HOMOCYSTEINE
IN
CORONARY ARTERY DISEASE

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HOMOCYSTEINE IN CORONARY ARTERY DISEASE

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

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door

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“ Om het losschieten van een knoop te voorkomen, heb je frictie nodig, en om frictie te doen ontstaan moet er enigerlei druk zijn. Deze druk, in samenhang met de plek in de knoop waar die ontstaat, heet de kneep. De betrouwbaarheid van een knoop lijkt enkel en alleen af te hangen van de kneep”

Uit: the Ashley book of knots

Voor Lucie

Contents

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Chapter

Introduction

Atherosclerotic disease is the leading cause of death in Western society. Established risk factors as hypercholesterolaemia, hypertension, diabetes mellitus and smoking only explain a part of the number of patients with cardiovascular disease. More recently, hyperhomocysteinemia has also been considered as an additional risk factor for vascular disease[1,2] .
Patients with severe hyperhomocysteinemia or classical homocystinuria caused by a deficiency in cystathionine β-synthase, a rare inborn error of metabolism, suffer from serious vascular disease[3]. Also, a blockade of homocysteine remethylation caused by defects of methylenetetrahydrofolate reductase or methionine synthase activities can lead to very high levels of plasma homocysteine which results in a high risk of arterial and venous vascular disease[4]. This observation has brought up the hypothesis that homocysteine is toxic to the vascular wall and that mild hyperhomocysteinemia is a possible risk factor for coronary artery disease [5]. Over the past twenty years retrospective and prospective studies have demonstrated an association between hyperhomocysteinemia and arterial vascular disease[6]. The relationship between homocysteine and vascular disease is based on:

a) the observations of premature vascular disease in patients with homocystinuria
b) the relationship between plasma homocysteine and both clinical vascular disease as well as preclinical atherosclerotic disease
c) the relationship between homocystinuria in children and premature vascular disease in their parents or relatives
d) improvement of cardiovascular disease or surrogate endpoints after homocysteine lowering therapy.

However it still remains unclear whether elevated homocysteine levels itself are causally related to the increased cardiovascular risk or whether homocysteine is just a marker of the process involved in atherosclerotic disease[7].

Evidence is accumulating that elevated homocysteine causes endothelial dysfunction but the mechanisms responsible for endothelial dysfunction in

hyperhomocysteinemia are poorly understood[8]. Several investigators have shown that homocysteine reduces the bioavailability of NO and enhances smooth muscle cell proliferation, both of which are important markers of athero-thrombotic disease [8]. Recently it was hypothesized that hyperhomocysteinemia may stimulate the formation of asymmetrical dimethylarginine, an endogenous inhibitor of NO synthase[9] . The relationship between hyperhomocysteinemia and endothelium-derived hyperpolarizing factor is less clear.
A meta-analysis of 12 studies revealed that folic acid lowered total plasma homocysteine levels by 25%[10]. Addition of vitamin B12 led to a further reduction of 7%. Vitamin B6 did not lower fasting homocysteine levels but uncontrolled studies have shown that post-load homocysteine levels were reduced by 21% to 42 % [10,11].

Aim of this thesis:

This thesis focuses on the relationship between homocysteine and coronary artery disease with emphasis on coronary endothelial function and progression of atherosclerosis. Secondly we investigated the relationship between genetic determinants of hyperhomocysteinemia focusing on possible optimal therapeutic strategies.
With regard to the relationship of homocysteine and coronary artery disease, the following questions were addressed:

1) Is hyperhomocysteinemia related to the extent and progression of atherosclerosis in coronary vessels?
2) Is hyperhomocysteinemia associated with coronary endothelial function?
3) Can homocysteine-lowering therapy in hyperhomocysteinemic patients improve coronary endothelial function?
4) Can homocysteine-lowering therapy in normo-homocysteinemic patients improve coronary endothelial function?
5) What is the optimal treatment regime with regard to homocysteine-lowering?

With regard to genetic determinants of hyperhomocysteinemia the following questions were raised:

1) Is the common thermolabile mutation in the MTHFR gene, 677 C→T associated with elevated plasma homocysteine and coronary artery disease?
2) Concerning patients with this mutation, is it possible to optimize the treatment regimen?

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Chapter

Homocysteine in coronary artery disease

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More than 80 cross-sectional, case-control and prospective cohort studies have been published dealing with the relationship between homocysteine and arterial vascular disease [1]. Most but not all studies demonstrated a relationship between elevated plasma homocysteine levels and an increased risk for vascular disease. In 1976 Wilcken, et al. were the first to report on 25 patients with coronary artery disease of whom 7 patients had elevated homocysteine levels after loading with methionine in the range of obligate heterozygotes of cystathionine β-synthase [2]. In 1985, Boers reported that patients with premature peripheral vascular disease and cerebrovascular arterial occlusive disease had post load hyperhomocysteinemia in 28% of the cases[3]. These findings were confirmed in 1991 by Clarke et al., who showed that post-load hyperhomocysteinemia is associated with premature coronary artery disease, cerebrovascular disease and peripheral arterial disease[4]. A meta analysis of 27 reports from 1988 to1994 on 2500 patients with coronary artery disease, 900 with cerebrovascular disease and 700 with peripheral disease showed a calculated summary odds ratio as an estimation of the relative risk in persons with elevated homocysteine levels of 1.7 (95% CI 1.5-1.9) for coronary artery disease, 2.5 (95% CI 2.0-3.0) for cerebrovascular disease and 6.8(95% CI 2.9-15.8) for peripheral arterial disease[5].

The European Concerted Action Project on hyperhomocysteinemia and vascular disease was started to provide robust confirmation of hyperhomocysteinemia being associated with arterial occlusive disease and to determine possible interactions with the established risk factors[6]. Fasting and post-load homocysteine levels in the upper quintile of the distribution of levels were considered hyperhomocysteinemic. In this population of 750 cases and 800 controls with relative low cut-off points, the odds ratio for arterial occlusive disease was 2.2 (95% CI: 1.7-2.7) of fasting plasma homocysteine levels and 2.1 (95% CI: 1.7-2.7) of post load hyperhomocysteinemia. These odds ratios did not change after adjustment for other risk factors suggesting that mild hyperhomocysteinemia is an independent risk factor for arterial vascular disease. In contrast to earlier meta analyses, the odds ratios for coronary artery disease,

cerebrovascular disease and peripheral disease were not different. More recent meta-analysis demonstrated that stronger associations were observed in retrospective studies where homocysteine was measured in blood collected after the onset of disease than in prospective studies among individuals who had no history of cardiovascular disease when blood was collected [7]. With regard to the consistency of the finding of a positive association between hyperhomocysteinemia and premature vascular disease, it is important to note that to date, more than 20 prospective studies of the topic have been published. Among these, the population-based, nested, case-control studies showed that a 5 μmol/L increment in total plasma homocysteine results in a 20–30% increase in cardiovascular risk, which is substantially lower than the 60–90% risk enhancement shown in the retrospective case-control studies[7]. The prospective studies also suggested that the risk is highest during short-term follow-up and is attenuated after 3–4 years. Notably, total plasma homocysteine is a particularly strong predictor of cardiovascular events or death in subjects with pre-existing illness.

The meta-analysis of the Homocysteine Studies Collaboration Group demonstrated that after adjustment for known cardiovascular risk factors and regression dilution bias in the prospective studies, a 25% lower homocysteine level was associated with a 11% (OR, 0.89; 95% CI: 0.83-0.96) lower risk on coronary artery disease and a 19% (OR, 0.81; 95% CI: 0.69-0.95) lower risk on stroke [8]. So, all meta-analyses confirm that elevated plasma homocysteine is a modest independent predictor of coronary artery disease and of stroke risk in healthy populations.

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Chapter

Genetic determinants of hyperhomocysteinemia and cardiovascular risk

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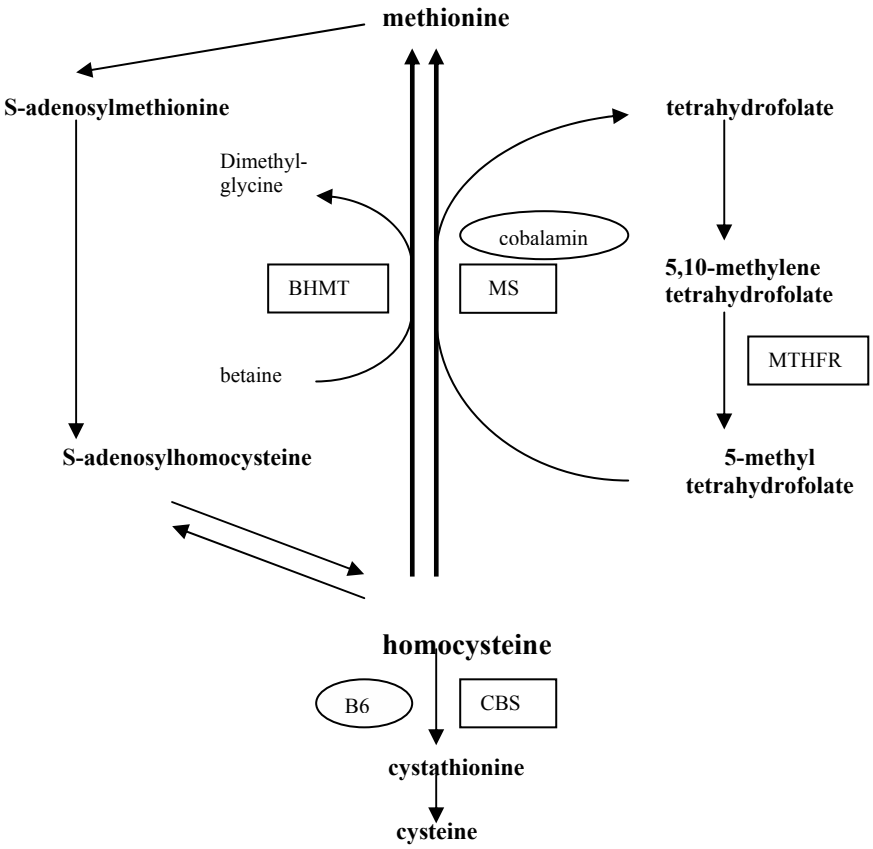
Homocysteine metabolism

Homocysteine is a sulphydryl-containing amino acid derived from the metabolic demethylation of methionine, an essential amino acid. Two pathways, the transsulphuration pathway and the remethylation pathway are involved in the conversion of homocysteine.



Enzymes involved in homocysteine metabolism

1) *CBS: cystathionine β-synthase*
2) *MS: methionine synthase*
3) *MTHFR: methylene tetrahydrofolate reductase*
4) *BHMT: betaine-homocysteine methyltransferase*

Vitamin B6 and cobalamin are co-factors for cysthathionine *β*-synthase and methionine synthase respectively. Folate and betaine are co-substrates

Homocysteine can be irreversibly degraded to cystathionine by cysthathionine β-synthase (CBS) which requires pyridoxal-5-phosphate, an active form of vitamin B6 in order to function adequately[6]. Cystathionine is further converted to cysteine by γ-cystathionase. The transsulphuration pathway has a limited distribution and is found primarily in organs like kidney and liver. Homocysteine can also be metabolized via two methionine-conserving remethylation pathways. A substantial portion of homocysteine is remethylated in the liver or the kidney using betaine-homocysteine methyltransferase (BHMT) with betaine as methyl donor. The second remethylation pathway uses methionine synthase (MS) in the conversion of homocysteine to methionine. In this reaction 5-methyltetrahydrofolate, formed out of 5,10- methylene tetrahydrofolate by methylene tetrahydrofolate reductase (MTHFR) serves as methyl donor. MS requires the co-factor cobalamin (vitamin B12) for the methyl transport from 5-methyltetrahydrofolate to homocysteine. MS is activated by S-adenosylmethionine [7]. The MTHFR dependant methionine synthase remethylation pathway is located in almost all tissues.

Association between cystathionine β-synthase deficiency and cardiovascular risk

One of the main regulating enzymes in homocysteine metabolism is cystathionine β-synthase (CBS). Mutant CBS enzyme is an important determinant of hyperhomocysteinemia and is biochemically characterized, in homozygous form, by very high levels of plasma homocysteine, homocystinuria, hypermethionemia and hypocysteinemia. This inborn error of metabolism is inherited as an autosomal recessive trait and is the most common cause of homocystinuria with a worldwide incidence of 1:344.000 living births with the highest incidence in Ireland at 1:65.000[1]. The reported world wide incidence has been based on new-born screening and may be an underestimation of the true incidence. A recent study showed that the incidence of mutated CBS gene in homozygous form may be as high as 1:20.500 in a Danish population[2]. Until

now, about 100 different mutations have been described in the CBS gene[3], which is located on chromosome 21q22.3[4,5]. Relatively common mutations in homozygous form in West-European countries responsible for the classical homocystinuric phenotype are 833T→C with the amino acid substitution isoleucine to threonine(I278T), which confers vitamin B6 responsiveness and in Celtic populations the 919G→A with the amino acid change glycine to serine(G307S), which represents severe pyridoxine non-responsiveness. Together, they represent almost 75% of the homocystinuric alleles analysed[3]. Approximately 50% of the patients who are CBS deficient respond to pyridoxine, and this responsiveness is constant within sibships[6]. Patients who are homozygous for CBS deficiency have a very high risk for arterial and venous vascular disease. Pooled data on homozygous CBS deficient patients showed that before the age of 30 more than half of the patients had a severe vascular event[6]. Recent studies have revealed that providing homocysteine lowering therapy for these patients reduces the risk of a major vascular event with about 90 %[7].
In two early studies, CBS activity was studied in cultured fibroblasts of mild hyperhomocysteinemic patients with vascular disease[8,9]. In these patients a reduced activity in CBS activity was found resembling heterozygosity for classical homocystinuria and it was proposed that such carriership was the cause of mild hyperhomocysteinemia. In later studies the association between reduced activity of CBS and vascular patients with mild hyperhomocysteinemia could not be reproduced [10-12]. Next, molecular genetic studies demonstrated that heterozygosity for mutant CBS is at the most a very minor cause for mild hyperhomocysteinemia in vascular patients[12-15]. Furthermore, the calculated number of individuals heterozygote for CBS deficiency was shown to be too low to account for the high incidence of hyperhomocysteinemia in patients with premature vascular disease[16].

Association between methylenetetrahydrofolate reductase deficiency and cardiovascular risk

Patients with severe deficiencies of methylenetetrahydrofolate reductase (MTHFR) display a wide range of clinical features such as neurological abnormalities, mental retardation and premature vascular disease[2]. Severe MTHFR deficiency is the most common inborn error in folate metabolism, still very rare with an estimated world wide incidence of 1:3.000.000 living births[17]. This inborn error of folate metabolism is inherited as an autosomal recessive trait. Severe MTHFR deficiency is biochemically characterized by hyperhomocysteinemia and homocystinuria in the presence of hypomethionemia. The MTHFR gene has been mapped to 1p36.3 and in homocystinuric patients 24 mutations have been identified until now [18-20].

In 1988, Kang demonstrated a new MTHFR variant that is very sensitive to heating at 46˚C [21] and in 1995, Frosst et al. identified the very common thermolabile mutation in the MTHFR gene, 677 C→T converting an alanine to a valine codon, which mutation leads to the thermolability of the enzyme [22]. Subjects who are homozygous for this mutation have residual activities after heating that are approximately 30% of those for controls. It is important to note that this mutation also decreases specific activity of MTHFR at normal body temperature. Prevalence of this polymorphism in homozygous form is between 0% and 25% depending upon the population in which prevalence was determined [23]. Kang et al. described that MTHFR 677 C→T mutation was more common among patients with coronary artery disease compared to controls [24]. If elevated homocysteine levels are responsible for the increased risk for vascular disease than the thermolabile MTHFR genotype, accompanied by higher homocysteine levels, should lead to an increased risk for vascular disease. In one of the largest case-control studies on 735 patients with coronary artery disease and 1250 controls the calculated odds ratio was 1.21 (95% CI: 0.87-1.68) suggesting that the risk for premature vascular disease is not associated with the MTHFR TT genotype[25]. A recent meta analysis of 23 studies indeed revealed no significant increased risk of vascular disease among subjects with the TT

genotype compared to subjects with the CC genotype [26]. The calculated odd's ratio was 1.12 (95% CI: 0.92-1.37). Nevertheless, patients with TT genotype had a plasma homocysteine concentration which was 2.6 µmol/l higher than patients with CC genotype. Therefore, the expected odd's ratio for vascular disease of patients with TT genotype compared to CC genotype, based on their homocysteine levels, should be about 1.26. This is well within the confidence interval of the calculated odds ratio in the above mentioned meta-analysis. It is remarkable that the association of this mutation with an increased risk of vascular disease differs between continents. Among the European studies, 6 out of 11 report an odd's ratio more than 1 compared to only 2 out of 8 studies in the United States and Canada. A possible explanation might be that it is much more common to fortify grain products and to take multivitamin supplements in the United States and Canada[27]. As shown by several studies, the mutation is associated with mild hyperhomocysteinemia mainly in subjects with low-normal folate status which makes it likely that the TT genotype emerges as a risk factor for vascular disease mainly in populations with a low-normal folate intake[28] . Homozygosity for another MTHFR 1298A→C mutation, converting glutamate to alanine, in the population is also very common with a prevalence of 10%, although, an association between hyperhomocysteinemia and this mutation was not found [29,30].

Association between other enzymes involved in homocysteine metabolism and cardiovascular risk

Until now seven mutations of methionine synthase (MS) have been described [31-33] . There is, however, little evidence that MS polymorphisms play an important role in mild hyperhomocysteinemia. Methionine synthase reductase (MTRR), a recently identified enzyme, is involved in homocysteine metabolism by reductive activation of MS [34]. Recently, a very common missense mutation has been identified in the MTR gene. Homozygosity for this mutation, MTRR 66 A→ G, amino acid substitution isoleucine to methionine, is prevalent in 25-30 % and is associated with an increased risk for spina bifida. An association with

hyperhomocysteinemia or vascular disease has not been observed [35]. Until now 13 mutations of MTRR are identified[34,36].
So far, 3 mutations have been described in the betaine homocysteine methyltransferase (BHMT) gene [37] but mutant BHMT whose remethylation reaction is limited to liver and kidney seems not to be associated with hyperhomocysteinemia[37].

Treatment of inborn errors of metabolism leading to severe and moderate hyperhomocysteinemia

Severe hyperhomocysteinemia

Homozygous CBS deficiency and homozygosity for severe MTHFR mutations will lead to severe hyperhomocysteinemia, with fasting homocysteine levels above 50 μmol/l in children and 100 μmol/l in adults and cause, irrespectively of the underlying enzymatic defect, a very high risk of premature arteriosclerotic and thrombotic events.
Clinical treatment of patients, homozygous for CBS deficiency, should aim on reduction of the major biochemical abnormalities in these patients. For the 50% of the patients that are vitamin B6 responsive, vitamin B6 in pharmacological doses in combination with folic acid or vitamin B12 or both is the treatment of choice. Patients who are non-pyridoxine responsive are advised to use a methionine restricted and cysteine supplemented diet mostly in combination with some pyridoxine, folic acid and vitamin B12 supplementation. In the treatment of the latter patients, the use of betaine is also an option, especially when maintaining a dietary restriction is not achievable[38].
Patients with severe MTHFR deficiency are treated with the high doses of folate and riboflavin, a co-factor in MTHFR activity [39] and patients with folate poor or non-responsiveness MTHFR deficiency with large doses betaine [40].
It is therefore obvious that for patients with severe hyperhomocysteinemia genetic diagnosis in addition to assessment of enzymatic activity is imperative for adequate tailoring of treatment and for genetic counselling.

Moderate hyperhomocysteinemia

Moderate hyperhomocysteinemia can be the result of genotypes which cause a reduced but not total deficient enzyme deficiency with homocysteine levels between 40 µmol/l and 100 µmol/l in adults often combined with a reduced folate status [41]. Also combinations of heterozygosity for mutant CBS and mutant MTHFR can cause moderate elevated homocysteine levels. Concerning patients with moderate hyperhomocysteinemia, it is therefore advisable to perform genetic diagnosis and assess enzymatic activity for optimal treatment and for genetic counselling.

