzuurgehalte in het bloed, of mogelijk zelfs een andere aan homocysteïne gerelateerde stof die hart- en vaatziekten veroorzaakt. In tegenstelling tot onze eigen studie lieten de meeste andere studies geen duidelijk verband zien tussen de MS variant en het risico op coronaire hartziekten We weten echter ook nog

niet of de variant van het MS enzym slechter of misschien juist beter werkt dan het normale MS enzym. Verder hebben we aangetoond dat mensen met de MTHFR variant niet meer foliumzuur nodig hebben dan mensen met het normale MTHFR enzym om eenzelfde procentuele daling in homocysteïne (m.a.w. zelfde snelheid van daling in homocysteïne) te bereiken. Zij moeten waarschijnlijk wel langer dan 12 weken foliumzuursupplementen gebruiken om dezelfde lage homocysteïnespiegels te bereiken als mensen met het normale MTHFR enzym. Al lijkt het er niet op dat homocysteïneverlaging door foliumzuursupplementen een gunstig effect heeft op het stollingsproces, de resultaten van onze meta-analyse suggereren wel dat een verhoging van de inneming van foliumzuur door de algemene bevolking het aantal gevallen van coronaire hartziekten zou kunnen verlagen. De gemiddelde dosis extra foliumzuur die nodig is voor een maximale daling van het homocysteïnegehalte wordt geschat op ten minste 400 µg per dag. Het is moeilijk om deze extra hoeveelheid via de dagelijkse normale voeding te bereiken (bijvoorbeeld met een voeding rijk aan groente en fruit). Het is echter aannemelijk dat iedere stijging in inneming van foliumzuur toch een voordeel voor de volksgezondheid zou kunnen betekenen. De inneming van foliumzuur zou substantieel verbeterd kunnen worden door ervoor te zorgen dat het in de voeding aanwezige foliumzuur beter door de darm zou kunnen worden opgenomen. Daarnaast kan het nodig zijn om voedsel met foliumzuur te verrijken of om foliumzuursupplementen te gebruiken.

Appendix

MTHFR 677C>T polymorphism and coronary heart disease – exploring effect modification by classical CHD risk factors

Mariska Klerk, Petra Verhoef, Frans J. Kok, and Evert G. Schouten

Introduction

In observational studies, individuals with elevated levels of plasma homocysteine tend to have moderate increases in risk of coronary heart disease (CHD). The MTHFR 677C>T polymorphism is a genetic alteration in an enzyme involved in folate metabolism that causes elevated homocysteine concentrations. In a pooled meta-analysis of individual participant data from 40 observational studies, we assessed the relation of the MTHFR 677C>T polymorphism with CHD risk. We showed that the MTHFR 677TT genotype is associated with a 16% increase in risk of CHD (see chapter 2).[1] This result provides support for the hypothesis that impaired folate metabolism, leading to elevated homocysteine levels, plays a causal role in the occurrence of CHD. However, continent specific analyses showed marked differences between the results obtained in Europe, compared with North American populations. The MTHFR 677TT genotype was significantly associated with a 14% increase in CHD risk in Europe. However, in North America an inverse, although not significant, association with CHD risk was observed. This heterogeneity could probably largely be explained by interaction between the MTHFR 677C>T polymorphism and folate status (see chapter 2). However, the difference between the associations observed in Europe and North America might additionally be explained by effect modification by factors other than folate status. Two studies showed a synergistic effect of the MTHFR 677TT and classical CHD risk factors.[2;3] Another study, however, showed that the risk of CHD conferred by the MTHFR 677TT genotype was larger in cases without other CHD risk factors, than in cases with other CHD risk factors.[4] Furthermore, it has been suggested that the strong positive association between MTHFR 677C>T polymorphism and CHD found in Japanese studies might be explained by the generally lower cholesterol levels and lower BMI in these populations compared with Western countries.[5]

We explored whether differences in CHD risk profile between European and North American populations might have contributed to the observed heterogeneity with respect to the association between the MTHFR 677C>T polymorphism and CHD risk in our pooled meta-analysis.