Mild hyperhomocysteinemia

There is convincing evidence that mild hyperhomocysteinemia, with fasting homocysteine levels above 15 µmol/l, is a risk factor for premature vascular disease. It is important to note that mildly elevated homocysteine levels can be caused by genetic as well as environmental factors. Several lifestyle determinants like folate intake, smoking and coffee intake can modulate homocysteine levels causing mildly elevated plasma homocysteine levels. It is therefore inaccurate to assume that the increased risk for premature vascular disease associated with mild hyperhomocysteinemia can always be attributed to a single genetic mutation. A meta-analysis of 12 studies revealed that folic acid lowered total plasma homocysteine levels by 25% [42]. Addition of vitamin B12 led to a further reduction of 7%. Vitamin B6 did not lower fasting homocysteine levels but uncontrolled studies showed that post-load homocysteine levels were reduced 21% to 42 % [43,44]. It might therefore be useful to add vitamin B6 in case of elevated post-load homocysteine levels. Until now the treatment of mildly elevated homocysteine levels is independent on a possible underlying genetic defect.

Table 1

Gene	locus	mutation	ref.
CBS	21q22.3	919 G→A	[3]
CBS	21q22.3	833 T→C	[3]
MTHFR	1p36.3	several mutations	[18-20]
MTHFR	1p36.3	677 C→T	[22]

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4

Chapter

Homocysteine, atherosclerosis and endothelial dysfunction

Several studies have been published on the relationship between homocysteine and vascular disease. However, unlike the overwhelming evidence from epidemiological studies, that elevated total plasma homocysteine levels are an independent risk factor for cardiovascular disease, the mechanism responsible for this increased risk remains unclear.

Homocysteine and the development of atherosclerotic lesions.

In a post-mortem study of two children with severe hyperhomocysteinemia, Mc Cully et al. reported on the existence of severe atherosclerotic disease[1]. The lesions involved the large, medium sized and small arteries. Since then many studies have been performed to identify possible adverse effects of homocysteine on the vascular wall.

Several in-vitro studies have been published upon adverse effects of homocysteine on functional properties of cultured endothelial cells. Anti-coagulant properties of cultured endothelial cells, expressed as protein C activation, are inhibited when they are exposed to exogenous homocysteine[2,3]. The expression of pro-coagulants like tissue factor activity and Factor V activity are induced[4,5]. Exogenous homocysteine stimulates the growth and proliferation of cultured smooth muscle cells[6,7]. The bioavailability of endothelium-derived nitric oxide is impaired by exogenous homocysteine[8]. An important concern about many of the above mentioned studies is the fact that the required homocysteine concentration used in these studies are more than tenfold higher than the plasma concentration of homocysteine in patients. Another limitation is that in most studies the free thiol-form of homocysteine is used which normally only represents a small fraction of plasma homocysteine in patients.

Homocysteine and anatomic changes in coronary arteries

Several animal studies have been performed, investigating the effect of elevated homocysteine levels on the development of atherosclerotic lesions in the arteries. With baboons, rats and rabbits, parenteral injection of homocysteine or

homocysteine thiolactone led to desquamation of endothelial cells and produced structural changes in arteries resembling early atherosclerotic lesions[9,10,11]. In a more recent study using minipigs with moderate hyperhomocysteinemia, structural changes in large conduit arteries as in resistance vessels could be demonstrated[12].

Despite the fact that in different animal models a relationship between atherosclerotic lesions and homocysteine could be determined, coronary angiographic studies show only a weak correlation between the number of coronary stenoses or the number of involved coronary vessels and elevated plasma homocysteine levels[13,14,15,16]. Important shortcomings of the above mentioned studies are that the investigators used visual estimation of lesion severity to define whether a vessel was significantly diseased. Visual estimation of lesion severity overestimates the percent stenosis compared to estimation of lesion severity using quantitative coronary angiography[17]. A study using quantitative coronary angiography to evaluate the severity of atherosclerosis in coronary arteries in relationship with plasma homocysteine levels is presented in chapter 6 of this thesis.

Homocysteine and endothelial function

Evidence is accumulating that elevated homocysteine causes endothelial dysfunction[18]. Mechanisms responsible for endothelial dysfunction in hyperhomocysteinemia are poorly understood. Several investigators demonstrated that homocysteine reduces the bioavailability of NO and enhances smooth muscle cell proliferation, both of which are important markers of athero-thrombotic disease[18]. Recently, it was hypothesized that hyperhomocysteinemia may stimulate the formation of asymmetrical dimethylarginine, an endogenous inhibitor of NO synthase[19]. Homocysteine may also impair the function of endothelium-derived hyperpolarizing factor, a vasodilatator substance released by the vascular endothelium[20]. De Vriese et al. demonstrated that folates can restore impaired endothelium-derived hyperpolarizing factor instantly[21] .

Endothelial function related to nitric oxide release can be assessed in humans non-invasively, using flow-mediated dilatation of the brachial artery[22]. An association between hyperhomocysteinemia and impaired flow mediated vasodilatation of the brachial artery was observed for the first time in children homozygous for cystathionine β-synthase deficiency who have very high levels of plasma homocysteine[23]. Two other studies demonstrated an impaired flow mediated vasodilatation of the brachial artery in subjects with mild to moderate hyperhomocysteinemia[24,25]. However, not all the studies with humans show a relationship between an increased total plasma homocysteine levels and impaired endothelial function using a fore arm model[26,27,28]. Some investigators have focused on different forms of homocysteine in plasma demonstrating that reduced homocysteine is related to endothelial dysfunction but not free oxidized homocysteine or protein bound homocysteine[29].

The clinical relevance of flow-mediated vasodilatation was recently demonstrated by Gokce et al. They demonstrated that flow-mediated vasodilatation of the brachial artery predicts short-term cardiovascular events following vascular surgery[30]. Modena et al. demonstrated that improvement of more than 10% of the flow-mediated vasodilatation in the fore-arm was able to predict events[31] . It is important to note that the variance of flow-mediated vasodilatation in an individual patient is larger than the differences described in the above mentioned studies. It is therefore difficult to evaluate a therapeutical intervention in an individual patient using the fore-arm model. Factors known to impair endothelial function in the coronary circulation are also associated with impaired flow mediated dilatation in the brachial artery[32,33]. Acetylcholine is widely used as a standard substance to test endothelial function in human coronary arteries[34]. In subjects with normal coronary vessels, acetylcholine induces vasodilatation of epicardial coronary vessels and an increase of coronary blood flow (CBF) of 50% or more[35]. In patients with atherosclerosis, or in the presence of risk factors for vascular disease like hypercholesterolaemia, hypertension, diabetes mellitus or smoking, acetylcholine induces paradoxical vasoconstriction of epicardial conductance vessels, expressed as a decrease in

mean segment diameter (MSD) and minimal obstruction diameter (MOD), and a smaller increment of CBF[34]. An increase of CBF less than 20% after the administration of acetylcholine in coronary vessels is associated with increased cardiovascular mortality[36,37]. Endothelial cells synthesize and release vasoactive mediators and thereby modulate vascular tone. Nitric oxide (NO) and prostacyclin are the best characterized vasodilatators and are largely responsible for endothelium dependant vasodilatation in epicardial coronary vessels. However, not all endothelium-dependant relaxation can be explained by nitric oxide and prostacyclin. Compelling evidence is provided for the existence of another factor, endothelium-derived hyperpolarizing factor that is especially important in resistance vessels[38]. In humans with coronary atherosclerosis, endothelial vasodilatator function is not confined only to epicardial conductance vessels but extents also to the coronary resistance vessels. CBF in the absence of obstructive lesions is regulated mainly by the coronary resistance vessels[39]. Abnormal CBF changes during administration of endothelium dependant vasodilatators like acetylcholine in patients without obstructive coronary artery disease are associated with cardiovascular morbidity and mortality[35,40]. Since homocysteine is associated with cardiovascular mortality and morbidity[14,41], these changes in CBF may therefore explain, at least partially, the role of homocysteine in cardiovascular disease.

Recently the effects of homocysteine-lowering treatment on endothelial function using flow-mediated vasodilatation of the brachial artery was studied. Although some investigators could demonstrate a beneficial effect of folic acid on flow-mediated vasodilatation[42,43] the results are not unequivocal [44,45]. In this thesis we describe the effect of homocysteine-lowering treatment on coronary endothelial function

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5

Chapter

Pharmacokinetic study on the utilization of 5-methyltetrahydrofolate and folic acid in patients with coronary artery disease

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Introduction

More than 80 cross-sectional, case-control and prospective cohort studies have been published upon the relationship between homocysteine and arterial vascular disease [1]. Despite overwhelming epidemiological evidence confirming that hyperhomocysteinemia is an independent risk factor for vascular disease, the exact causal relationship remains to be proven. Methylenetetrahydrofolate reductase (MTHFR) is a regulating enzyme in folate-dependant homocysteine remethylation because it catalyses the reduction of 5,10 methylenetetrahydrofolate to 6[S] 5-methyltetrahydrofolate (5-MTHF), the natural circulating form of folate in plasma. 6[S]5-MTHF serves as methyl donor for the conversion of homocysteine to methionine. In 1995 Frosst et al. identified a common mutation in the MTHFR gene, a transition at nucleotide 677(C→T) converting an alanine to a valine [2]. Patients who are homozygous for this variant have an elevated total plasma homocysteine [3]. Recently it was demonstrated convincingly that these subjects, 10 to 20% of the population, have an increased cardiovascular risk [4]. Many studies have demonstrated that elevated homocysteine can be lowered by the intake of folic acid in doses ranging from 400 μg to 10 mg/day [5]**.** Recently, we demonstrated a beneficial effect of homocysteine-lowering therapy using long-term folate therapy, 5 mg, on coronary endothelial function in hyperhomocysteinemic patients, a surrogate endpoint for cardiovascular events [6]. In addition, Doshi et al. demonstrated that 5-methyltetrahydrofolate (5-MTHF) had a direct beneficial effect on endothelial function, independent of plasma homocysteine levels [7]. These results suggest that high concentrations of the natural isomer of 5-MTHF in plasma may be beneficial in cardiovascular disease. Whether these effects are related to a reduced activity of the MTHFR enzyme or due to a better bioavailability of 5-MTHF is unclear. Orally administered folic acid needs to be reduced and converted to

tetrahydrofolate before it can become metabolic active. In subjects homozygous for the MTHFR 677 C→T mutation, it may be speculated that the direct administration of 5-methyltetrahydrofolate instead of folic acid can facilitate the remethylation of homocysteine into methionine. Otherwise, it is possible that the efficacy of 5-MTHF is better independently of a reduced activity of the MTHFR enzyme. 5-MTHF is available as a mixture of 6[S] and 6[R] racemates. Although, it is assumed that only 6[S] 5-MTHF is bio-active, the possible biological effects of 6[R] 5-MTHF are not clear. The aim of this study was to determine the pharmacokinetic properties of a single dose of orally administered 6[R,S] 5-MTHF, a commercially available racemic mixture of the two diastereoisomers of 5-MTHF, versus folic acid in cardiovascular patients with homozygosity for the MTHFR polymorphism (TT genotype) compared to patients with the wild type genotype (CC).

Methods

Patient selection:

This study is a open controlled, 2 way, 2 period randomised cross over study with a one week run in and a one week wash out period. Patients with established coronary heart disease were screened by DNA analysis for the presence of the MTHFR 677C→T polymorphism . Genomic DNA was extracted from peripheral blood lymphocytes by a standard procedure, and mutation analysis was performed essentially as described by Frosst et al.[2]. All patients have given informed consent. Twelve patients with the TT genotype MTHFR and twelve patients with wild type MTHFR enrolled in the study.

The groups were matched for age, sex and body weight. Inclusion of patients required a body weight within 20% of normal values according to the Metropolitan Height and Weight Tables. No clinically important abnormal physical findings were tolerated. In all subjects enrolled in the

study at baseline and after four weeks, blood samples were analysed for liver and renal function, haematology, urinalysis and vitamins. Patients with a myocardial infarction in the past 3-month were excluded, as well as patients with relevant history of liver disease, renal disease and gastrointestinal disease. Medication involved in homocysteine metabolism, the use of vitamins or blood donation in the past three months were not allowed**.** Users of significant amounts of tobacco (10/day), alcohol (> 40 gr/day) or drugs were excluded.
Patients did not take food or drinks, apart from water, 12 hours before administration of the folic acid or 5- MTHF and up to 4 hours after the administration of the medication. In the period of the study patients followed a stable diet which guaranteed a stable intake of 200 μg folate a day (the recommended daily allowance). The study medication consisted of a single oral dose of folic acid (folina^{R}), 5 mg or 6 [R,S] 5-MTHF (Prefolic^{R}), 5 mg.

Study schedule

Each patient underwent a pre-study assessment within 2 weeks before run in period. During the first study period the patients received a single oral dose of either 5 mg folic acid or 5 mg 6 [R,S] 5-MTHF, dissolved in 50 ml distilled water according to the randomisation list. Patients were blinded for treatment**.** A wash out period of one week followed after which the patients crossed over to the other treatment schedule. Administration of the folic acid or 5-MTHF to the fasting patients was performed between 07.00 and 09.00 a.m.

Blood samples:

Venous blood samples were taken at the following times: 0 (pre-dose), 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 hours following the administration of either folic acid or 6[R,S] 5-MTHF. The blood samples were collected in heparinized tubes and put on ice immediately and within 30 minutes

centrifuged at 3000 x g for 15 minutes at 4ºC. The supernatant plasma was transferred to a polypropylene tube containing sodium ascorbate and stored at -20^0 C.

Analytics:

The plasma concentrations of the [6R] and [6S] 5 MTHF diastereoisomers were determined by a validated stereoselective method. After an ion-exchange step of purification from the biological matrix, 5-MTHF diastereoisomers are resolved by a stereospecific enzymatic reaction, using 5-MTHFR. At the end of the reaction, aliquots of the samples were analysed by reverse phase high performance liquid chromatography (HPLC) with fluorescence detection. With this method the concentrations in plasma of the diastereoisomers in the low baseline range (3-4 $ng.ml^{-1}$) are determined with an inter-assay coefficient of variation of 10%. The limit of quantification for 6[R] 5-MTHF is kept at 5 $ng.ml^{-1}$ [8]. The following parameters were calculated from the plasma concentrations of [6R] and [6S] 5-MTHF acid stereoisomers obtained up to 12 hours after administration of single dose folic acid or 5-MTHF: Cmax ($ng.ml^{-1}$)(the highest concentration of the diastereoisomer in plasma), t max (hours)(the time when Cmax. is achieved), C0-12z ($ng.ml^{-1}$)(the concentration of the diastereoisomer at sampling time z), AUC 0-12z (The area under the curve of the plasma concentration versus time up to sampling time z), AUC∞ (the area under the curve of the plasma concentration versus time using the equation: $AUC\infty=AUCz+Cz.\lambda z^{-1}$) and $t^{1/2}$ (hours) Plasma homocysteine levels were determined at baseline, 2 hours, 4 hours and twelve hours after taking the vitamins, using high performance liquid chromatography as described by te Poele et al [9].

Statistics:

Baseline characteristics are summarised by appropriate descriptive statistics. Pharmacokinetic parameters are analysed using variance analysis considering the following factors in the model: treatment period, category and sequence. The treatment effect and the treatment category interaction are tested at a 0.05 level. A log transformation is carried out on AUCz, AUC∞ and Cmax before performing ANOVA. For presentation the point estimates (mean, SD and 95% confidence intervals) are converted back in the original scale.

Results

In 157 patients screened for the TT MTHFR mutation, we found 14 patients who were homozygous for this mutation. Of 27 patients initially included 24 patients completed the study. Three patients were excluded from the study due to a protocol violation, two patients with a TT genotype and one patient with the CC genotype. Baseline characteristics of the participating patients are listed in Table 1.

Table 1
Baseline characteristics

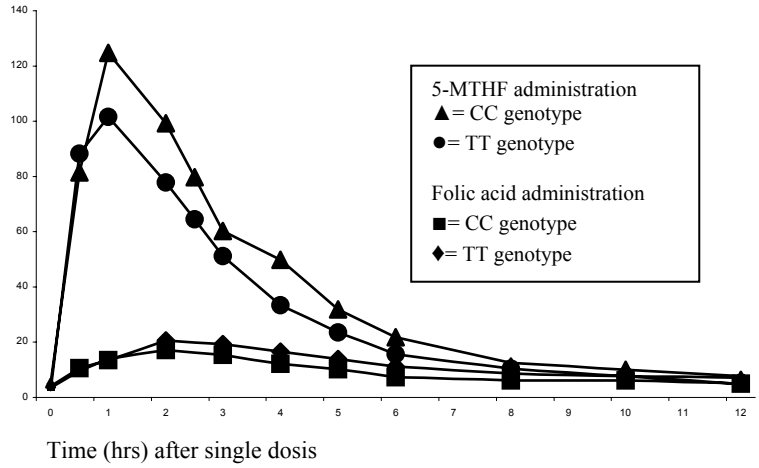
	N=10(TT)	N=14(CC)	p value†
Age (years)	56.9 (range 46-64)	57.2 (range 46-66)	0.91
Sex (male/female)	8/2	11/3	0.55
Homocysteine (µmol/l)	26.8 (18.1)*	15.4 (3.5)	<0.05
Folate	13,5 (5.7)	19.1 (17.8)	0.35
Vitamin B12	225 (112)	230 (88)	0.89
Vitamin B 6	49 (17)	44 (12)	0.47
Total cholesterol (mmol/l)	5.69 (1.41)	5.16 (0.76)	0.25
Triglycerides (mmol/l)	2.05 (1.1)	2.11 (1.0)	0.89
Glucose (mmol/l)	5.7 (1.8)	7.2 (2.4)	0.11
Kreatinine (µmol/l)	101 (11.6)	101 (39.0)	0.99

*SD between brackets, † Student T test for equality of means or Chi square test where appropriate

Mean fasting total plasma homocysteine levels (tHcy) in the patients TT genotype was 26.8 µmol/l (SD 18.2) compared to 15.4 µmol/l (SD 3.5) in the patients with CC genotype ($p<0.05$). Total plasma folate was 13.5 nmol/l (SD 5.7) in the group with TT polymorphism compared to 19.1 nmol/l (SD 17.8) in the group with CC polymorphism ($p=0.35$). No other significant differences were found between the two groups.

One week after the completion of the study plasma folate levels were increased from 13.5 to 24.5 nmol/l in the group with TT polymorphism compared to no change in the group with the CC polymorphism ($p<0.05$). Vitamin B12 and vitamin B6 did not change during follow-up. The administration of the single dosage of 5 mg folic acid or 5 mg 5-MTHF did not influence plasma homocysteine levels during the follow-up of 12 hours, irrespective of the patients genotype. In both groups, homocysteine levels did not change at follow-up after one week.

Figure 1: Plasma level 6[S] 5-MTHF (ng.ml^{-1})



Genotype and treatment
6[S] 5-MTHF plasma concentration (ng.ml^{-1}) in patients with MTHFR CC genotype (▲) or TT genotype (●) following the administration of 6[R,S] 5-MTHF.
6[S] 5-MTHF plasma concentration (ng.ml^{-1}) in patients with MTHFR CC genotype (■) or TT genotype (♦) following the administration of folic acid.

Pharmacokinetic properties of 6[R,S] 5-MTHF versus folic acid

The main pharmacokinetic parameters following both treatment strategies for the 6[S] 5-MTHF and 6[R] 5-MTHF diastereoisomers are listed in Table 2. All pharmacokinetic parameters demonstrate that the bioavailability of 6[R,S]5-MTHF acid is higher compared to folic acid. The peak concentration of the natural diastereoisomer 6[S] 5-MTHF following the administration of 5-MTHF is more then seven times higher compared to the peak concentration of 6[S] 5-MTHF following the administration of folic acid , 129 ng/ml (SD 42.4) versus 14.1 ng/ml (SD 9.4)($p<0.001$) respectively. The peak concentration of the diastereoisomer 6[R] 5-MTHF following the administration of 5-MTHF compared to folic acid is 144 ng/ml (SD 75) versus 42 ng/ml (SD 40)($p<0.001$) respectively.

Table 2

Pharmacokinetic parameters for 6[S] 5-MTHF after administration of either folic acid or 5-MTHF

	Folic acid **n=24**	5-MTHF **n=24**	**p value†**
Ke	0.17 (0.1)*	0.42 (0.18)	p<0.001
T1/2	4.9 (2.5)	2.7 (2.6)	p<0.01
Tmax	2.3 (0.8)	1.25 (0.53)	p<0.0005
Cmax	14.1 (8.9)	129 (33)	p<0.0001
AUC 0-12	73 (39)	383 (113)	p<0.0001
AUC 0-∞	96 (48)	405 (117)	p<0.0001

*SD between brackets, † Student T- test for equality of means

Influence of MTHFR 677C→T genotype on the pharmacokinetic properties of 6[R,S] 5-MTHF

The main pharmacokinetic parameters of the biological active 6[S] 5-MTHF diastereoisomer for both treatment strategies in relationship with the patients' genotype are listed in Table 3. No significant differences in pharmacokinetic parameters exist between patients with the TT genotype and patients with CC genotype. Figure 1 shows the 12 hours curve follow-up of 6[S] 5-MTHF in ng/ml after oral administration of folic acid and 5-MTHF in both genotype

groups. Data on the 12 hours curve follow-up of 6[R] 5-MTHF are essentially the same (data not shown)

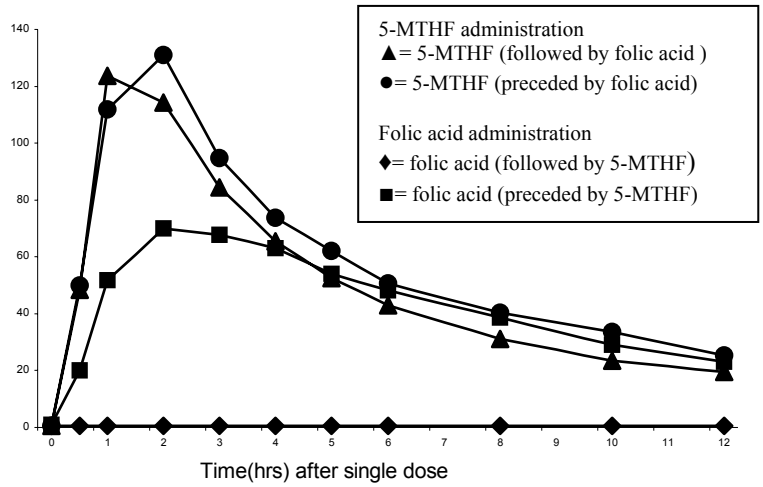
Table 3

Pharmacokinetic parameters for 6[S] 5-MTHF after administration of either folic acid or 5-MTHF in patients with (TT) and (CC) genotype

	Folic acid			5-MTHF		
genotype	**TT**	**CC**	**p value†**	**TT**	**CC**	**p value**
K_e	0.15(0.1)*	0.17(0.1)	0.56	0.32(0.1)	0.35 (0.2)	0.66
T ½	5.0(2.0)	4.8(2.9)	0.83	2.36(0.7)	2.82(3.5)	0.69
T max	2.4(0.8)	2.3(0.8)	0.66	1.3(0.5)	1.2(0.5)	0.88
C max	14(11)	14(7)	0.83	101 (29)	107(36)	0.66
AUC 0-12	72(39)	74(41)	0.90	303 (105)	329 (121)	0.58
AUC 0-∞	99(37)	94(55)	0.82	312 (111)	340 (123)	0.35

* SD between brackets, † ANOVA one way analysis

Figure 2: plasma level of 6[R] 5-MTHF (ng/ml^{-1})



Sequence of treatment:
6[R] 5-MTHF plasma concentration (ng.ml^{-1}) in patients after folic acid intake in the first period (♦), 6[R] 5-MTHF plasma concentration (ng.ml^{-1}) in patients after folic acid intake in the second period (■)
6[R] 5-MTHF plasma concentration (ng.ml^{-1}) in patients after 6[R,S] 5-MTHF intake in the first period(▲) and patients after 6[R,S] 5-MTHF intake in the second period(●).

Pharmacokinetic properties of 6[R,S] 5-MTHF in relationship with the treatment sequence

The sequence of treatment with folic acid or 5-MTHF (either folic acid or 6[R,S] 5-MTHF as the first drug) did not influence the results of the pharmacokinetic parameters for the 6[S] 5-MTHF diastereoisomer (data not shown). The washout curve for the 6[R] 5-MTHF diastereoisomer showed that in the case that folic acid was given in the first period the metabolic inactive 6[R] 5-MTHF diastereoisomer was not present in the plasma (detection limit < 5 ng/ml). However, if folic acid was given in the second period, levels of 6[R] 5-MTHF became clearly detectable in plasma. This was irrespective of the patients genotype (Figure 2).

Discussion

This study demonstrated a higher bioavailabity of orally administrated racemic 6[R,S] 5-MTHF, 5 mg compared to folic acid, 5mg. The effects were not influenced by the MTHFR 677TT genotype of the patient. Plasma homocysteine levels did not alter following the administration of a single dosage of either folic acid or 6[R,S] 5-MTHF acid.

Pharmacokinetic properties of folic acid and 6[R,S] 5-MTHF

In our study a prompt rise of 6[S] 5-MTHF, the natural isomer possessing biological activity [10] , within 1 to 3 hours following the administration of folic acid or 6[R,S] 5-MTHF was shown with peak concentrations who were sevenfold or more higher than baseline concentrations. Our data, with respect to the pharmacokinetic profile, are consistent with a previous study evaluating the effect of oral administration of folic acid in patients with the TT polymorphism compared to patients with the CC polymorphism where comparable rises of the 6[S] 5-MTHF diastereoisomer have been seen[11]. It is to be expected that 5-MTHF levels are proportionally lower following the administration of folic acid compared to 6[R,S] 5-MTHF because 6[R,S] 5-MTHF is directly available following absorption in the

intestinal cell while folic acid needs to be metabolised first into tetrahydrofolate. Depending upon folate supply tetrahydrofolate can be transported into the portal circulation, converted to 6[S] 5-MTHF or be stored in tissue as a tetrahydrofolate. This prolonged biochemical pathway can be a possible explanation for the difference in the pharmacokinetic parameters. Some studies used 5-MTHF as pharmacological agent to improve endothelial function is vascular patients [7]. It can be speculated that these high levels of circulating metabolic active folates may be responsible for the observed direct improvement of endothelial function .

Folates in relation to genotype:

Our data demonstrates that there are no differences with respect to the pharmacokinetic properties of both folic acid and 6[R,S]5-MTHF in relation to the patients' genotype. Mutations in MTHFR have been associated with a lower proportion of 5-MTHF and a higher proportion of formylated folates [12]. This, because of a decreased MTHFR activity resulting in a lower rate of reduction of 5,10-methyleneTHF to 5-MTHF, leading to increased availability of 5,10-methylene-THF for oxidation to the formylated folate forms. Our results suggest that the activity of MTHFR in vivo is not the rate-limiting step in the conversion of folic acid to 5-MTHF.

The sequence of therapy:

The sequence of administration of either folic acid or 6[R,S]5-MTHF did not influence pharmacokinetic parameters with respect to the plasma level of the physiologically active form 6[S] 5-MTHF. Since oral applied 5-MTHF consists of a racemic mixture of 6[S]5-MTHF and 6[R] 5-MTHF it can be explained that the 6[R] 5-MTHF isomer can be detected in the plasma. Compared to plasma levels of 6[S] 5-MTHF, plasma levels of 6[R] 5-MTHF reach higher peak concentrations and still have a higher plasma concentration 12 hours after the intake. These findings suggest that this isomer has a slower clearance from the

plasma, possibly due to the non-natural form of 5-MTHF. Our findings are consistent with a previous study investigating the effects of high dosage 6[R,S] 5-MTHF demonstrating a higher plasma concentration and slower plasma clearance of 6[R] 5-MTHF compared to 6[S] 5-MTHF [13]. In that study it was demonstrated that protein binding of 6[R] 5-MTHF is 88% compared to 56% of the 6[S] 5-MTHF with a decreased renal clearance as a consequence.

If folic acid was given as the first drug, 6[R] 5-MTHF could not be detected in the plasma. This is to be expected since folic acid will only be metabolised into its natural active isomer. However, when folic acid was given in the second period (one week after the administration of the racemic 6[R,S] 5-MTHF), 6[R] 5-MTHF could be detected in the plasma, even at relative high levels (figure 2). A previous study revealed that the clearance rate of 6[R] 5-MTHF is about 4 times slower than for the 6[S] 5-MTHF [14]. However, this can not explain the fact that one week after a single bolus 6[R,S] 5-MTHF we found relative high levels of 6[R] 5-MTHF only after we supplied folic acid. The 6[R] 5-MTHF isomer was not detectable at baseline, thus before the administration of folic acid. It seems therefore less likely that the difference in plasma protein-binding between 6[S] 5-MTHF and 6[R] 5-MTHF explains this phenomenon since the 6[R] 5-MTHF isomer was not detectable in plasma at baseline. Our results suggest that the 6[R]5-MTHF isomer is stored in the body until it is released following the administration of a relative large dosage of folic acid. Folates are stored in tissues, mainly in the liver where folates are bound tight by cytosolic and mitochondrial folate binding proteins. This binding may not be stereospecific. The biological effects of 6[R] 5-MTHF binding are unclear. Mader et al. reported that no serious short-term side-effects were seen in patients receiving high doses of 6[R,S]5-MTHF[13]. Since 6[R] 5-MTHF is not metabolised, it can be speculated that it may inhibit regulatory enzymes related to folate and homocysteine metabolism. Secondly , the

bioavailabity of 6[S] 5-MTHF may be reduced due to competition with the 6[R] 5-MTHF diastereoisomer.

Genotype of the patient and homocysteine levels

Several investigators have demonstrated that subjects who are homozygous for the TT polymorphism have an elevated tHcY only when plasma folate is low [3, 15, 16, 17]. Plasma folate levels in the group of patients with TT polymorphism were relatively low compared to folate levels in the CC group. Obviously, the administration of a single dosage of folic acid or 6[R,S] 5-MTHF did not alter homocysteine levels during the study suggesting that long-term treatment is mandatory.

Conclusion:

Our results demonstrates that 6[R,S] 5-MTHF has a different pharmacokinetic profile compared to folic acid, irrespective of the patients' MTHFR 677 C→T genotype. Although the clinical significance of these differences require further investigation, the prompt sevenfold rise in the concentration of the natural isomer may be a promising feature of 5-MTHF in the treatment of vascular disease. The clinical consequences of relative high plasma levels of the non-natural isomer, 6[R] 5-MTHF, following the administration of 6[R,S] 5-MTHF are unknown. Since our study suggests that this isomer is stored in the body, further investigations of the clinical long-term effects of the racemic 6[R,S] 5-MTHF acid are necessary to evaluate possible detrimental effects.

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6

Chapter

Thermolabile methylenetetrahydrofolate reductase in coronary artery disease

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Circulation. 1997;96 : 2573-7

Introduction

A recent meta-analysis by Boushey et al. of 27 independent studies in which plasma homocysteine concentrations were quantitatively related to atherosclerotic disease demonstrated that mild hyperhomocysteinemia is an independent and graded risk factor for cerebral, peripheral, and coronary artery disease [1]. Elevated homocysteine concentrations may originate from nutritional deficiencies in cofactors or co-substrates of enzymes involved in homocysteine metabolism or from molecular defects in genes coding for enzymes crucial in this metabolism.[2,3,4,5].

The enzymes pivotal in homocysteine metabolism are cysthationine β synthase (CBS), the first enzyme in homocysteine transsulfuration, and methylene tetrahydrofolate reductase (MTHFR), which is involved in the folic acid dependent remethylation of homocysteine to methionine. Both genes have been cloned and characterized,[6,7] and several mutations have been reported [8,9,10,11,12] in patients with a homozygous deficient phenotype. Heterozygotes for either CBS or MTHFR deficiency often have elevated homocysteine concentrations. However, the frequency of heterozygosity for CBS and MTHFR deficiency is too low to account for the frequency of mildly elevated homocysteine levels in patients with cardiovascular disease.[13] MTHFR is a flavoprotein that reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating form of folate. In 1988, Kang et al [5] described a new MTHFR variant with thermolabile properties. Individuals with this MTHFR variant have decreased specific MTHFR activity in lymphocytes (<50% of the control mean), have increased thermolability after preincubation at 46°C, and may have elevated plasma homocysteine concentrations. In other studies, the same group reported an increased incidence of this MTHFR variant in patients with coronary artery disease [14] compared with control subjects (17% versus 5%, respectively), and they were able to correlate the incidence of thermolabile MTHFR to the severity of coronary artery disease.[15] In a Dutch study, this thermolabile MTHFR was found to be the

cause of abnormal homocysteine metabolism in 11 of 39 hyperhomocysteinemic vascular patients (28%)[16]. Recently, Frosst and coworkers17 were able to identify a relatively common 677C→T mutation in the MTHFR gene, which substituted a conserved alanine by a valine residue. Individuals who are homozygous for this mutation often have elevated homocysteine concentrations,[17 18] especially in combination with a low folate status.[19 20] In the present study, we investigated the prevalence of the 677C→T mutation in a well-defined population of 735 male cad patients and in 1250 population-based control subjects and assessed the association of this mutation to serum homocysteine concentration. Several studies have investigated the homozygous 677C→T mutation as a risk factor for cad but with conflicting results,[21, 22, 23, 24, 25, 26] probably because of the relatively small numbers of individuals included in each study separately. We therefore performed a meta-analysis of eight case-control studies reporting data on the MTHFR genotype distribution to estimate the relative risk of the homozygous (+/+) genotype for coronary artery disease.

Methods

Study Population

We studied 735 male patients with angiographically assessed coronary artery disease enrolled in the REGRESS, which was conducted under auspices of the Interuniversitary Cardiology Institute The Netherlands. REGRESS is a double-blind, placebo-controlled, multicenter trial designed to assess the effects of lipid-lowering therapy with pravastatin on progression and regression of coronary artery disease in 885 male patients with normal to moderately elevated serum cholesterol levels (4 to 8 mmol/L). The overall study design and inclusion criteria of patients have been described extensively[27]. A large control group was constructed, consisting of individuals recruited from several published [28, 29] and unpublished Dutch case-control studies (L.A.J.K., H.J.B., Van der Put, Den Heijer, and Rosendaal, unpublished results), which resulted in a control

group consisting of 1250 unrelated population-based control subjects. All 677C→T mutation analyses were performed in our laboratory.
For a summary quantitative risk assessment of the 677CT mutation in coronary artery disease, we evaluated eight international case-control studies,[21, 22, 23, 24, 25, 26, 29] including the present one. In this analysis, we confined ourselves to case-control studies in which MTHFR genotype distributions among both coronary artery disease patients and control subjects either
were given or could be calculated from their data. We calculated the MTHFR genotype distribution and odds ratios of the (+/+) genotype for coronary artery disease in each study separately and for all studies combined.

MTHFR Genotype Analysis

Genomic DNA was extracted from peripheral blood lymphocytes by standard procedure, and mutation analysis was performed essentially as described by Frosst et al.[17] Electrophoresis in a 4% agarose gel followed by ethidium bromide staining and UV illumination allowed detection of mutated alleles.

Homocysteine Determination

After an overnight fast, blood was drawn from the coronary artery disease patients for an assessment of fasting homocysteine concentrations, and serum was stored at -70°C until analysis. Homocysteine concentrations were determined by high-performance liquid chromatography with use of a 150x4.6-mm Hypersil ODS column in a high-performance
liquid chromatography analyzer (Thermo Separation Products) after the thiol groups were bound to a fluorescent label (SBD-F).30

Statistical Analysis

Odds ratios and 95% confidential intervals(CI) were calculated as an estimate of the relative risk of the different genotypes in coronary artery disease.[31] Differences in genotype distributions were calculated by 2 analysis. To assess the relationship between the 677C→T transition and homocysteine concentrations,

we calculated median homocysteine concentrations in different genotype groups. Differences between homocysteine concentrations in these genotype groups were assessed by one-way ANOVA, followed by pairwise t tests on log-transformed data. All probability values are two-tailed, and a value of $P<.05$ was considered statistically significant.

Results

MTHFR Genotype Analysis

The overall frequency for the (+) allele was 31.8% among patients and 29.5% among control subjects. The numbers of individuals homozygous for the 677CT transition were 70 of 735 coronary artery disease patients (9.5%) versus 106 of 1250 controls (8.5%; Table 1). The genotype distributions in both groups of individuals are consistent with those calculated from the Hardy-Weinberg equilibrium. The odds ratios as an estimate of the relative risk of the (+/+) and (+/-) genotypes relative to the risk of the (-/-) genotype for coronary artery disease were 1.21 and 1.14, respectively (Table 1). We also assessed the relative risk of the (+/+) genotype in relation to the risk of individuals with both other genotypes. In this model, the risk of the homozygous (+/+) genotype in coronary artery disease was 1.14 (95% CI, 0.83 to 1.56).

Table 1.

Distribution of 677C→T MTHFR variant among patients with coronary artery disease and control subjects

Coronary artery disease patients (n=735), n (%)	Control Subjects (n=1250), n (%)	Odds Ratio (95% CI)
+/+ 70 (9.5)	106 (8.5)	1.21 (0.87-1.68)
+/- 328 (44.6)	527 (42.2)	1.14 (0.94-1.38)
-/- 337 (45.9)	617 (49.4)	1.01 *

*Reference category: odds ratio=1.0.

Association Genotype and Total Homocysteine

Homocysteine concentrations were measured in 515 of 735 coronary artery disease patients. The numbers of individuals in the three different MTHFR genotype groups in this subset of individuals were 51 (9.9%), 233 (45.2%), and 231 (44.9%) for the (+/+), (+/-) and (-/-) genotype, respectively, which are not substantially different from the MTHFR genotype distribution observed in the entire patient group (2=0.137, P=NS). As homocysteine concentrations in the different genotype groups showed a skewed distribution (data not shown), homocysteine concentrations are expressed in median (range) values. Individuals with the homozygous (+/+) genotype have the highest homocysteine concentrations and (-/-) individuals have the lowest, whereas heterozygous (+/-) individuals have intermediate homocysteine levels.

Table 2.

Association between MTHFR genotype and fasting serum homocysteine concentrations in patients with coronary artery disease

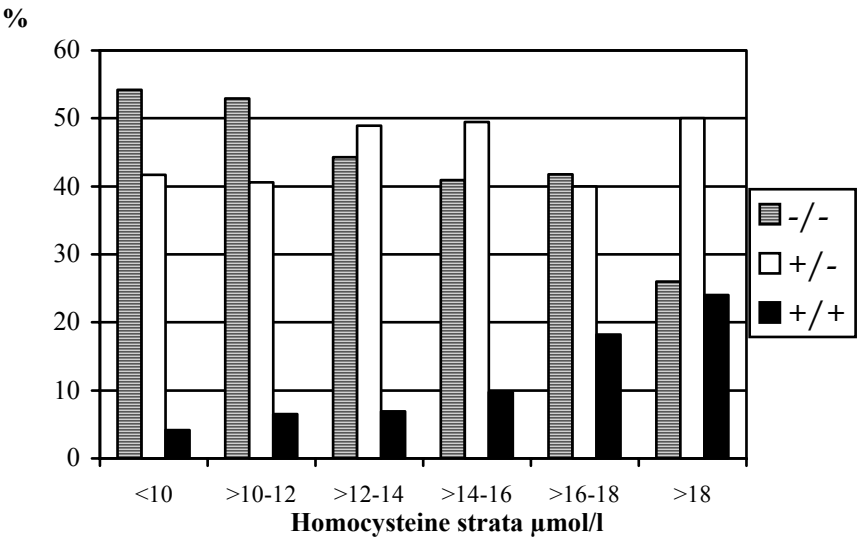
MTHFR Genotype	Fasting Homocysteine µmol/l [1]
+/+ (n=51)	15.4 (8.7-56.9)[2]
+/- (n=233)	13.4 (7.0-42.9)[3]
-/- (n=231)	12.6 (6.5-30.2)

Homocysteine concentrations are expressed as median values. Range is given in parentheses. Homocysteine concentrations are expressed as median values. Range is given in parentheses. [1] P<.001 (ANOVA with log-transformed data).
[2] P<.002 (t test) for +/+ versus +/- and -/- genotypes. [3] P<.05 (t test) for +/- versus -/- genotype

Both homozygotes (+/+) and heterozygotes (+/-) have significantly elevated homocysteine concentrations compared with (-/-) individuals, demonstrating a significant effect of the homozygous (+/+) as well as heterozygous (+/-) genotype on homocysteine levels (Table 2). We also assessed the MTHFR genotype distribution in different homocysteine strata (Fig 1). The frequency of

the homozygous 677CT mutation showed a gradual increase from 4% in the lowest homocysteine stratum (homocysteine <10 µmol/L) to 24% in the highest stratum (homocysteine >18 µmol/L), again indicating the association between homocysteine concentrations and the homozygous (+/+) genotype.