Methods

The analyses were performed using data of our meta-analysis of individual participant data from 40 observational studies (11,162 cases and 12,758 controls), that investigated the association between the MTHFR 677C>T polymorphism and CHD risk. A detailed description of the study selection and data collection is presented in chapter 2. Data on CHD risk factors (age, sex, BMI, hypertension, hypercholesterolemia, diabetes, current smoking and alcohol use) were available for 10-45% of the studies, depending on the risk factor. The meta-analysis comprised 22 European studies and 10 North-American. Data of 8 studies from various other continents were not taken into account in these additional analyses.

A certain factor could only explain the difference in CHD risk between Europe and North America if three criteria were fulfilled: 1) there had to be a difference between European and North American studies with regard to that factor, 2) the factor had to be an effect modifier of the association between the MTHFR 677C>T polymorphism and CHD risk, and 3) the direction of the effect modification and the difference under 1 combined had to be a logical explanation for the observed difference in CHD risk between the continents.

To address the first two issues, we performed the following analyses. First, means and frequencies of CHD risk factors in cases and controls were calculated for Europe and North America. Second, we explored possible interaction between the MTHFR 677C>T polymorphism and CHD risk factors, by calculating ORs within strata of CHD risk factors. In addition, per study means and the frequencies of CHD risk factors in controls were calculated and plotted against the corresponding OR of CHD for TT versus CC genotype. Also correlations between each of the CHD risk factors and the ORs were assessed by calculating Spearman correlation coefficients.

Results

Table A.1 shows means and frequencies of CHD risk factors for cases and controls, separately for Europe and North America. In both cases and controls, there were no clear differences in mean age or BMI, frequency of males or prevalence of diabetes between European and North American populations. However, the prevalence of hypercholesterolemia and frequency of current smoking and alcohol use was higher in Europe compared with North America. Furthermore, in North American controls compared with European controls, the prevalence of hypertension was slightly higher.

Table A.1 Means and frequencies of CHD risk factors in cases and controls, separately for Europe and North America

	Cases		Controls	
	N	**Mean ± SD or %**	**N**	**mean ± SD or %**
MTHFR 677C>T genotype (%)				
Europe	6207	-	8343	-
North America	3146	-	2532	-
Age (years)				
Europe	4376	56 ± 11	6106	51 ± 13
North America	3105	57 ± 10	2514	53 ± 11
BMI (kg/m^2)				
Europe	1672	26.6 ± 3.7	2268	25.7 ± 3.7
North America	1427	26.8 ± 4.3	1576	25.6 ± 4.1
Sex (% male)				
Europe	4952	80	6403	65
North America	3143	81	2532	62
Hypertension (%)				
Europe	3759	47	5311	17
North America	2201	43	2068	25
Hypercholesterolemia (%)				
Europe	3507	35	5116	18
North America	2219	23	2118	12
Diabetes (%)				
Europe	3738	16	5249	5
North America	2114	14	2147	4
Current smoking (%)				
Europe	3771	41	5299	28
North America	1640	25	2035	17
Alcohol use (%)				
Europe	1011	72	1817	75
North America	1565	65	1999	69

Table A.2 ORs of CHD for MTHFR 677TT versus CC, stratified for CHD risk factors

	OR	95% confidence interval
Age (years)		
≤ 55	1.17	1.05 to 1.31
> 55	1.24	1.01 to 1.52
BMI (KG/M^2)		
≤ 25	1.09	0.87 to 1.37
> 25	1.05	0.86 to 1.28
Sex		
Men	1.17	1.05 to 1.31
Women	1.24	1.01 to 1.52
Hypertension		
No	1.12	0.98 to 1.28
Yes	1.14	0.92 to 1.42
Hypercholesterolemia		
No	1.22	1.07 to 1.38
Yes	1.02	0.78 to 1.32
Diabetes		
No	1.08	0.96 to 1.22
Yes	0.90	0.59 to 1.36
Smoking		
No	1.09	0.95 to 1.26
Yes	1.14	0.93 to 1.39

Table A.2 shows the ORs of CHD for the TT versus the CC genotype within strata of CHD risk factors. For hypercholesterolemia and diabetes there was a difference between the ORs of the two strata, although confidence intervals were large and overlapping. TT subjects without hypercholesterolemia had increased risk of CHD, whereas TT subjects with hypercholesterolemia did not. However, since the prevalence of hypercholesterolemia was higher in Europe compared with North America, the observed interaction between the MTHFR 677C>T polymorphism and hypercholesterolemia could not explain the higher OR of CHD observed in Europe. A slight positive association between the TT genotype and CHD risk was observed in subjects without diabetes, whereas there was a slight inverse association in subjects with diabetes. However, interaction between the MTHFR 677C>T polymorphism and diabetes could not explain heterogeneity, because there were no differences in

prevalence of diabetes between Europe and North America. For the other CHD risk factors the differences in OR between the strata were so small in relation to the large confidence intervals, that we cannot speak of clear interaction between the risk factor and the MTHFR 677C>T polymorphism.

Figure A.1 combines the information of tables A.1 and A.2 by showing plots of study specific means or frequencies of CHD risk factors in controls against the corresponding OR of CHD for TT versus CC genotype. In this figure, European and North American studies can be distinguished. For most CHD risk factors, European and North American studies were about equally distributed over the range of the risk factor. However, in all North American studies the percentage of current smokers was < 25%, while in the majority of the European studies the percentage of current smokers was > 25%. None of the CHD risk factors, including smoking, was clearly correlated with the OR of CHD for TT versus CC genotype ($r<|0.5|$ and $P>0.10$). Therefore, these figures confirm that it is unlikely that the difference in association between the MTHFR 677TT genotype and CHD risk between European and North American studies would be explained by differences in CHD risk profile.