Figure 1



Legenda:

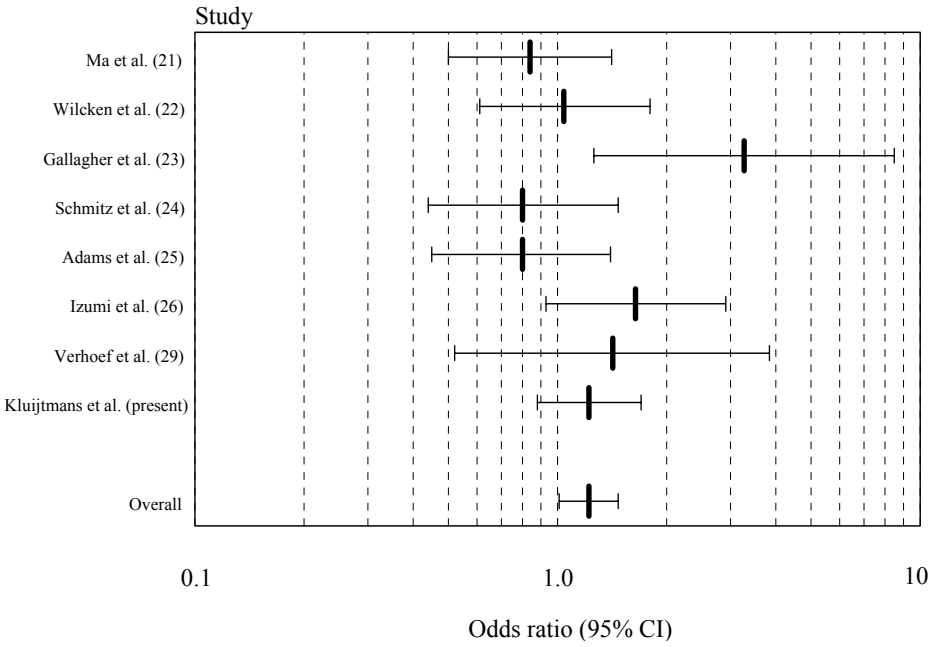
MTHFR genotype distribution in different homocysteine strata. Serum homocysteine concentration is expressed in micromoles per liter. In each stratum, the total number of individuals is set to 100%.

Thermolabile MTHFR in coronary artery disease

For a summary estimate of the relative risk of the homozygous (+/+) genotype, we performed a meta-analysis of studies reporting data on the MTHFR genotypes in patients with coronary artery disease. In this analysis, we confined ourselves to case-control studies in which MTHFR genotype distributions among coronary artery disease patients as well as control subjects either were given or could be calculated from their data. For each study, we calculated odds ratios and

95% C.I.s for the (+/+) genotype relative to the (-/-) genotype separately (Fig 2). The combination of all studies reported yielded a patient group consisting of 2476 individuals (299 +/+, 1097 +/-, and 1080 -/-) and a control group of 2481 individuals (257 +/+, 1090 +/-, and 1134 -/-). From the MTHFR genotype distribution in this combined study group, we calculated an odds ratio of 1.22 (95% CI, 1.01 to 1.47) for the homozygous (+/+) genotype and 1.06 (95% CI, 0.94 to 1.19) for the heterozygous (+/-) genotype, both relative to the (-/-) genotype. When the heterozygotes (+/-) and (-/-) individuals are combined, the odds ratio for coronary artery disease among those with the (+/+) genotype was 1.19 (95% CI, 1.00 to 1.42).

Figure 2



Odds ratios and 95% CIs for CAD associated with the (+/+) genotype relative to the (-/-) genotype. Reference number of each study is given in parentheses.

Discussion

In this study, we showed a correlation between the 677C→T mutation in the MTHFR gene and homocysteine concentrations in which homozygous (+/+) and even heterozygous (+/-) individuals exhibited significantly elevated homocysteine levels compared with (-/-) individuals. Furthermore, by combining all previously reported studies, we were able to demonstrate the significance of the homozygous (+/+) genotype as a risk factor for coronary artery disease. Many studies have explored the relationship between elevated homocysteine concentrations and an increased risk for atherosclerotic vascular disease. [2, 3, 32, 33, 34, 35] Recently, these studies have been summarized in a meta-analysis,[1] which led to the conclusion that elevations in homocysteine concentrations have to be considered as an independent and graded risk factor for different categories of arterial occlusive diseases. Several clinical studies supported this conclusion by establishing a quantitative relationship between coronary occlusion and homocysteine levels.[35, 36] On the basis of a linear relationship between homocysteine and the risk of coronary artery disease, Boushey et al [1] calculated an odds ratio for coronary artery disease of 1.6 (95% CI, 1.4 to 1.7) for a 5 μmol/L increase in homocysteine concentrations. Accordingly, a risk of 1.12 can be calculated for a 1 μmol/L increase in homocysteine. In our analysis of the association between MTHFR genotype and homocysteine concentrations, we observed an increase in median homocysteine concentrations of 0.8 μmol/L for heterozygous (+/-) and 2.8 μmol/L for homozygous (+/+) individuals relative to (-/-) individuals, which equals a risk for coronary artery disease of 1.10 and 1.34 for the heterozygotes (+/-) and homozygotes (+/+), respectively, relative to the risk of (-/-) individuals. These risk estimates calculated are well in line with the odds ratios for the homozygous (+/+) and heterozygous (+/-) genotypes observed in the present study (Table1). The frequency of the homozygous (+/+) genotype varies between different populations. The effect of this mutation on homocysteine concentrations depends on study design, inclusion criteria, ethnic background, age, and vitamin intake of

the population. Except for the study of Schmitz et al, [24] all recent studies on this MTHFR variant and hyperhomocysteinemia [18, 19, 21, 29, 37, 38] showed elevated homocysteine concentrations in homozygous (+/+) individuals. The present study supports these observations and indicates again that the homozygous (+/+) genotype is associated with elevated homocysteine concentrations (Table 2 and Fig 1). Folate status is considered an important environmental modulator of homocysteine levels only in homozygous (+/+) individuals.[18, 19, 20, 21] The effect of the homozygous (+/+) genotype on homocysteine concentrations might therefore differ between separate studies as a result of a different intake of folate. A possible adjustment for plasma folate could not be performed in this study, because blood folate levels were not determined. In the present study, we were also able to demonstrate a statistically significant effect of the heterozygous (+/-) genotype on homocysteine concentrations. This is in line with the results reported by Harmon et al. ,[38] who observed elevated plasma homocysteine concentrations in heterozygous (+/-) individuals in the top 50% of the homocysteine distribution. On the basis of specific and residual MTHFR activities measured in isolated lymphocytes, this observation was not unexpected, because we have shown that heterozygous (+/-) individuals have significantly decreased specific and residual MTHFR activities compared with nonaffected (-/-) individuals.[18, 20] MTHFR-dependent homocysteine remethylation, in which 5-methyltetrahydrofolate (the product of the reaction catalyzed by MTHFR) serves as methyl donor, is present in nearly every cell of the human body. Therefore, any significantly deleterious effect in MTHFR enzyme activity will be reflected in elevation of homocysteine concentration in these cells. Previous studies were unable to detect an effect of the heterozygous (+/-) genotype on homocysteine concentrations, probably because of the relatively limited number of individuals included in those studies. Kang et a. [l5] were the first to report on a thermolabile MTHFR variant in two patients with coronary artery disease and hyperhomocysteinemia. Subsequent studies by the same group [14, 15, 39] showed an association between this thermolabile MTHFR and (the severity of) coronary artery disease. In a large

study among coronary artery disease patients and healthy control subjects, Kang et al.[5] detected thermolabile MTHFR in 36 of 212 cases (17%) versus 10 of 202 control subjects (5%).[14] In the present study, we observed a much lower frequency of the thermolabile (+/+) genotype among Dutch coronary artery disease patients and a higher frequency among Dutch population-based control subjects. Several possible explanations for this phenomenon should be considered. In the study by Kang et al.,[5] the frequency of thermolabile MTHFR was assessed biochemically and was not based on genotyping of the 677C→T mutation. Because of the wide range in MTHFR activities in homozygous (+/+) and heterozygous (+/-) individuals,[18, 20] some individuals with a biochemically determined thermolabile MTHFR might not have been homozygotes (+/+), but heterozygotes (+/-) for the thermolabile allele or carriers for other mildly defective MTHFR alleles. In addition, Kang et al. used in their studies a control group consisting of healthy controls with no history or clinical evidence of arterial occlusive disease. In our study, control subjects were recruited from the general population, possibly including individuals with a positive history of coronary artery disease, which may dilute an eventual effect of the homozygous (+/+) genotype. For a summary estimation of the relative risk of the homozygous (+/+) genotype, we analyzed eight different case-control studies presenting data on the MTHFR genotype distribution in coronary artery disease patients. From these studies,[21, 22, 23, 24, 25, 26, 29] only an Irish study [23] observed a significant odds ratio for the homozygous (+/+) genotype in coronary artery disease. In all other studies, the odds ratios for the homozygous (+/+) genotype were not significantly increased. By combining all studies, we were able to calculate a significant odds ratio of 1.22 (95% CI, 1.01 to 1.47) for the homozygous (+/+) genotype relative to the (-/-) genotype in coronary artery disease, an odds ratio comparable to that obtained in the present study on the risk of thermolabile MTHFR in REGRESS. This overall result indicates that the thermolabile (+/+) genotype itself is a modest but significant genetic risk factor for coronary artery disease, a risk that is likely modulated by environmental factors, especially folate status.[18, 19, 20, 21]

In conclusion, we demonstrated for the first time, that both homozygotes (+/+) and heterozygotes (+/-) for the 677C→T mutation in the MTHFR gene have significantly elevated homocysteine concentrations relative to (-/-) individuals. The odds ratios observed for both (+/+) and (+/-) genotypes for coronary artery disease are graded and in concordance
with the risk calculated from a large quantitative study on homocysteine as a risk factor for coronary artery disease. By performing a meta-analysis, we were able to show that the homozygous (+/+) genotype is a genetic risk factor for coronary artery disease.

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7

Chapter

Longterm low-dose folic acid reduces plasma total homocysteine concentration in patients with cardiovascular disease.

The European Concerted Action Project Folic Acid Dose-Finding Study

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Introduction

Moderately elevated plasma concentrations of the sulphur amino acid homocysteine are associated with increased risk of occlusive cardiovascular disease[1]. Elevated plasma total homocysteine concentrations(tHcy) may be reduced by supplemental synthetic folate given as folic acid[2], by vitamin B6 [3] or, in rare cases of vitamin B 12 deficiency, by supplemental vitamin B12[4]. Several randomised control trials designed to test the hypothesis that lowering plasma tHcy reduces risk of cardiovascular disease are currently underway.[5] The present study was designed to identify the minimum dose of folic acid that optimally lowers moderately elevated plasma tHcy levels in patients with cardiovascular disease. Of secondary importance was the issue of whether supplemental vitamin B6 and B12 have significant additional effects in terms of reducing plasma tHcy levels. After the present study was initiated, a meta-analysis of twelve other randomised trials indicated that a 25% reduction in plasma tHcy levels could be achieved with daily dietary folic acid supplements of between 0.5 and 5mg[6] . Supplemental vitamin B12 (0.5mg mean daily intake) reduced tHcy levels by a further seven per cent[6]. However, many of the trials were small[3] and involved highly selected,[7] young and healthy subjects [8,9]. In those trials involving patients, some had venous thrombosis[10] and in those with established vascular disease, the lowest dose of supplemental folic acid used was 0.5 mg[6].

Subjects and Methods

Subjects

One hundred and twenty patients from four European centres with both diagnostic clinical and investigative evidence of vascular disease were recruited. Assuming a 30 % reduction in plasma tHcy concentration, sample size calculations indicated that a study of 100 patients would have 80% power at a 5% level of significance. Patients with clinical and investigative evidence of vascular disease were eligible for this study. Exclusion criteria were the use of

medication or vitamin preparation involved in homocysteine metabolism. Also, patients with systemic illness, chronic alcohol abuse or pregnancy were excluded. The duration of the study was three months and entailed a total of five visits for each patient. Local ethics committee approval was granted in advance of the study.

Methods

The study involved a factorial design as shown in Table1. This design included the incorporation of a vitamin B6 limb and the possibility to study the independent effect of vitamin B12 (400µg) on plasma tHcy levels. Within each centre, each eligible patient was randomised to one of ten treatment groups, resulting in twelve patients in each group. Each of the ten groups was characterised by a specific dose of folic acid (200ug, 1mg and 5mg), vitamin B12 (400µg), vitamin B6 (30 mg), and placebo, alone, or, in combination. All tablets were packaged identically in keeping with the double blind nature of the study. Following an initial screening visit, a blood sample for plasma tHcy and for methylene tetrahydrofolate reductase (MTHFR) genotyping was taken after an overnight fast with the patient seated at 45 degrees for 15 minutes before sampling at the first visit. This sample was centrifuged and stored, protected from light, at -20°. The tHcy sample was repeated at the second visit, two weeks later. At this second visit, sufficient medication for five weeks was provided but each patient was asked to return in four weeks. Further medication was given at the third and fourth visit. The extra seven days' supply was provided to allow for subjects who failed to return for review on schedule i.e. every four weeks. Two further blood samples were taken and stored for tHcy estimations at the third, and eight weeks later, at the fifth visit. Plasma tHcy concentrations were analysed centrally in laboratories in Bergen, Norway, while the analysis for the MTHFR 677 C→T genotype was carried out in Nijmegen, The Netherlands.

Table 1

10 Study groups

Placebo	Vitamin B12 400 µg
Folic acid 200 µg	Folic acid 200 µg +vitamin B12
Folic acid 1 mg	Folic acid 1 mg + vitamin B12
Folic acid 5 mg	Folic acid 5 mg + vitamin B12
Folic acid 1 mg + vit. B6 30 mg	Folic acid 1 mg + vitamin B12 + vit. B6 30 mg

Statistical Methods

The initial analysis describes the average percent change in tHcy for various treatment arms. Further analysis considered the log transformed tHcy levels. Results were obtained using an analysis of covariance that allowed us to describe the association between percentage change in tHcy and baseline tHcy having adjusted for treatment regimen and similarly to describe the variation in percentage change in tHcy at visit three/five and treatment regimen (folic acid, vitamin B6 or vitamin B12) having adjusted for baseline tHcy levels. Average tHcy levels at different time points were compared using a paired t-test of log transformed tHcy concentration.

Results

Using log transformed data, no significant difference in tHcy concentrations between the first and second visits ($p=0.38$) was found and baseline tHcy was defined as the average of the first two readings. There was a significant decrease in tHcy concentration between the baseline measurement and the third visit ($p<0.0001$) and a smaller, although still statistically significant decrease, between the third and last tHcy measurement ($p=0.02$). Tables 2 and 3 indicate the mean proportional change in tHcy concentration according to treatment arm. For those groups receiving folic acid, there was a significant difference in tHcy

concentration between the groups at visit 3 (p=0.004) but not at visit 5 (p==0.31). The effect of vitamin B12 in terms of proportional tHcy reduction was not significant at visit three (p=0.34) but was significant by the time of the last visit (p=0.05).

Table 2

Mean change (%) in total plasma homocysteine at visit three

	vitamin B12 400µg	no vitamin B12	combined groups
Placebo	-1.0 %	-1.5 %	-1.3% (95% CI: -3.7- 1.1)
Folic acid 200 µg	-15.4 %	-9.6%	-12.5% (95% CI: -14.6 - -10.4)
Folic acid 1 mg	-22.7%	-20.0%	-22.4% (95% CI: -23.3- --20.4)
Folic acid 5 mg	-24.9%	-23.6%	-24.2% (95% CI: -26.4- -22.0)
Folic acid 1 mg/ Vitamin B6 30 mg	-23.8%	-22.7%	-23.3% (95% CI: -21.3- --25.3)

Table 3

Mean change (%) in total plasma homocysteine at visit five

	vitamin B12 400µg	no vitamin B12	combined groups
Placebo	-5.8 %	2.1 %	-1.9% (95% CI: -4.0- 1.2)
Folic acid 200 µg	-23.7 %	16.1%	-20.0% (95% CI: -22.7- -17.3)
Folic acid 1 mg	-22.6%	-19.1%	-22.5% (95% CI: -24.5- -20.3)
Folic acid 5 mg	-26.5%	-25.0%	-25.7% (95% CI: -28.1- -23.3)
Folic acid 1 mg/ Vitamin B6 30 mg	-26.8%	-21.5%	-24.2% (95% CI: -26.2- -22.0)

One half of the patients who were randomised to receive l mg folic acid also received 30mg of vitamin B6. An ANCOVA that included baseline log tHcy, folic acid (4 categories), Vitamin B12 (2 categories) and B6 (2 categories), revealed no significant difference in the average percent change in tHcy

concentration between those who received vitamin B6 given at 30mg daily and those who did not. Since vitamin B6 was given to those patients already receiving l mg of folic acid, the possibility that vitamin B6 has an effect on tHcy concentrations when given with other doses of folic acid cannot be excluded. Vitamin B6 was not included in subsequent analysis. There was a significant association between baseline tHcy and proportional change in tHcy levels at visit three ($p<0.0001$) and at visits five ($p=0.02$) (Table 4). Increased baseline tHcy concentrations were associated with a more rapid proportional decline in tHcy concentration.

Table 4

Predicted change (%) in tHcy at visit 3 and 5 (mean change and 95% CI)
Results adjusted for baseline total plasma homocysteine and vitamin B12 treatment

	Combined B12 arms at visit 3 (mean and 95% CI)	Combined B12 arms at visit 5 (mean and 95% CI)
Placebo	-2.5% (95% CI –8.5- 3.9)	-1.1% (95% CI: -8.3- 6.5)
Folic acid 200 µg	-13.6 % (95%CI –18.9- --7.9)	-19.5% (95% CI: -25.4- -13.1)
Folic acid 1 mg	-24.0%(95%CI-27.7- -20.1)	-22.5% (95% CI: -26.9- -17.8)
Folic acid 5 mg	-26.1%(95% CI-30.8- --21.1)	-25.7% (95% CI: -31.3- -19.6)

Among those receiving folic acid there was a significant association between folic acid dose and the proportional decline in tHcy at visit three ($p<0.0001$), but not visit five ($p=0.22$). The lowest dose of folic acid (200µg) achieved a reduction in tHcy concentration at visit five which was not significantly different from that achieved by the highest dose at the third visit: 26.1(95CI: 21.1 -30.8) compared to 19.5(95CI: 13.0 - 25.4). There was a significant association between vitamin B12 and the additional proportional reduction at the fifth visit 7.0% (95C.l.: 0.0 -13.0) but not at week three (95 CI:-3 -13.0) Because the methylene tetrahydrofolate reductase 677 C→T mutation is one of the factors responsible for the interindividual variation in the response to tHcy lowering therapy the frequency of this genotype was estimated. In total, twelve homozygotes were

identified (8.6%) and, at most, two homozygotes for the genotype were found in 4 treatment groups.

Discussion

Plasma total homocysteine (tHcy) concentration is inversely related to plasma folate concentration [11,13] and to dietary intake of folate and vitamin B6 [12]. This inverse relationship is weaker for vitamin B6 and vitamin B12 levels [12]. The lowest plasma tHcy concentration is associated with a folate intake of approximately 400μg/day [12] but recent data suggest that the intake among adults age 65 years and older is actually between 200 and 250 μg/day [14,15]. The present study indicates that, among patients with established cardiovascular disease, daily exposure to the lowest dose of supplemental folic acid (200μg) for 12 weeks achieves a reduction in plasma tHcy that is not significantly different from that achieved with 1mg of folic acid for four weeks. A daily supplemental dose of 200μg synthetic folic acid with 400 μg vitamin B12 is sufficient to reduce plasma tHcy concentrations by approximately 20 per cent in this population with established cardiovascular disease and high baseline tHcy concentrations. This suggests that among typical patients with atherosclerotic vascular disease, it is possible to saturate folate stores with daily low-dose folic acid and vitamin B12 supplements. Providing higher daily doses achieves little further reduction in plasma tHcy concentration. The 30mg dose of vitamin B6 used in this study had no significant effect on elevated fasting tHcy concentration. This does not exclude an effect at higher doses and does not discount the possible value of reducing post-load tHcy concentrations (not measured in this study) with vitamin B6 [16]. In addition, there is evidence that vitamin B6 deficiency is related to vascular disease risk independently of tHcy concentration [17,18]. One other general practice-based study among healthy patients indicates that an additional folate intake of 200 μg/day reduces plasma tHcy by 10% [19]. In one further study, plasma tHcy was reduced by 20% with an additional 350 μg/day of dietary folic acid [20]. The reduction in plasma tHcy concentration achieved by a 200 μg daily dose of synthetic folic acid is important

for three reasons. Firstly, achieving maximal tHcy reduction with the lowest effective dose of folic acid may reduce the risk of potential adverse effects resulting from higher intakes of folic acid [21]. Secondly, a daily folic acid increment of 200μg approximates to what is achievable by optimal dietary means alone [5]. However, a recent study illustrates the well-described reduced bioavailability of dietary folate [22] and suggests that the most effective means of lowering tHcy concentration is with fortified foods or synthetic folic acid supplements [23]. An approximate 300 μg increase in daily folic acid from folate-fortified cereals resulted in a plasma tHcy reduction of 24 per cent. However, the consumption of folate-rich foods that achieved a daily folate increase of over 400 μg resulted in a nine per cent reduction in plasma tHcy concentrations. Folic acid supplements did not achieve a tHcy lowering effect in excess of that achieved by folic acid fortified cereals [23]. Thirdly, a 20 per cent reduction in plasma tHcy concentration among populations with a mean level of 12 μmol/L might result in a cardiovascular risk reduction of 25 percent [24]. By a variety of methods, an increased intake of folic acid would prevent between four and 14 per cent of coronary heart disease deaths in the United States population [23]. Enrichment of flour with folic acid has been undertaken in the United States since 1998 and one early report suggests a significant reduction in the prevalence of low folate states [25]. Whether or not this increase in folate intake will reduce the risk of first occurrence of cardiovascular disease in the entire population remains to be seen. The present study suggests that potentially significant benefits might be achievable with low dose folic acid supplementation in populations of patients with established cardiovascular disease.

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8

Chapter

Hyperhomocysteinemia is not associated with the extent and progression of coronary atherosclerosis in men with coronary artery disease

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Introduction

Atherosclerosis remains the leading cause of death in Western society. Established risk factors as hypercholesterolaemia, hypertension, diabetes mellitus and smoking cannot fully explain the great number of patients with cardiovascular disease. Different inborn errors in methionine metabolism lead to severe hyperhomocysteinemia or homocystinuria. Such patients suffer from serious vascular disease[1]. These observations led to the hypothesis that homocysteine is a toxic agent for the vascular system. Since then a large number of epidemiological studies have been published on the relationship between mild hyperhomocysteinemia and coronary artery disease. It has now been demonstrated that an elevated fasting total plasma homocysteine level (tHcy) in patients with vascular disease is associated with increased cardiovascular mortality[2,3]. In a recent meta-analysis elevated homocysteine is also considered a modest independent predictor of coronary artery disease in healthy populations [4]. The mechanism responsible for the toxic effect of homocysteine remains however unclear. Several studies demonstrated a correlation between tHcy levels and the extent and severity of coronary artery disease[5,6,7]. However these results are not unequivocal [2,8]. None of these studies used quantitative coronary angiography (QCA) for the evaluation of the extent and severity of coronary artery disease. Previous angiographic follow up studies using quantitative coronary angiography could not demonstrate a convincing relationship between classic risk factors as smoking, blood pressure, total cholesterol, LDL-cholesterol and HDL- cholesterol and progression of atherosclerosis[9,10]. The effects of elevated plasma homocysteine levels on progression of atherosclerosis are unclear. Aim of this study was to evaluate the relationship of tHcy with the extent of coronary artery disease and the relationship of tHcy with anatomical changes in the coronary vessels in patients with symptomatic coronary artery disease.

Methods

Study population:

The study population was recruited from the Regression Growth Evaluation Statin Study (REGRESS) population, a double blind, placebo controlled, multicenter study conducted in the Netherlands to assess the effects of 2 years of treatment with the HMG-CoA reductase inhibitor pravastatin on progression and regression of angiographically documented coronary atherosclerosis[11]. All male patients, < 70 years old, who were scheduled to undergo coronary angiography for clinical reasons were eligible for participation in this study. If the total cholesterol value was between 4.0 mmol/l and 8.0 mmol/ and the coronary angiography showed at least one lesion that narrowed the lumen in a coronary vessel by ≥50% the patients were qualified for this study. Patients with previous PTCA and CABG, patients with congestive heart failure, pacemaker implant and cardiac disease requiring valve replacement were excluded. Also, patients with diabetes mellitus, uncorrected hypo- or hyperthyroidism, renal disease (creatinine ≥ 150 μmol/l), hepato-biliary or pancreatic disease were excluded. Between December 1989 and December 1991, 1068 patients were initially screened before participation in the REGRESS study. Finally 885 patients were randomized. Patients received pravastatin, 40 mg once daily or placebo and during the treatment period patients were asked to stay on a stable diet. Of 606 patients a EDTA-plasma sample was available for tHcy determination**.**

Clinical events

The following clinical events were analyzed: Myocardial infarction (fatal and nonfatal). To establish a diagnosis of a new myocardial infarction two of the three following criteria had to be met: characteristic angina> 30 minutes, new ischemic Q waves or ST-T wave changes in the ECG and elevation of creatine-kinase MB fraction to > 3 times the upper limit of normal. Coronary heart disease death : no known non-athererosclerotic cause and presence of cardiac symptoms within 72 hours of death. Non-scheduled PTCA or CABG. Stroke and

transient ischemic attack: motor or sensory dysfunction more (stroke) or less (TIA) than 24 hours.

Quantitative coronary angiography

The influence of tHcy on the extent and progression of atherosclerosis was assessed using quantitative coronary angiography at baseline and after two years. For quantitative coronary angiography the coronary vessels were divided in 13 segments according to the American Heart Association classification.[12] Mean Segment Diameter (MSD) and Minimum Obstruction Diameter (MOD) of all segments were measured using quantitative coronary angiography. MSD reflects diffuse changes in coronary segments or arteries while MOD reflects the degree of narrowing of an atherosclerotic lesion. A stenosis of ≥ 50% was considered significant. Patients with a stenosis more than 50% in two vessels or more are considered to have multivessel disease. Because PTCA and CABG procedures may influence progression considerably we excluded in the primary analysis lesions and segments modified or conceivably modified by PTCA or CABG. In addition, patients were categorized with regard to MOD and clinical events as progressors, stable patients and regressors. Progressors are patients with at least one lesion worsening by ≥0.4 mm or the development of a new lesion that reduces the lumen diameter by ≥ 0.4 mm. If a patient had suffered a clinical cardiovascular event, he was considered to be a progressor, irrespective of the angiographic outcome.

Biochemical Measurements

After an overnight fasting an EDTA-plasma sample of venous blood was drawn from the patients. Plasma total homocysteine was measured by high performance liquid chromatography[13]. Patients were divided in quartiles according to their homocysteine level. For calculation of the relative risk of progression of atherosclerosis in hyperhomocysteinemia, we considered a tHcY of ≥ 15 µmol/l as abnormal. Lipid laboratory tests were carried out at the Lipid Reference

Laboratory and comprised total cholesterol, HDL- cholesterol and triglycerides. LDL-cholesterol was calculated according to the Friedewald formula.

Statistical analysis:

Analysis of data was performed using one-way analysis of variance or Chi-Square test where appropriate. Differences between homocysteine-quartile groups were tested for trend. Spearman rank correlations were reported for the association between homocysteine levels and MSD and MOD at baseline and for the association between change in MOD, MSD and percent stenosis during follow-up. Angiographic changes were calculated using covariance analysis with baseline levels as covariate and homocysteine levels as factor. Cumulative event incidence were calculated with Kaplan-Meier curves, and compared using the log-rank test.

Results

Of the 885 patients included in the REGRESS study, homocysteine levels were available in 606 patients. Angiographic follow-up was available in 438 patients. Baseline characteristics of these groups are presented in table 1. Patients not included in this study had slightly more often a history of hypertension (33%) than patients included in the study (26%) ($p=0.027$). Patients in whom a plasma homocysteine sample was available and who had a second angiography, total cholesterol (5.96 mmol/l SD 0.87) was slightly lower compared to the patients initially included in REGRESS (6.04 mmol/l SD 0.86) $p<0.05$. During follow-up HDL-cholesterol increased significantly more in the 606 patients eligible for this study compared to patients in whom no homocysteine sample was available. Otherwise there were no significant nor large differences in baseline characteristics.

Table 1

Baseline characteristics

	All Patients (N=885)	Patients with homocysteine sample (n=606)	Patients with homocysteine sample and second angiography (n=438)	p value† p1‡	p2§
Age (years)	56.2 (8)*	56.4 (8)	55.6 (8.0)	0.44	0.53
Body Mass Index	26.0 (2.7)	25.9 (2.6)	25.9 (2.7)	0.10	0.15
Systolic BP (mmHg)	135 (19)	135(18)	134 (18)	0.97	0.79
Diastolic BP (mmHg)	81 (10)	81 (10)	82 (10)	0.72	0.79
Ejection fraction (%)	70 (13)	70 (12)	71 (12)	0.73	0.84
Baseline MOD (mm)	1.76 (0.35)	1.75 (0.35)	1.80 (033)	0.28	0.98
Baseline MSD (mm)	2.74 (0.37)	2.73 (0.38)	2.76 (0.37)	0.18	0.53
Total cholesterol (mmol/l)	6.04 (0.86)	6.00 (0.86)	5.96 (0.87)	0.07	0.017
HDL-cholesterol(mmol/l)	0.93 (0.23)	0.92 (0.22)	0.93 (0.22)	0.27	0.37
LDL-cholesterol(mmol/l)	4.31 (0.78)	4.28 (0.78)	4.25 (0.78)	0.23	0.10
Triglycerides(mmol/l)	1.78 (0.76)	1.76 (0.76)	1.73 (0.75)	0.29	0.14
Creatinine(µmol/l)	96.4 (15.7)	96.8 (16.5)	96.9 (16.8)	0.30	0.30
Smoking (%)	27.7%	26.2%	26.9%	0.09	0.17
History of hypertension (%)	27.8%	25.6%	26.9%	0.027	0 .09
Familial heart disease (%)	48.6%	48.3%	47.4%	0.76	0.59
History of myocardial infarction (%)	47.4%	46.4%	45.3%	0.35	0.24

*Values are reported as a mean with SD between brackets
† P-value of one way analysis of variance, or chi-square test where appropriate.
‡ p1 for the difference between all patients and patients in whom a plasma homocysteine sample was available,
§ p 2 for the difference between all patients and patients with in whom a plasma homocysteine sample was available and also a second angiography.

Of the 606 patients included in this study mean age was 56.6 years (SD 8.0). Median tHcy was 13.2 µmol/l with a range of 6.5-67.7 µmol/l. Patients were divided in quartiles according to their homocysteine level. The quartiles ranged from 6.5 µmol/l to 11.3 µmol/l (1), 11.4 µmol/l-13.2 µmol/l (2), 13.5 µmol/l-15.5 µmol/l (3) and 15.6 µmol/l-67.7 µmol/l (4). Baseline characteristics of the four quartiles are shown in table 2. As expected homocysteine levels are correlated with age ($p<0.001$). Triglycerides between quartiles differed significantly ($p=0.021$). No other differences between quartiles existed. Table 3 shows the extent of cardiovascular disease of the four quartiles. When

considering a stenosis of ≥ 50% significant, approximately 60% of the patients had multivessel disease at baseline. The number of vessels involved in the

Table 2

Baseline characteristics patients with homocysteine sample at baseline

Homocysteine levels in quartiles (µmol/l)	**6.5-11.3 (n=152)**	**11.4-13.2 (n=153)**	**13.3-15.5 (n=154)**	**15.6-67.7 (n=147)**	**p value†**
Age (years)	53.5 (7.6)*	55.7 (7.5)	58.1 (8.0)	58.4 (8.1)	<0.001
BMI	26.0 (2.5)	25.8 (2.4)	26.0 (3.0)	25.8 (2.5)	0.92
Systolic BP (mmHg)	133 (19)	135 (19)	137 (19)	134 (16)	0.24
Diastolic BP (mmHg)	80 (11)	81 (9)	81 (10)	82 (10)	0.23
Ejection fraction (%)	70 (12)	70 (12)	71 (12)	70 (13)	0.81
Total cholesterol (mmol/l)	5.96 (0.8)	6.08 (0.8)	5.90 (0.9)	6.06 (0.9)	0.67
HDL-cholesterol (mmol/l)	0.93 (0.24)	0.95 (0.19)	0.91 (0.22)	0.90 (0.22)	0.10
LDL-cholesterol (mmol/l)	4.25 (0.8)	4.38 (0.7)	4.18 (0.8)	4.32 (0.86)	0.97
Triglycerides (mmol/l)	1.74 (0.8)	1.64 (0.7)	1.78 (0.7)	1.89 (0.8)	0.021
Smoking (%)	28.9%	23.5%	22.1%	30.6%	0.87
History of hypertension(%)	21.7%	21.7%	33.8%	25.2%	0.16
Familial heart disease(%)	48.7%	48.4%	46.8%	49.7%	0.97
History of myocardial(%) infarction	45.4%	43.8%	49.4%	46.9%	0.66

*Values are reported as a mean with SD between brackets
† P-value of one way analysis of variance, or chi-square test where appropriate

Table 3

Extent of coronary artery disease in 606 patients

Homocysteine levels in quartiles (µmol/l)	**6.5-11.3 (n=152)**	**11.4-13.2 (n=153)**	**13.3-15.5 (n=154)**	**15.6-67.7 (n=147)**	**p value***
Extent of coronary artery disease					0.24
1 vessel	66 (43.7%)	56 (36.6%)	64 (41.6%)	53 (36.6%)	
2 vessels	50 (33.1%)	58 (37.9%)	51 (33.1%)	49 (36.6%)	
3 vessels	35 (23.2%)	39 (25.5%)	39 (25.3%)	43 (29.7%)	

* exact chi-square test for linear trend

atherosclerotic process did not differ among quartiles (p=0.24). When considering a tHcY of ≥ 15 μmol/l as abnormal, the relative risk (RR) for patients with tHcY ≥15 μmol/l to have multivessel disease is 1.21, p=0.54, (95%CI: 0.78-1.58) .

Table 4 shows the clinical events of the patients according to their homocysteine levels. The number of cardiovascular deaths is less than 1% irrespective of homocysteine levels. Only 92 patients suffered from a cardiovascular event during follow up. The number of events did not correlate with tHcy quartiles (p=0.97). Also, when we compared the number of events in the placebo and pravastatin groups separately no differences were found concerning the number of events in the four homocysteine quartiles (p=0.87 and p=0.60 respectively). The RR for patients with tHcY of ≥15 μmol/l to suffer a cardiovascular event is 1.01, (95% CI: 0.62-1.62) p=0.96.

Table 4

clinical events

Homocysteine levels in quartiles (μmol/l)	**6.5-11.3 (n=152)**	**11.4-13.2 (n=153)**	**13.3-15.5 (n=154)**	**15.6-67.7 (n=147)**	**p value ***
non-fatal myocardial infarction	2	1	3	3	0.46
fatal myocardial infarction	0	0	0	1	0.25
cardiovascular death	1	1	1	1	0.99
death unknown cause	1	1	2	0	0.83
non-scheduled PTCA	13	9	13	8	0.48
non-scheduled CABG	5	10	12	6	0.69
Stroke	1	0	0	1	0.99
Non vascular death	2	1	0	1	0.52

* exact chi-square test for linear trend

Of 606 patients enrolled in this study, a second coronary angiography was available in 438 patients. Complete angiographic data of the remaining 168

patients were not available for analysis due to missing of a pair of matching angiographies (n=77) or due to the fact that all coronary segments were considered to be influenced by a performed PTCA or CABG(n=91). In the first homocysteine quartile 38 (25%) patients had no second angiography, in the second quartile 32 (21%), in the third 37 (24%), and in the fourth quartile 51 (35%). This difference between quartiles was statistically significant (p=0.048). During follow-up lipid values in the placebo group remained unchanged. Lipid values in the pravastatin group decreased significantly. Other baseline risk factors did not change significantly during follow-up. Changes in lipid values during follow-up differed not between the patients with and without a second angiography. Changes in MSD and MOD were not associated with different quartiles of homocysteine in all patients (p=0.39, p=0.86 respectively) (Table 5).

Table 5

Angiographic changes during follow-up

Homocysteine levels in quartiles (µmol/l)	**6.5-11.3**	**11.4-13.2**	**13.3-15.5**	**15.6-67.7**	**p value†**
All patients (n=438)					
Change MSD, mm	-0.06 (0.18)	-0.09 (0.20)	-0.10 (0.20)	-0.09 (0.20)	0.18
Change MOD, mm	-0.11 (0.44)	-0.10 (0.22)	-0.12 (0.27)	-0.09 (0.21)	0.68
Placebo group (n=216)					
Change MSD, mm	-0.06 (0.19)	-0.13 (0.21)	-0.14 (0.23)	-0.13 (0.20)	0.13
Change MOD, mm	-0.07 (0.16)	-0.14 (0.23)	-0.15 (0.26)	-0.11 (0.20)	0.30
Pravastatin group (n=219)					
Change MSD, mm	-0.05 (0.18)	-0.05 (0.18)	-0.06 (0.16)	-0.05 (0.21)	0.94
Change MOD, mm	-0.15 (0.57)	-0.06 (0.20)	-0.08 (0.28)	-0.06 (0.22)	0.25

*Values are reported as a mean with SD between brackets
† p-value of covariance analysis with baseline levels as covariate, and homocysteine quartile as factor testing the trend in the quartiles.

The mean change in MSD was -0.08 mm (SD 0.19) for tHcy < 15 µmol/l compared to –0.10 mm (SD 0.20) for tHcY ≥15 µmol/l, p=0.43, mean difference

0.017 mm (95%CI: -0.025 - 0.054). The mean change in MOD was -0.11 mm (SD 0.33) for tHcy < 15 µmol/l compared to –0.10 mm (SD 0.21) for tHcY ≥15 µmol/l, p=0.84, mean difference 0.006 (95%CI: -0.057 - 0.070).
Change in MSD, change in MOD and change of percent stenosis did not correlate with tHcy (Spearman rank correlation factor r=0.05, r=0.01, r=0.02 respectively**).** 282 patients out of 438 patients were progressors (64%), progression was not related to quartiles of tHcy (p=0.37). The relative risk of progression for patients with tHcY≥15 µmol/l is 1.14 (95%CI 0.81-1.58), p=0.77. When we compared the effects of tHcy levels for the placebo group and the pravastatin treated group separately, again no differences were found. The relative risk was also calculated for baseline total cholesterol ≥6.0 mmol/l (RR 1.67, 95% CI 1.15-2.43, p=0.007), LDL cholesterol ≥ 4.3 mmol/l RR 1.38, 95% CI 0.95-2.01, p=0.11), HDL cholesterol < 0.9 mmol/l (RR 1.15 95% CI 0.79-1.67, p=0.43), triglycerides ≥ 1.6 mmol/l (RR 1.13, 95% CI 0.78-1.64, p=0.48), hypertension (yes/no) (RR 0.98 95% CI 0.64-1.5, p=0.73) and smoking (yes/no) (RR 1.16 95% CI 0.76-1.76, p=0.11).

Discussion

This is one of the largest prospective study on the relationship between tHcy levels and progression of atherosclerosis in coronary vessels in patients with symptomatic coronary artery disease. This study showed no association between tHcy and the extent of coronary atherosclerosis and no significant correlation between tHcy levels and progression of atherosclerosis. In this study only baseline total cholesterol was significantly related to progression of atherosclerosis. In REGRESS none of the other clinical baseline risk factors in patients were related to progression. In contrast to our findings there is strong evidence in both experimental and clinical investigations that hyperhomocysteinemia is associated to clinical vascular disease. There is growing evidence both in vitro and in vivo studies that homocysteine causes endothelial injury and therefore may have an adverse impact on anti-thrombotic and vasomotor effects of the vessel wall [14,15]. Recently we could demonstrate

that homocysteine-lowering treatment improved coronary endothelial function in hyperhomocysteinemic patients[16].