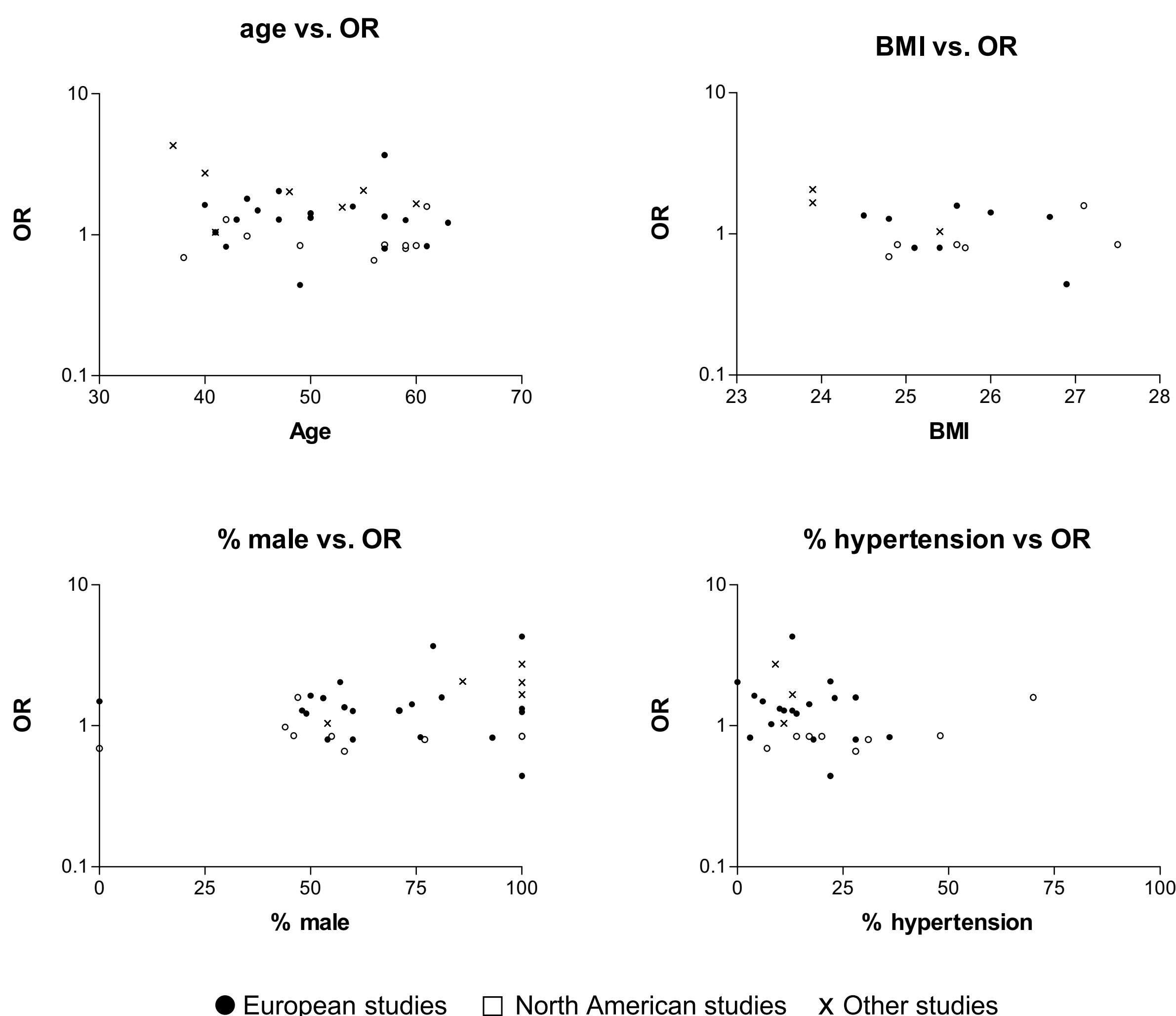

Figure A.1 Study specific means or frequencies of CHD risk factors versus the OR of CHD for TT versus CC genotype