Homocysteine in relationship to cardiovascular events:

The number of cardiovascular events during a relative short period of follow-up was too low to draw conclusions about a possible relationship between elevated tHcY and cardiovascular events. Based on the data of this study the odds ratio estimating the risk to suffer a vascular event in patients with a homocysteine level of ≥ 15 µmol/l was only 1.01 (95%CI 0.62-1.62). These data are conflicting with the data of Nygård et al [2] and Taylor et al.[3] who showed, that tHcy is related to cardiovascular events and mortality. A possible bias in Nygårds study was that patients with a low ejection fraction were responsible for more than 50% of the mortality suggesting that the lower ejection fraction, at least in part, may be responsible for this excess in mortality. Patients in our study were relatively younger, and patients above 70 years excluded**.** Also, patients suffering from diabetes mellitus were excluded. Some of these differences in may explain the fact that the number of events in our study was low, irrespective of the patients' homocysteine levels. In our study, a significantly higher number of patients in the quartile with the highest homocysteine levels had no second angiography. This aspect could bias the results of our study although the number of clinical events differed not among quartiles. Since cardiovascular events are correlated with changes in serial quantitative coronary angiography, it seems less likely that this aspect has important drawbacks on our study[17] .

Homocysteine and coronary atherosclerosis:

Data upon possible relation between homocysteine and the extent of coronary artery disease are not unequivocal. Some investigators found a graded effect of homocysteine levels on coronary artery disease [5,6,7] while other investigators found no relation [8]. This may be explained by the fact that the investigators used different methods to assess the extent of coronary artery disease, none of them based on quantitative coronary angiography. It is well known that visual

estimation of the degree of coronary stenosis is subject to significant operator variability[18]. The use of quantitative coronary angiography has several drawbacks when we are investigating the effect of homocysteine on progression of coronary atherosclerosis. Due to a lack of follow-up in a substantial portion of the patients initially included, partly because of a clinical event, the effects of homocysteine on change in MSD and MOD can be underestimated in this study. In this study, in the group of patients with the highest plasma homocysteine levels, the number of patients without a second angiography is significantly higher. However, since the correlation coefficients for the association between tHcY levels and change in MOD or MSD show no association it seems less likely that our results have been biased by the lack of follow up in a part of this group. It is also possible that a follow up period of two years for detection of atherosclerotic changes related to homocysteine is too short. Observational studies with repeated coronary angiography have shown that mainly the time between two consecutive angiographies, and not baseline variables like lipid levels, was associated with progression of atherosclerotic disease [9,10,19,20]

Study limitations

The results of our study, as in all angiographic studies, can be biased since follow-up angiography was not available in all patients who were initially included in this study. It is possible that changes in risk factors during follow-up influenced the results of our study. However lipid values in the placebo group and smoking habits in both groups did not change significantly. It seems less likely that changes in other risk factors have biased our findings. Although the number of patients included in this angiographic study is high, a two year follow up to investigate the effect of homocysteine on progression of atherosclerosis is probably too short. The number of events is too low to draw conclusions upon a possible relationship between elevated plasma homocysteine and cardiovascular events. We have no data on folic acid and vitamin B12 levels of the patients included in this study. However, irrespective of the cause of

hyperhomocysteinemia in the study patients the effect of homocysteine on vascular disease remains probably the same.

Conclusion

The findings in this prospective angiographic study suggest that tHcy is not associated with angiographic progression of atherosclerosis nor with the extent of coronary artery disease. Since elevated plasma homocysteine levels are clearly associated with an increased cardiovascular risk, the mechanism responsible for this increased risk is remains unclear.

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9

Chapter

Coronary endothelial dysfunction is related to elevated plasma homocysteine levels in patients with coronary artery disease

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Freek WA Verheugt. Submitted

Introduction

Hyperhomocysteinemia is a strong and graded risk factor for premature cardiovascular disease. Until now more than 80 studies have been published upon the association between homocysteine and vascular disease[1]. The pathophysiological mechanisms responsible for this increased risk remain unclear. Evidence is accumulating that elevated homocysteine causes endothelial dysfunction[2]. Endothelial dysfunction is a key mechanism in the current hypothesis of atherothrombosis. Recently we could demonstrate that homocysteine –lowering treatment in hyperhomocysteinemic patients improves coronary endothelial function[3] . Most but not all human studies show a relationship between increased total plasma homocysteine levels (tHcY) and impaired endothelial function using a fore-arm model [2]. Although factors known to impair endothelial function in the coronary circulation also are associated with impaired flow mediated dilatation in the brachial artery[4,5] , the direct relevance of this technique for long term cardiovascular risk is uncertain. Aim of this study was to investigate the relationship between elevated tHcY and acetylcholine-induced changes in coronary blood flow (CBF) in non-stenotic coronary vessels in patients with coronary artery disease.

Methods

Patient selection

Patients with objectively confirmed ischemic heart disease who were referred for elective PTCA to our catheterization laboratory were eligible for this study. Endpoints were acetylcholine-induced changes in endothelium dependent coronary blood flow. Inclusion criteria were age between 18 and 70 years and at least one coronary vessel without a significant stenosis (stenosis < 20%) . We considered a tHcY of ≥ 15 μmol/l as abnormal. Patient groups were matched for gender, smoking habits, history of hypertension, family history of vascular

disease, lipid levels and actual medication. Patients who used medication or vitamins involved in homocysteine metabolism were excluded from this study as were patients with diabetes mellitus and patients with uncontrolled hypertension. Patients with renal failure reflected as a creatinine level of more than 150 µg /l or serious liver failure were also excluded. The ethical committee of our institution approved the protocol. Informed consent was obtained in all patients.

Coronary angiography

Long acting nitrates were withdrawn 24 hours before the procedure and replaced by short acting nitrates if necessary. After the performance of the elective PTCA a new guiding catheter 7 Fr. (Medtronic) was placed in a non-dilated coronary artery without a significant stenosis (< 20%). A Doppler guide wire (0.014 inch diameter), (Flowire, Endosonics Incorporated) within a 2.2 F coronary infusion catheter (Medtronic) was advanced in the vessel of interest just distal to a landmark of a proximal epicardial segment with a diameter of at least 2.0 mm. Assessment of endothelium dependent vasodilatation and coronary flow was performed by subsequent selective infusions of acetylcholine with a total amount of 10^{-8} M, 10^{-7} M, 10^{-6}M respectively. Acetylcholine was dissolved in NaCl 0.9% and infused with a Terumo® pump at a rate of 2 ml/min during 3 minutes. For assessment of endothelium independent vasodilatation nitroglycerin 200 µg was then injected in the coronary vessel as a bolus. Before infusion of acetylcholine baseline angiography was performed using Hexabrix® with biplane technique. Then acetylcholine infusion started. After each infusion of acetylcholine a coronary angiography followed.

Assessment of coronary blood flow

Doppler flow velocity spectra were analyzed to determine time-averaged peak velocity. Coronary blood flow (CBF), assuming laminar flow, is the cross sectional area times average peak velocity times 0.5 [6]. Endothelium-dependent coronary flow reserve is calculated as percent change in CBF in response to

acetylcholine. Normal coronary endothelium dependent function is defined as an increase of CBF of 50% or more [7].

Quantitative coronary angiography

Analysis of baseline and follow-up angiographies was performed using the CAAS II QCA software (Pie Medical, Maastricht, The Netherlands). Analysis of MOD and MSD started at the proximal landmark of the defined epicardial segment and continued until the second important landmark of the segment. For assessment of CBF the cross sectional area was measured over 5 mm in the segment 5 mm to 10 mm distal to the tip of the Flowire.

Laboratory measurements

After an overnight fast a sample of venous blood was drawn on EDTA from the patients and put on ice immediately. Plasma total homocysteine was measured by high performance liquid chromatography[8]. Also samples for vitamin and lipid analysis were analyzed.

Statistics

A sample size of 18 subjects would be required to detect an improvement in CBF of 100% (SD 50%), with 90% power at a 0.05 significance level. Data are expressed as mean ± standard deviation (SD). Analysis of data was performed using one-way analysis of variance (ANOVA) or chi-square test where appropriate. Response differences in CBF between groups following two subsequent amounts of acetylcholine infusion (10^{-7}M and 10^{-6}M) were compared using a multivariate repeated measures model (General Linear Model, SPSS 10.1). Spearman rank correlation coefficients are given for the relationship between tHcY and maximal change in CBF.

Results

The study population eligible for this study consisted of 38 patients, 34 men and 4 women. Mean age was 54.4 years (SD 7.0) and mean tHcY was 14.2 (SD 4.2).

19 patients had a tHcY of < 15 µmol/l (mean tHcY: 10.5 µmol/l (SD 2.1) and 19 patients had a tHcY of ≥15 µmol/l (mean tHcY: 17.9 µmol/l, (SD 1.7). Baseline characteristics of the two groups are shown in table 1. Folic acid levels were significantly higher in patients with low tHcY levels, 13.6 nmol/l (SD 1.9) compared to 10.6 nmol/l (SD1.4) in patients with high tHcY($p<0.001$). Levels of vitamin B12 were also significantly higher in patients with low tHcY, 433 pmol/l (SD 55) compared to 380 pmol/l (SD 70) in patients with high tHcY($p<0.05$). No other differences in baseline characteristics between groups existed.

Table 1

Baseline characteristics

Homocysteine level	< 15 µmol/l	≥15 µmol/l	P value*
Age (years)	56.2	53.0	0.15
Male/female	17/2	17/2	1.0
Smoking (yes)	4	5	0.72
Hypertension (yes)	4	5	0.72
Hyperlipidemia	16	16	1.0
Family history	11	9	0.54
Statin therapy	16	15	0.98
Homocysteine (µmol/l)	10.6 (2.2)	17.9 (1.7)	<0.0001
Total Cholesterol (mmol/l)	5.41 (0.4)	5.68 (1.0)	0.30
LDL-cholesterol (mmol/l)	3.55 (0.4)	3.91 (0.8)	0.11
HDL-cholesterol (mmol/l)	1.04 (0.18)	1.01 (0.19)	0.65
Triglycerids (mmol/l)	1.6 (0.2)	1.6 (0.6)	0.94
Folic acid (nmol/l)	13.9 (1.9)	10.6 (1.6)	<0.001
Vitamin B12 (pmol/l)	433 (55)	380 (70)	<0.05
Creatinine (µmol/l)	97 (6)	92 (9)	0.11
Glucose (mmol/l)	5.32 (0.4)	5.18 (0.7)	0.55

*p value using one way analysis (ANOVA) or Chi Square test where appropriate

†between brackets: SD

Response of CBF to acetylcholine (table 2):

Baseline CBF(ml/min) was 54.7 ml/min (SD 24) in patients with normal plasma homocysteine levels compared to 49.2 ml/min (SD 13) in hyperhomocysteinemic patients p=0.39; (95% C.I –7.4 ml/min to +18 ml/min). In 21 out of 38 patients the increase of coronary blood flow following subsequent dosages of acetylcholine was abnormal, 16 patients with elevated plasma homocysteine

levels and five patients with a normal tHcY. In subjects with normal tHcY, CBF (ml/min) increased at the maximal dosage of AcH from 54.7 ml/min to 114.2 ml/min (SD 71) compared to an increase from 49.2 ml/min to 51.9 ml/min (SD 26) in hyperhomocysteinemic patients ($p<0.01$) (95% C.I. 42-163). Figure 1 demonstrates the response in CBF (%) to subsequent amounts of AcH and nitro-glycerine. Response differences in CBF (%) between the two groups following two subsequent amounts of acetylcholine infusion (10^{-7}M and 10^{-6}M) demonstrated a mean increase in CBF of 10% (SD 40) for patients with high tHcY compared to a mean increase of 88 % (SD 91) for patients with a normal tHcY ($p<0.001$) (95% C.I. 59-124) (Repeated Measures Model).

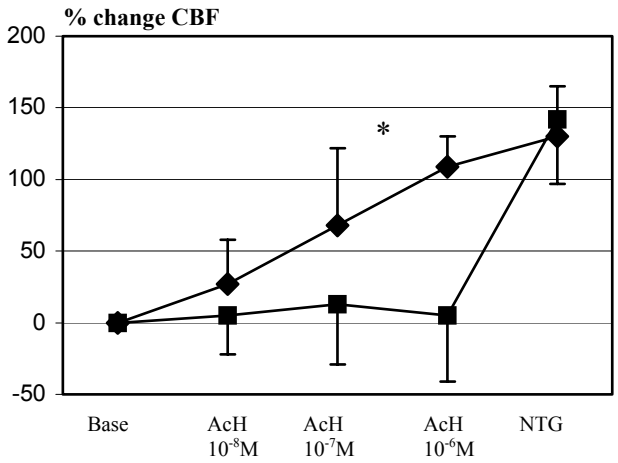

Figure 1
Effect of acetylcholine and nitroglycerin on percent change in coronary blood flow in both groups. ◆=total plasma homocysteine < 15 µmol/l. , ■= total plasma homocysteine ≥15 µmol/l. *p< 0,001 for the response differences in CBF (%) between the two groups following two subsequent amounts of acetylcholine infusion (10^{-7}M and 10^{-6}M) (95% C.I. 59%-124%) (Repeated Measures Model).

When corrected for folic acid or vitamin B12 the difference is still significant ($p<0.05$ and $p<0.001$ respectively). Odds ratio (OR) for the association of hyperhomocysteinemia and coronary endothelial dysfunction was 14.9 (95% C.I. 3.0-74.0) $p<0.01$).

Spearman rank correlation coefficients for the association of tHcY and maximal change in CBF(%) was –0.43 ($p<0.005$) (Figure 2). When corrected for folate and vitamin B12, spearman rank correlation coefficient was –0.30 ($p=0.042$). Endothelium independent vasodilatation following the administration nitro-glycerine 200 µg was comparable for both groups ($p=0.94$).

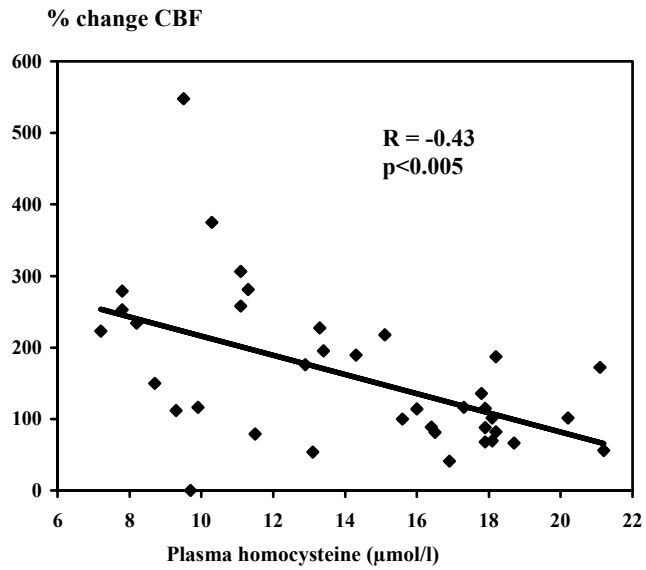

Figure 2
Scatter plot depicting the maximal change in coronary blood flow (CBF) (%) following acetylcholine (10^{-6}M).Spearman rank correlation coefficient r=-0.43, $p<0.005$ for the association of plasma homocysteine levels and maximal change in coronary blood flow

Response of MSD and MOD to acetylcholine (table 2):

Mean segment diameter (MSD) at baseline was comparable between the groups($p=0.67$). Mean MSD decreased from 2.46 mm (SD 0.53) to 2.11 mm (SD 0.72) following the administration of AcH 10^{-6} M.

Table 2

Coronary flow and epicardial diameter following infusion of acetylcholine

Homocysteine level	< 15 µmol/l	≥15 µmol/l	P value*
CBF	**(ml/min) % change**	**(ml/min) % change**	
Baseline	54.7 (24) †	49.2(13)	
AcH 10^{-8} M	69.3(35) 26.8%	51.7(18) 5.3%	0.03
AcH 10^{-7} M	91.5(54) 67.9%	56.6 (18) 14 %	<0.01
AcH 10^{-6} M	114.2(71) 108.7%	51.9(26) 5.4%	<0.001
MSD	**(mm) % change**	**(mm) % change**	
baseline	2.52(0.6)	2.39(0.4)	
AcH 10^{-8} M	2.48(0.6) -2.5%	2.34(0.5) -2.4%	0.57
AcH 10^{-7} M	2.40(0.6) -4.8 %	2.18(0.5) -9.1%	0.52
AcH 10^{-6} M	2.09(0.8)-17.2 %	2.06(0.5) -14.0 %	0.39
MOD	**(mm) % change**	**(mm) % change**	
Baseline	1.91(0.5)	1.86(0.3)	
AcH 10^{-8} M	1.85(0.5) -3.2 %	1.73(0.4) -7.3 %	0.20
AcH 10^{-7} M	1.82(0.5) -4.3 %	1.61(0.4) -13.6 %	0.06
AcH 10^{-6} M	1.54(0.7) -19.6 %	1.55(0.4) -16.7 %	0.52

*p value one way analysis of variance (ANOVA) (for the difference in mean % change)
† between brackets: SD

Figure 3

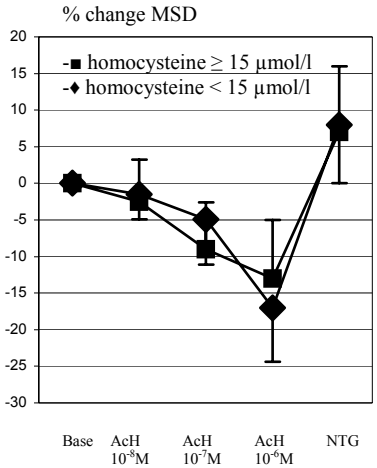

Figure 4

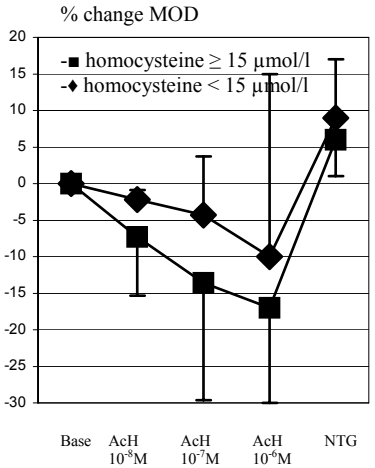

Figure 3 demonstrates changes in MSD following subsequent dosages of AcH and nitro-glycerine for both groups. Changes in MSD (%) following the administration of to subsequent amounts of AcH (10^{-7}M and 10^{-6}M) were comparable for both groups (p=0.39). Minimal obstruction diameter (MOD) at baseline was comparable between the groups (p=0.67). Mean MOD decreased from 1.85 mm (SD 0.41) to 1.53 mm (SD 0.55) following the administration of AcH 10^{-6}M. Figure 4 demonstrates changes in MOD following subsequent dosages of AcH and nitro-glycerine for both groups. Changes in MOD (%) following the administration of subsequent amounts of AcH (10^{-7}M and 10^{-6}M) were comparable for both groups (p=0.52). No differences occurred following the administration of nitro-glycerine.

Discussion

This study demonstrated a strong and graded effect of tHcY on coronary endothelial function, expressed as change in coronary blood flow.

Acetylcholine induced changes in CBF and cardiovascular prognosis

Acetylcholine is widely used as standard substance to test endothelial function in human coronary arteries [9]. In subjects with normal coronary vessels, acetylcholine induces vasodilatation of epicardial coronary vessels and an increase of CBF of 50% or more [7]. In patients with atherosclerosis, or in the presence of risk factors for vascular disease like hypercholesterolaemia, hypertension, diabetes mellitus or smoking, acetylcholine induced paradoxical vasoconstriction of epicardial conductance vessels, expressed as a decrease in MSD and MOD, and a smaller increment of CBF [9]. Coronary endothelial dysfunction, expressed as a change in CBF, is associated with increased cardiovascular events [10,11,12].

In our study, the patients in the group with a normal tHcY showed a mean increase of CBF following two subsequent dosages of AcH of 88% while in the

group of patients with an abnormal tHcY there was only 10 % increase of CBF suggesting that the prognosis of patients with high tHcY is unfavorable compared to patients with a normal tHcY and a normal reaction to acetylcholine. We also demonstrated a decrease in MSD and MOD suggesting that baseline endothelial function in both groups was abnormal. However, in contrast to changes in CBF, the relationship between paradoxical vasoconstriction of epicardial vessels, represented as change in MSD and MOD, and cardiovascular mortality and morbidity is less clear.

Homocysteine and endothelial function

Endothelial cells synthesize and release vasoactive mediators and thereby modulate vascular tone. Nitric oxide (NO) and prostacyclin are the best characterized vasodilatators and are largely responsible for endothelium dependant vasodilatation in epicardial coronary vessels. However, not all endothelium-dependant relaxation can be explained by NO or prostacyclin. Some studies have provided compelling evidence for the existence of another factor, endothelium-derived hyperpolarizing factor (EDHF) that is especially important in resistance vessels [13]. In humans with coronary atherosclerosis, endothelial vasodilatator function is not confined only to epicardial conductance vessels but extents also to the coronary resistance vessels. Coronary blood flow in the absence of obstructive lesions is regulated mainly by the coronary resistance vessel [14]. Mechanisms responsible for endothelial dysfunction in hyperhomocysteinemia are poorly understood. Several investigators have shown that homocysteine reduces the bioavailability of NO and enhances smooth muscle cell proliferation, both of which are important markers of atherothrombotic disease [2]. Recently it was hypothesized that hyperhomocysteinemia may stimulate the formation of asymmetrical dimethylarginine, an endogenous inhibitor of NO synthase [15] . The relationship between hyperhomocysteinemia and EDHF is unclear.

Several investigators have focused on the relationship between tHcY and endothelial function using the flow-mediated forearm model. Recently, Doshi et

al. demonstrated that improvement of flow-mediated vasodilatation occurred within four hours after the ingestion of folic acid while homocysteine in plasma remained unchanged [16]. They suggested that folic acid improves endothelial function in coronary artery disease acutely by a mechanism largely independent of homocysteine. This may be in contradiction with the findings by some other investigators of a positive correlation between tHcY and flow-mediated vasodilatation [17,18,19] although these results are not unequivocal [20,21]. Although a study in coronary vessels can be biased due to the selection of patients with coronary artery disease (for obvious ethical reasons), our study adds important evidence to the hypothesis that hyperhomocysteinemia induces coronary endothelial dysfunction.

Coronary blood flow and acetylcholine

Factors known to impair endothelial function in the coronary circulation expressed, as changes in MSD and MOD are also associated with impaired flow mediated dilatation in the brachial artery [4,5]. However, the relationship between change in CBF and impaired flow mediated dilatation of the brachial artery is less clear. In all patients, we found a paradoxical vasoconstriction in the epicardial vessels, expressed as a decrease in MSD and MOD, after administration of acetylcholine. However, in patients with a normal tHcY level there was a normal increase in CBF and in patients with an elevated tHcY a blunted CBF increment. Our results suggest that homocysteine has therefore primarily an adverse effect on endothelial function in the coronary resistance vessels.

Abnormal coronary blood flow changes during administration of endothelium dependant vasodilatators like acetylcholine in patients without obstructive coronary artery disease are associated with cardiovascular morbidity and mortality [7,22]. Since tHcY is associated with cardiovascular mortality and morbidity[23,24] , these changes in CBF may therefore explain, at least partially, the role of homocysteine in cardiovascular disease.

Influence of folic acid and vitamin B12

In our study tHcY is inversely related to blood levels of folic acid ($r=-0.69$, $p<0.0001$) and vitamin B12 ($r=-0.55$, $p<0.001$), as has been reported before [25]. It is possible that low folate and not elevated tHcY levels are responsible for the abnormal reaction to acetylcholine. However, when corrected for folate or vitamin B12 there is still a significant association between acetylcholine-induced changes in CBF and tHcY suggesting that these changes are at least in part homocysteine dependant.

Conclusions

We concluded that change in coronary blood flow as an expression of coronary endothelial function is strongly and gradually related to total plasma homocysteine levels in patients with coronary artery disease.

Study limitations:

This study can be biased due to the relative small number of patients. Also, the selection of patients with established coronary artery disease (for ethical reasons) may have biased our results. However, since baseline characteristics and baseline CBF, MSD and MOD were comparable for both groups the results of this study may add evidence to the concept that elevated plasma homocysteine levels are related to coronary endothelial function.

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10

Chapter

Coronary endothelial function in hyperhomocysteinemia: improvement after treatment with folic acid and cobalamin in patients with coronary artery disease

Frank F Willems, Wim RM Aengevaeren ,Godfried HJ Boer , Henk J Blom, Freek WA Verheugt. Journal of the American College of Cardiology 2002;40:766-72

Introduction

Hyperhomocysteinemia is an important risk factor for premature cardiovascular disease. Until now more than 80 studies have been published upon the association between homocysteine and vascular disease [1]. Homocysteine levels can be reduced 25% to 30 % using folic acid and supplementation of vitamin B12 provides additionally 7% reduction. This reduction is higher in hyperhomocysteinemic patients and patients with a low pretreatment folate status [2]. The pathophysiological mechanisms responsible for the increased risk remain unclear. Generally it is assumed that homocysteine is toxic to the vascular wall and causes endothelial dysfunction [3]. Endothelial dysfunction is a key mechanism in the current hypothesis of atherothrombosis and it is shown that functional impairment of the endothelial function defined as an impairment of endothelium-dependent coronary blood flow precedes significant arterial vessel disease [4]. Until now it is unknown whether homocysteine-lowering therapy will improve prognosis in patients with elevated homocysteine levels and coronary artery disease. Some investigators have shown that folic acid improves endothelial function in the forearm model in patients with coronary artery disease [5]. However until now it is unclear whether homocysteine-lowering therapy improves coronary endothelial function. The aim of this study was to evaluate the effect 6 months of therapy with folic acid 5mg in combination with cobalamin 400 µg on coronary endothelial function in hyperhomocysteinemic patients with symptomatic coronary artery disease.

Methods

Patient selection

This study is a double blind randomized, placebo controlled study with a follow up of 6 months investigating the effect of folic acid 5 mg and cobalamin on coronary endothelial function. Cobalamin 400 µg was added to avoid unopposed folic acid treatment in undiagnosed vitamin B12 deficiency. Endpoints were

ACH-induced changes in endothelium-dependent coronary blood flow at baseline and after 6 months of therapy. Other endpoints were change in mean segment diameter (MSD), and in minimal obstruction diameter (MOD) of a pre-defined segment of a coronary vessel, after infusion of ACH.
Patients with objectively confirmed ischemic heart disease who were referred for elective PTCA between April 1997 and June 1999 to our catheterization laboratory were eligible for this study. Inclusion criteria were a plasma homocysteine level of 15.5 μmol/l or more, age between 18 and 70 years, and at least one coronary vessel without a significant stenosis (stenosis < 50%). Patients who used medication or vitamins involved in homocysteine metabolism were excluded from this study as were patients with diabetes mellitus and patients with uncontrolled hypertension. During follow up patients were asked to make no changes in their diet or exercise levels. Patients in whom a coronary stent was placed received 160 mg of acetylsalicylic acid instead of 80 mg in the first month. Otherwise, the medication was kept unchanged when possible. Patients with kidney failure reflected as a creatinine level of more than 150 μg /l or serious liver failure were also excluded. Patients returned after 2, 8, 12 and 23 weeks for reassessment of lipid profile, vitamin levels, homocysteine levels and clinical signs of recurrent angina pectoris. The ethical committee of our institution approved the protocol. Written informed consent was obtained in all patients.

Coronary angiography

Long acting nitrates were withdrawn 24 hours before the procedure and replaced by short acting nitrates if necessary. All procedures were performed under the same conditions in time and location. After the performance of the elective PTCA a new guiding catheter 7 Fr. (Medtronic) was placed in a non-dilated coronary artery without a stenosis (≤ 50%). A Doppler guide wire (0.014 inch diameter), (Flowire, Endosonics Incorporated) within a 2.2 F coronary infusion catheter (Medtronic) was advanced in the vessel of interest just distal to a landmark of a proximal epicardial segment, defined as an important side branch

of a coronary vessel, with a diameter of at least 2.0 mm. After achieving a stable velocity signal, adenosine 18 μg was administered through the guiding catheter for maximal hyperemia and endothelium-independent coronary flow velocity was measured. Assessment of endothelium-dependent vasodilatation and coronary flow was performed by selective infusions of ACH with a total amount of 10^{-8} M, 10^{-7} M, 10^{-6}M respectively. ACH was dissolved in NaCl 0.9% and infused with a Terumo® pump at a rate of 2ml/min during 3 minutes. Nitroglycerin 200 μg was then injected as a bolus.
Before infusion of ACH baseline angiography was performed using Hexabrix® with biplane technique. Then ACH infusion started. After each infusion of ACH a coronary angiography followed. This procedure was performed at base line and after 6 months of treatment.

Assessment of coronary blood flow

Doppler flow velocity spectra were analyzed to determine time-averaged peak velocity. Volumetric coronary blood flow (CBF) is the cross sectional area times average peak velocity times 0.5[6]. The cross sectional area is the mean area over a distance of 5 mm in the segment 5 mm to 10 mm distal to the tip of the Flowire. Endothelium-dependent coronary flow reserve is calculated as percent change in CBF in response to ACH. Normal coronary endothelium-dependent function is defined as an increase of CBF of 50% or more [7]. The endothelium-independent coronary flow velocity reserve (CVR) was calculated by dividing the average peak velocity by the baseline average peak velocity after 18 μg adenosine injection.

Quantitative coronary angiography

Analysis of baseline and follow-up angiographies was performed using the CAAS II QCA software (Pie Medical, Maastricht, The Netherlands). Analysis of MOD and MSD started at an important side branch of the epicardial vessel and continued until the second important side branch of the segment. Care was taken

to ensure that the same segment of the coronary vessel was examined at baseline and follow-up. Therefore the films were examined in the same session to ensure analysis of the identical portion of the vessel. The investigators were blinded for the treatment regimen during analysis of the coronary angiography.

Laboratory measurements

After an overnight fasting a sample of venous blood was drawn on EDTA tube from the patients and put on ice immediately. Samples were stored at -20ºC and analyzed within one week. Plasma total homocysteine was measured by high performance liquid chromatography. After 6 months a second homocysteine sample was taken to investigate the effects of folic acid and cobalamin on homocysteine levels. Also samples for vitamin analysis and lipids were analyzed at baseline and follow up.

Statistics

Data are expressed as mean ± standard deviation (SD). Analysis of baseline data was performed using Chi-Square test or Mann-Whitney test where appropriate. Response differences in CBF, MSD and MOD, following two subsequent amounts of acetylcholine infusion (10^{-7}M and 10^{-6}M) were compared in two conditions (before treatment and after treatment) using a double multivariate repeated measures model (General Linear Model, SPSS 10.1).

Results

In the period of 2 years, eighteen patients were recruited in the study, three patients were excluded during follow up because of symptomatic re-stenosis in the PTCA vessel, two in the folic acid group, one in the placebo group. Thus, follow-up coronary angiography could be obtained in 15 patients, seven in the folic acid group and eight in the placebo group. Two women and 13 men

completed the study. Mean age was 52.2 years (range 40-66 years). Of the study group baseline homocysteine levels were 17.9 µmol/l (range 15.6-21.2). Other baseline characteristics of the treatment and placebo group are listed in table 1.

Table 1

Baseline characteristics

	Folic acid/cobalamin group (n=7)	placebo group (n=8)	p value
Age (mean) (yrs)	53.4 (range 40-63)	51.3 (range 42-66)	0.54
Male/female patients	7/0	7/1	0.73
Smoking	2	1	0.44
Hypertension	1	2	0.61
Hyperlipidemia	6	6	0.61
Family history	5	2	0.07
Beta blockers	6	8	0.27
Calc.antagonist	4	3	0.45
Nitrates	3	3	0.83
Acetylsalicylic	7	8	1.0
ACE inhibitors	1	2	0.61
Statins	4	5	0.19
Homocysteine,µmol/l	17.1 (0.9)*	18.7 (2.0)*	0.15
Total cholesterol, mmol/l	6.07 (1.3)	5.56 (0.9)	0.40
HDL-cholesterol, mmol/l	1.06 (0.11)	0.93 (0.19)	0.23
LDL-cholesterol, mmol/l	4.29 (1.1)	3.96 (0.5)	0.61
Triglycerides, mmol/l	1.7 (0.8)	1.7 (0.5)	1.0
Folic acid, nmol/l	11.2 (1.3)	10.3 (1.4)	0.09
Vitamin B12, pmol/l	415(95)	345 (38)	0.12
Glucose (fasting), mmol/l	5.08 (0.7)	5.40 (1.0)	0.69
Kreatinine, µmol/l	93 (10)	92 (13)	0.54

*Between brackets: SD.

In the folic acid/cobalamin group in 4 patients the right coronary artery (RCA) was used for analysis, in 2 patients the ramus circumflexus (RCX) and in 1 patient the left anterior descending artery (LAD). In the placebo group in 4 patients the RCA was used for analysis, in 3 patients the RCX and in 1 patient the LAD. No significant differences between groups were found. In the folic acid/cobalamin group total plasma homocysteine decreased 31.5 % from

17.1µmol/l (SD 0.91) to 11.7 µmol/l (SD 1.59) (p<0.0001) as compared to no change in the placebo treated group. In the folic acid/cobalamin group plasma folic acid improved from 11.2 nmol/l (SD 1.3) to 26.9 nmol/l (SD 3.4) (p<0.0001) and plasma vitamin B12 improved from 415 pmol/l (SD 95) to 463 pmol/l (SD119) No changes occurred in the placebo treated group. All other biochemical parameters remained unchanged (table 2).

Table 2

Changes in risk factors

	Folic acid cobalamin group (n=7)	**p value***	**placebo group (n=8)**	**p value ***
Homocysteine, µmol/l				
Baseline	17.1 (0.91)†	<0.0001	18.7 (1.97)	0.92
Follow-up	11.7 (1.59)		18.8 (2.21)	
Serum folate, nmol/l				
Baseline	11.2 (1.3)	<0.0001	10.3 (1.4)	0.90
Follow-up	26.9 (3.4)		10.4 (1.3)	
Vitamin B12, pmol/l				
Baseline	415 (95)	0.42	346 (38)	0.98
Follow-up	463 (119)		348 (41)	
Total cholesterol, mmol/l				
Baseline	6.07 (1.3)	0.63	5.66 (0.9)	0.97
Follow-up	5.77 (0.9)		5.55 (0.6)	
HDL cholesterol, mmol/l				
Baseline	1.06 (0.12)	0.87	0.93 (0.19)	0.68
Follow-up	1.04 (0.13)		0.99 (0.19)	
LDL cholesterol, mmol/l				
Baseline	4.24 (1.15)	0.62	3.87 (0.69)	0.98
Follow-up	3.97 (0.81)		3.85 (0.46)	
Triglycerides, mmol/l				
Baseline	1.69 (0.85)	0.94	1.67 (0.34)	0.60
Follow-up	1.66 (0.72)		1.54 (0.38)	
Mean arterial pressure , mm Hg				
Baseline	105 (16)	0.92	111 (14)	0.68
Follow-up	107 (9)		108 (8)	

* p value for difference between baseline and follow up with Mann-Whitney test.
† between brackets: SD

In all patients with complete follow up, medication during follow up was not changed. No significant differences in changes in CBF, MSD and MOD were seen at baseline (table 3).

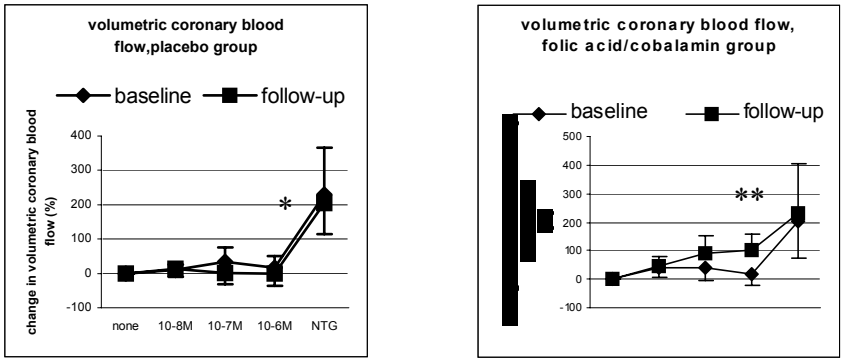

Figure 1

Effect of acetylcholine and nitroglycerin on percent change in volumetric coronary blood flow in both groups.
◆=Baseline, ■= follow-up. * p=0.15 for the difference between baseline and follow up at maximal dosage of acetylcholine (placebo group). ** p< 0,05 for the difference between baseline and follow up at the maximal dosage of acetylcholine (folic acid/cobalamin group).
P<0,005 for the difference at baseline and follow up between the placebo group and the folic acid/cobalamin group with General Linear Model

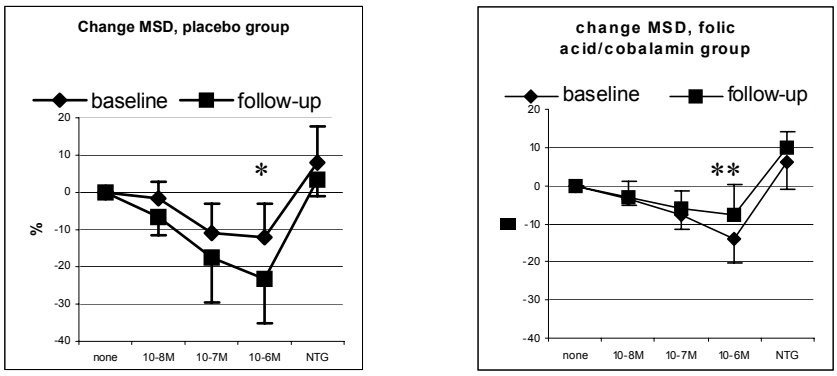

Figure 2

Effect of acetylcholine and nitroglycerin on percent change in mean segment diameter in both groups.
◆=Baseline, ■= follow-up.* p=0.09 for the difference between baseline and follow up at maximal dosage of acetylcholine (placebo group). **p= 0.10 for the difference between baseline and follow up at the maximal dosage of acetylcholine (folic acid/cobalamin group).
P=0.15 for the difference at baseline and follow up between the placebo group and the folic acid/cobalamin group with General Linear Model

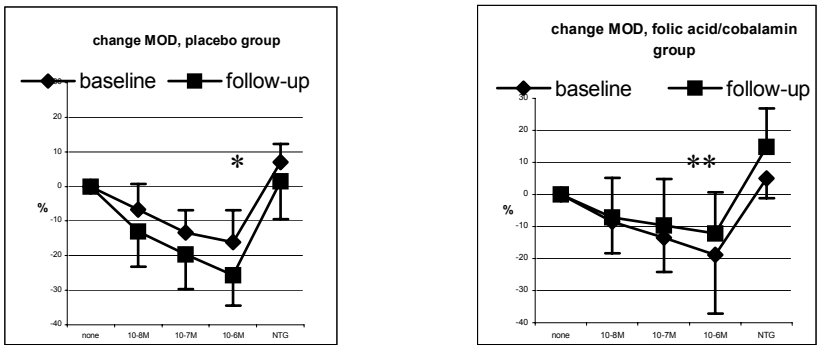

Figure 3:
Effect of acetylcholine and nitroglycerin on percent change in minimal obstruction diameter in both groups.◆=Baseline, ■= follow-up. * p = 0.06 for the difference between baseline and follow up at maximal dosage of acetylcholine (placebo group). ** p =0.26 for the difference between baseline and follow up at the maximal dosage of acetylcholine (folic acid/cobalamin group).