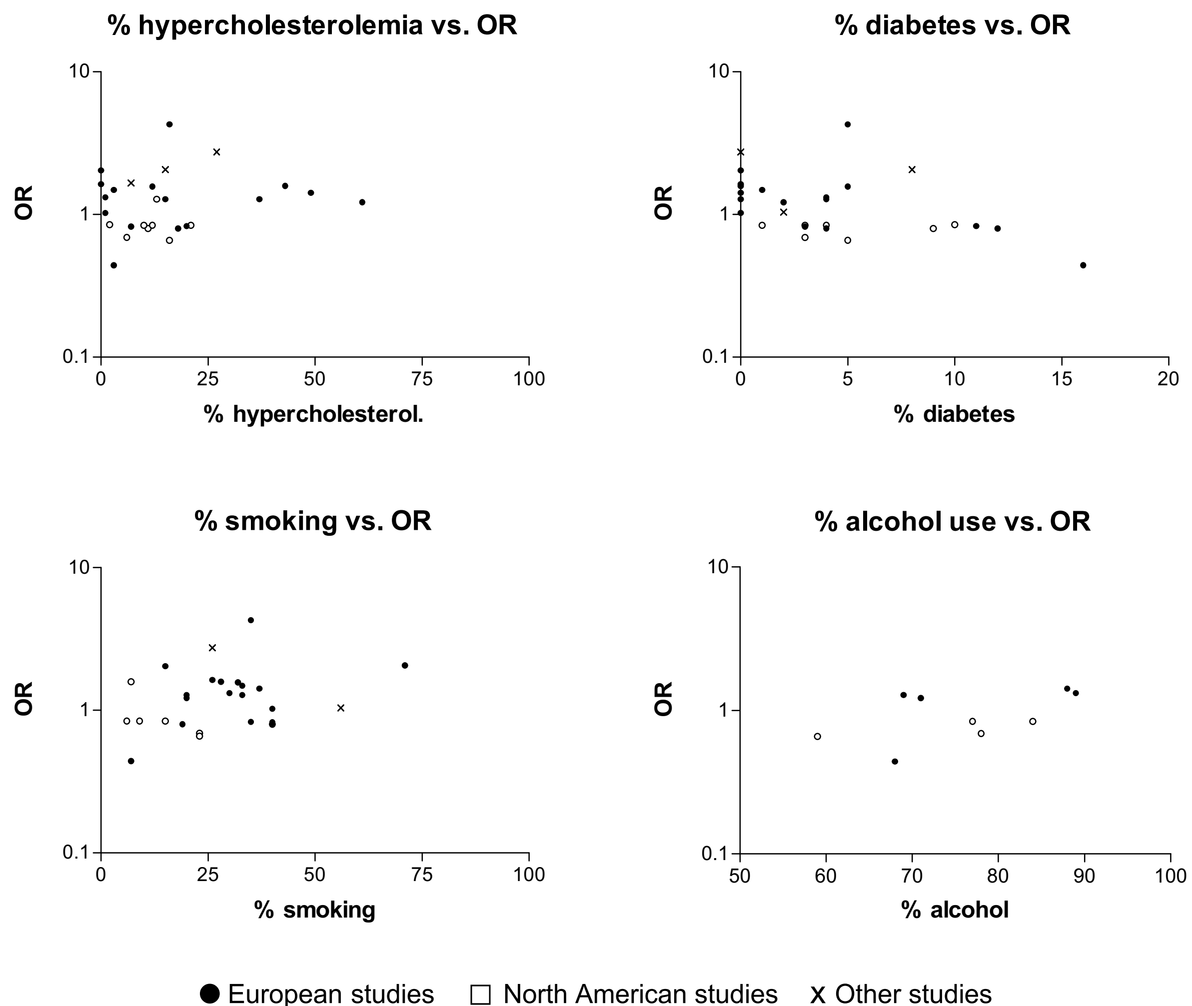

Figure A.1 *continued*

Discussion

Stratified analyses, in a pooled meta-analysis of observational studies investigating the association between the MTHFR 677C>T polymorphism and CHD risk, suggested that risk of CHD risk was increased in TT subjects without hypercholesterolemia or diabetes. However, the prevalence of hypercholesterolemia was higher in Europe compared with North America, and there were no differences in prevalence of diabetes between the two continents. Therefore, these interactions could not explain why the the MTHFR TT genotype is associated with increased risk in Europe, but not in North America.

The observed interactions for hypercholesterolemia and diabetes might be explained by the hypothesis that the MTHFR 677TT genotype is only associated with increased CHD risk in absence of stronger CHD risk factors as suggested by two previous papers.[4;5] Two other papers, however, suggested the opposite, i.e. a synergistic effect of the MTHFR 677C>T polymorphism and conventional risk factors.[2;3] Since the power for these stratified analyses was only small, the observed interactions for hypercholesterolemia and diabetes might just be due to chance.

Power calculation revealed that we needed more than > 10, 000 cases and an equal amount of controls to be able to give a valid estimate of the crude association between the MTHFR 677C>T polymorphism and CHD risk. To have enough power to investigate interaction properly, we would need at least 4 times more cases and controls,[6] which are just not available at this moment.

In conclusion, our findings suggest that it is unlikely that interaction between the MTHFR 677C>T polymorphism and CHD risk factors might have contributed to the difference in association between the MTHFR 677TT genotype and CHD risk between European and North American studies as observed in this pooled meta-analysis of observational studies.

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About the author

Mariska Klerk was born on April 12 1972 in Vlaardingen, the Netherlands. In 1990, she passed secondary school, Atheneum at the 'Scholengemeenschap Groen van Prinsterer' in Vlaardingen. In the same year she started the study 'Nutrition Technology' at the Haagse Hogeschool. In 1991, she switched and started the study 'Human Nutrition' at the former Wageningen Agricultural University. As part of the study she conducted research projects at the former Department of Human Nutrition and at the Department of Integrated Food Science of the Wageningen Agricultural University. Furthermore, she had a practical training at TNO-Nutrition in Zeist, the Netherlands and Laboratoire de Nutrition Humaine in Clermont-Ferrand, France. In September 1996, she received her MSc-degree. After her graduation she worked on literature reviews for the Dutch Health Council, Coberco Research, and the Division of Human Nutrition & Epidemiology of the Wageningen University. In August 1998, she was appointed as a PhD-fellow at the Division of Human Nutrition & Epidemiology of Wageningen University on the NWO-project 'Mild hyperhomocysteinemia, hemostasis and coronary heart disease: the role of common mutation and vitamins'. She joined the education program of the Graduate School VLAG (advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences). In 1998 and 2000 she attended the Erasmus Epidemiology Summer Program in Rotterdam. She was the chair of the PhD-board (1998-2000), and PhD-representative in the executive board of the Division of Human Nutrition & Epidemiology (1999-2000).

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