Table 3

Baseline coronary flow parameters following infusion of acetylcholine

		Folic acid/ cobalamin group n=7	placebo group n=8	p value
Mean Change in Volumetric coronary blood flow (%)	10-8M	38.6 (29)*	10.8(15)	0.051
	10-7M	39.2 (46)	33.1 (42)	0.79
	10-6M	15.6 (36)	16.3 (35)	0.96
	NTG	204.3 (133)	228.7 (137)	0.73
Mean Change in Mean segment diameter (%)	10-8M	-3.5 (1.7)	-1.7 (4.5)	0.64
	10-7M	-7.6 (3.8)	-11.0(7.9)	0.06
	10-6M	-13.9(6.2)	-12.1(8.9)	0.08
	NTG	6.1 (7.2)	8.0 (9.7)	0.39
Mean Change in Minimal obstruction diameter (%)	10-8M	-6.7 (7.4)	-8.6 (9.8)	0.40
	10-7M	-13.4 (6.5)	-13.6(10.6)	0.28
	10-6M	-16.2 (9.4)	-18.9 (18.3)	0.37
	NTG	7.0 (5.2)	4.9 (6.0)	0.02

*SD between brackets

Changes in CBF, MSD and MOD are calculated following the administration of the highest dosage of ACH. Changes in CBF, MSD and MOD after infusion of ACH at baseline were abnormal in all patients. At follow-up, after infusion of ACH, CBF increased in the folic acid/cobalamin group from 39.5 ml/min (SD15) to 77.5 ml/min (SD33) ($p<0.01$). In the placebo treated group volumetric coronary blood flow decreased from 53.2 ml/min (SD18) to 40 ml/min (SD16) ($p=0.38$). CBF in the folic acid/cobalamin treated group improved 96% (SD 58), (95% C.I.: 44%-154%) ($p<0.05$) as compared to a decrease of 16 %(SD 35), (95% C.I.: –20%- +30%) ($p=0.15$) in the placebo treated group. $P<0.005$ for the change in CBF at baseline and follow-up between the folic acid/cobalamin group and the placebo group with the General Linear Model (Figure 1). Mean segment diameter (MSD) decreased from 2.19 mm (SD0.22) to 1.83 mm (SD 0.14) in the placebo treated group and increased from 1.99 mm (SD 0.15) to 2.12 mm (SD 0.14) in the folic acid/cobalamin group. MSD decreased 16.4% in the placebo treated group and increased 6.5 % in the folic acid/cobalamin group ($p=0.15$) (figure 2). Minimal obstruction diameter (MOD) decreased from 1.76 mm (SD0.13) to 1.43mm (SD 0.09) in the placebo group and increased from 1.41 mm (SD 0.16) to 1.60 mm (SD 0.14) in the folic acid/cobalamin group. MOD increased 13.4 % in the folic acid/cobalamin group and decreased 18.7% in the placebo treated group ($p=0.14$) (figure 3). Endothelium-independent vasodilatation as presented in CVR was 2.78 (SD 0,29) at baseline in the placebo group and 2.91 (SD 0.32) in the folic acid/cobalamin group. In both groups CVR did not change significantly at follow up.

Discussion

This is the first study on changes in endothelium-dependent coronary flow in hyperhomocysteinemic patients with symptomatic coronary artery disease 6 months after treatment with folic acid and cobalamin. This regime significantly improved volumetric coronary blood flow, MSD and MOD. Since clinical trials

evaluating the effect of homocysteine-lowering therapy on clinical endpoints in patients with mild hyperhomocysteinemia are not yet available, our research focused on endothelial dysfunction as key mechanism in homocysteine–induced vascular disease.

Homocysteine and endothelial function

So far, angiographic studies on the association between plasma homocysteine levels and coronary endothelial function have not been published but some human studies used the reactive hyperemia model of the brachial artery demonstrating that severe hyperhomocysteinemia is associated with reduced flow-mediated vasodilatation [8]. Also in patients with mild hyperhomocysteinemia the plasma homocysteine level was a significant predictor of reduced flow-mediated vasodilatation [9,10]. In the forearm model, folic acid prevented the impairment endothelium-dependent vasodilatation after methionine loading [11] and ameliorated endothelium-dependent vasodilatation in patients with familial hypercholesterolaemia and normal tHcy [12,13]. However, the effect of folic acid on endothelial function correlated with tHcy suggesting that folic acid is more effective in patients with high tHcy [14].

Endothelial function in epicardial vessels and resistance vessels.

ACH is widely used as standard substance to test endothelial function in human coronary arteries [15]. In subjects with normal coronary vessels, ACH induces vasodilatation of coronary vessels. In patients with atherosclerosis, or in the presence of risk factors for vascular disease like hypercholesterolaemia, hypertension, diabetes mellitus and smoking, ACH has been shown to induce paradoxical vasoconstriction [16]. All patients in the study group had severe coronary endothelial dysfunction following infusion of ACH at baseline. Changes in MSD after intracoronary infusion of ACH show a coronary endothelial dysfunction expressed as a decrease in MSD at baseline. The same is true for changes in MOD. Volumetric coronary blood flow in all patients was severely disturbed at baseline. Follow-up demonstrated a significant

improvement of volumetric coronary blood flow in the patients who used folic acid and cobalamin. The changes in MSD improved after infusion of ACH at follow-up in the folic acid/cobalamin group as compared to the placebo group. Changes in MOD improved also albeit not significantly.
Our patients were characterized by an abnormal response upon ACH infusion at baseline. Since improvement of ACH-induced vasoconstriction depends on the response at baseline we could expect a stronger improvement of endothelial response on ACH infusion after intervention [17].
Suwaidi et al. demonstrated that severe coronary endothelial dysfunction, defined as an increase of volumetric coronary blood flow less then 20% after infusion of ACH, in the absence of significant coronary artery disease, is associated with an increase in cardiac events in the follow up [4]. This supports the concept that coronary endothelial dysfunction is an important marker for future progression of coronary vascular disease. Assessment of CVR, demonstrated that, in our study, all patients at baseline and follow-up had a CVR within the normal range of 2.7 +/-0.6 testifying that the investigated coronary vessels at baseline and follow-up had no significant stenosis which could influence changes in volumetric coronary blood flow, changes in MSD and MOD [18]. Our results supports, that hyperhomocysteinemic patients, by using folic acid and cobalamin might improve their cardiovascular prognosis.

Mechanisms of endothelial dysfunction .
Many observational, case-control and prospective studies have been published upon the association of hyperhomocysteinemia and coronary artery disease [1]. However, these studies cannot exclude the possibility that hyperhomocysteinemia is rather a marker of vascular disease than being causally related to vascular disease. Mechanisms responsible for endothelial dysfunction in hyperhomocysteinemia are poorly understood. Several investigators have shown that homocysteine reduces the bioavailability of nitric oxide and enhances smooth muscle cell proliferation, both of which are important markers of atherothrombotic disease [3]. Recently it was hypothesized that

hyperhomocysteinemia may stimulate the formation of asymmetrical dimethylarginine, an endogenous inhibitor of nitric oxide synthase [19].

Folic acid and restoration of endothelial function.

Folic acid reduces homocysteine levels 25% and addition of cobalamin led to a further reduction of 7%[2,20]. In our study plasma homocysteine levels reduced 31 % after 6 months of treatment with folic acid 5 mg and cobalamin 400 µg compared to no change in the placebo treated group. The mechanism responsible for the effect of folic acid is unclear. Apart from lowering of homocysteine, folic acid may influence endothelial function via other mechanisms. Folates are also involved in endogenous restoration of tetrahydrobiopterin, an essential cofactor for nitric oxide synthase [21]. Tetrahydrobiopterin restores endothelial function in the forearm model in hypercholesterolemic patients [22]. Other studies suggest an anti-oxidant effect of folic acid [12]. Improvement of endothelial function by anti-oxidant supplementation has been studied before but, until now the results are variable and more extensive studies are needed to resolve the question whether anti-oxidant therapy is useful in patients with coronary artery disease [14,23].

Also the natural outcome of untreated homocystinuria patients, with fasting plasma homocysteine levels > 100 µmol/l and a 50% risk of suffering from a vascular event before the age of 30 years suggests a strong effect of homocysteine on vascular disease [24]. In these patients with severe hyperhomocysteinemia appropriate homocysteine-lowering therapy reduced the risk on vascular events significantly with more then 90%[25]. Our study adds evidence to the concept that homocysteine-lowering therapy is clinical beneficial in patients with elevated homocysteine levels.

Study limitations.

Despite the relative small number of patients significant differences in coronary endothelial function after intervention were observed in the same range as seen in studies with cholesterol-lowering therapy. However, although this study may

add evidence to the fact that treatment of hyperhomocysteinemia may be useful in vascular disease ongoing intervention trials with clinical endpoints are needed to answer the question whether treatment of hyperhomocysteinemia indeed reduces cardiovascular events.

Conclusions.

We conclude that volumetric coronary blood flow as an expression of coronary endothelial function improves significantly after 6 months of treatment with folic acid and cobalamin in hyperhomocysteinemic patients with symptomatic coronary artery disease.

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11

Chapter

Can 5-methyltetrahydrofolate improve coronary endothelial function instantly in patients with coronary artery disease?

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Submitted

Introduction

Evidence is accumulating that a disturbed homocysteine metabolism provokes endothelial dysfunction. Vascular endothelial dysfunction is an early sign of atherosclerotic disease and it is more and more used as a surrogate end point for an increased cardiovascular risk. Several studies have demonstrated a prompt beneficial effect of folates in vascular disease using the fore-arm model showing an improvement of endothelial function[1]. However, the relevance of the fore-arm model in cardiovascular prognosis is unequivocal. The results of these studies should therefore be judged critically when they are used as a surrogate endpoint for vascular events. On the contrary, studies evaluating coronary endothelial function can , when assessed with quantitative coronary angiography and doppler velocity measurements, predict cardiovascular prognosis [2,3]. In a recent study, we demonstrated a beneficial effect of long-term folate therapy in hyperhomocysteinemic patients with coronary endothelial dysfunction at baseline[4]. It is unclear whether folates can also improve coronary endothelial function immediately. We investigated whether 5-methyltetrahydrofolate (5-MTHF), the metabolic active form of folate, could instantly improve coronary endothelial function in patients with coronary artery disease in a randomised, placebo controlled study.

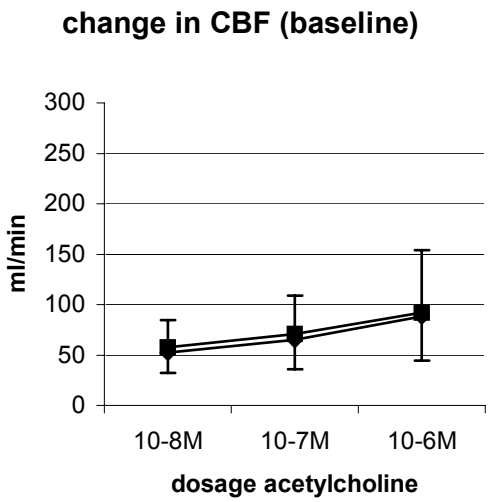
Methods

We investigated the effect of 5-MTHF 1 mg i.v. or placebo on coronary endothelial function. Based our previous studie [4], a sample size of 7 would have 80% power to detect a significant difference ($p<0.05$). Twenty patients, scheduled for elective PTCA, were randomized for this study. Coronary endothelial function, expressed as a change in coronary blood flow (CBF), was evaluated in a non-stenotic coronary vessel using acetylcholine (ACH) infusion in dosages of 10^{-8} M, 10^{-7}M and 10^{-6} M. CBF is determined using intracoronary

doppler velocity and quantitative coronary angiography. Endpoints were ACH-induced changes in CBF before, and 15 minutes after, the administration of 1 mg of 5- MTHF i.v. or placebo (NaCl 0.9%). Normal coronary endothelium-dependent function is defined as an increase of CBF of 50% or more [2]. Patients using medication or vitamins interfering with homocysteine metabolism were excluded from this study. Response differences (%) to the two highest amounts of AcH infusion (10^{-7}M and 10^{-6} M) were compared to the two conditions (baseline and follow-up) by using a double multivariate repeated measures model (General Linear Model, SPSS 10.1).

Results

20 patients were included, 15 men, 5 women. Baseline characteristics, such as risk factors, age and the use of medication, differed not among groups. Mean total plasma homocysteine level was 8.7 µmol/l, (SD 2.3). At baseline, following the administration of the maximal dosage of ACH, CBF increased from 58 ml/min, (SD 27) to 113 ml/min, (SD 87) in the 5-MTHF group compared to an increase from 48 ml/min (SD 21) to 101 ml/min, (SD 47) in the placebo group. 15 minutes after the administration of 5-MTHF or placebo, CBF increased from 58 ml/min, (SD 25) to 91 ml/min, (SD 64) in the 5-MTHF group compared to an increase from 42 ml/min, (SD 21) to 99 ml/min, (SD 48) in the placebo group. The mean increase of two subsequent amounts of acetylcholine compared to baseline was 107% (SD 90) in the 5-MTHF group and 116% (SD 80) in the placebo group (p=0.67) (GLM model of repeated measures). (figure 1)



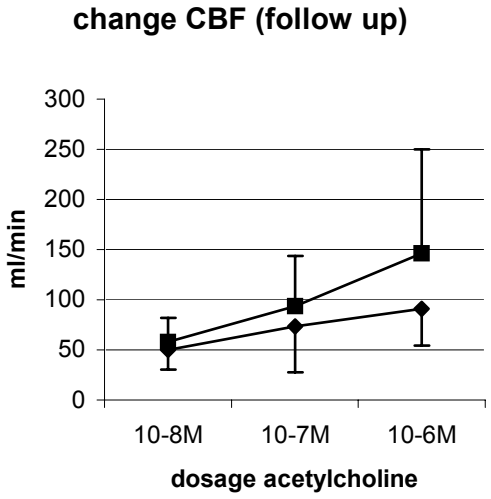


Figure 1:

Effect of acetylcholine on change in volumetric coronary blood flow at baseline and follow-up.
◆=placebo group, ■= MTHF group. P=0.94 for the difference at baseline between placebo group and MTHF group at the maximal dosage of acetylcholine. P=0.19 for the difference at follow up between placebo group and MTHF group at the maximal dosage of acetylcholine.

Discussion

This study could not demonstrate a prompt and significant effect of 5-MTHF on coronary endothelial function. Our results contrast with most other studies that are using endothelial function as a surrogate for cardiovascular events.[1,5] In our study, all patients had a normal increment of CBF suggesting that baseline coronary endothelial function was not severely disturbed. It has been demonstrated before that in patients with a normal endothelial function folates had no effect on endothelial function [5]. The exact mechanism for a possible beneficial effect of folates on endothelium is unclear. Some authors suggest that folates may have a beneficial effect on endothelial function independently of plasma homocysteine levels because homocysteine levels did not change during the study [1,5]. However, plasma homocysteine levels may only be a marker of a disturbed intracellular homocysteine metabolism. Intracellular 5-MTHF may restore this disturbed homocysteine metabolism because 5-MTHF is a co-substrate for homocysteine remethylation.

Conclusion

The administration of 5-MTHF i.v. did not instantly improve coronary endothelial function in patients with coronary artery disease. Considering the differences in the reported results between the studies evaluating endothelial function by using the fore-arm model and our study, we think that studies investigating the direct effects of folates on cardiovascular endpoints are now essential.

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Chapter

Summary and discussion

Chapter 1 is a general introduction of this thesis outlining the aim of our studies. In chapter 2 we review epidemiological evidence of the relationship between homocysteine and cardiovascular disease. Chapter 3 presents an introduction to homocysteine metabolism in relation to the genetics of hyperhomocysteinemia. Chapter 4 focuses on the impact of homocysteine for atherosclerosis and endothelial function.

In chapter 5 we describe the results of a study evaluating the pharmacokinetic properties of orally administered 6[R,S] 5-MTHF, the biological active diastereoisomer of folates, versus folic acid in subjects with homozygosity for the methylenetetrahydrofolate reductase (MTHFR) mutation 677 C→T, compared to patients with the wild type genotype. Our results demonstrate that 5-MTHF has a different pharmacokinetic profile with a better bioavailabity compared to folic acid, irrespective of the patients' genotype.

In chapter 6 the MTHFR 677C→T mutation is investigated as a genetic risk factor of coronary artery disease. The prevalence of the mutation is analysed in patients with documented coronary artery disease who participated in the Regression Growth Evaluation Statin Study (REGRESS) compared to a control population. The results demonstrate that in patients with homozygosity for this mutation the prevalence for coronary artery disease was higher compared to controls (9.5% compared to 8.5% respectively, odds ratio 1.21 (95% CI: 0.87-1.68). The results are however not statistically significant. In the meta-analysis that is performed on 2476 patients and 2481 controls, the summary estimate of the relative risk of the homozygous mutation yields an odds ratio of 1.22 (95% CI: 1.01-1.47) indicating that the patients homozygous for this mutation have a modest but significant 22% higher risk to develop coronary artery disease.

Chapter 7 describes the results of a study evaluating the long-term effects of a range of doses of folic acid with and without fixed dosages of vitamin B12 and vitamin B6. In 130 patients with established coronary artery disease, 200 µg folic acid given over a twelve-week period, achieved the same reduction in plasma homocysteine concentration as 1 mg folic acid, i.e. 26.1% (95% CI: 21.1-30.8) and 19.5% (95% CI 13.0-25.) respectively. The use of vitamin B12 achieved an

additional modest reduction of total plasma homocysteine at twelve weeks while vitamin B6 achieved no effect. Increased baseline total plasma homocysteine levels are associated with a more rapid decline in total plasma homocysteine levels after twelve weeks of therapy.

In chapter 8 we report on the results of a study investigating the effect of homocysteine on the extent and progression of coronary atherosclerosis. In 606 patients participating in the Regression Growth Evaluation Study, a prospective randomized double blind placebo-controlled study evaluating the effect of pravastatin, fasting total plasma homocysteine levels are determined. Quantitative coronary angiography is used at baseline and after two years to evaluate the extent and progression of atherosclerosis. Endpoints are change in mean segment diameter (MSD) and change in minimal obstruction diameter (MOD). This study demonstrates that the number of vessels involved in the atherosclerotic process is not related to total plasma homocysteine levels ($p=0.24$). Total plasma homocysteine levels are not related to the number of events during follow-up ($p=0.97$). Follow-up analysis of angiographic data was possible in 438 patients. Changes in MSD ($p=0.18$) and MOD ($p=0.68$) are not associated with total plasma homocysteine levels.

In chapter 9 we demonstrate the relationship between elevated total plasma homocysteine levels and coronary endothelial function, expressed as a change in coronary blood flow following infusion of acetylcholine in subsequent doses, in patients with coronary artery disease. A plasma homocysteine level of 15 µmol/l or more was considered abnormal. Coronary blood flow was determined using intracoronary doppler velocity and quantitative coronary angiography. As for hyperhomocysteinemic patients, the increase of CBF following two subsequent dosages of acetylcholine was abnormal (mean increment 1%) compared to a normal increment of coronary blood flow in the patients with normal homocysteine levels (mean increment 93%) ($p<0.001$). The results were independent of plasma folate and vitamin B12 levels.

In chapter 10 we report on the effect of six months of therapy with folic acid plus cobalamin on coronary endothelial function, expressed as a change in

coronary blood flow, in hyperhomocysteinemic patients with coronary artery disease. Fifteen patients scheduled for elective PTCA with plasma homocysteine levels of ≥16 μmol/l are randomized after the procedure for six months of treatment with folic acid 5 mg and cobalamin 400 μg daily or placebo. Coronary blood flow is determined using intracoronary doppler velocity and quantitative coronary angiography at baseline and after 6 months of treatment. In the folic acid/cobalamin treated group, coronary blood flow increased with 96% (SD 54), (95% C.I. 44%-154%) compared to a decrease of 16% (SD 35), (95% C.I.: –20%- +30%) in the placebo treated group ($p<0.005$).
Chapter 11: In this chapter we describe the results of a randomized, placebo controlled study investigating whether 5-methyltetrahydrofolate (5-MTHF), the metabolic active form of folic acid, can instantly improve coronary endothelial function in patients with coronary artery disease and normal plasma homocysteine levels.
Coronary blood flow is determined using intracoronary doppler velocity and quantitative coronary angiography. Endpoints are acetylcholine-induced changes in coronary blood flow before, and 15 minutes after, the administration of 1 mg of 5- MTHF i.v. or placebo (NaCl 0.9%). In the 5-MTHF treated group coronary blood flow increased 117% compared to an increase of 92% in the placebo group ($p=0.32$) demonstrating no significant effect of 5-MTHF on coronary blood flow.

Discussion

The hypothesis of a possible relation between hyperhomocysteinemia and atherosclerotic disease originated in 1969 when McCully described the presence of widespread vascular lesions in a child who died at an age of 7 weeks with homocystinuria [1]. Until now however, the question remains whether homocysteine is a causal factor in the development of premature atherosclerotic disease or just a marker of disease. With regard to the consistency of the findings of a positive association between hyperhomocysteinemia and premature vascular disease it is important to note that until recently, more than 20 prospective studies on this topic have been published. Among these, the

population-based, nested, case-control studies showed that a 5 μmol/L increment in total plasma homocysteine results in a 20–30% increase in cardiovascular risk, which is substantially lower than the 60–90% risk enhancement shown in the retrospective case-control studies[2]. Recently, the meta-analysis of the Homocysteine Studies Collaboration group demonstrated that after adjustment for known cardiovascular risk factors and regression dilution bias in the prospective studies, a 25% lower homocysteine level was associated with an 11% (OR, 0.89; 95% CI: 0.83-0.96) lower risk on coronary artery disease and 19% (OR, 0.81; 95% CI: 0.69-0.95) lower risk on stroke [3]. The mechanisms responsible for hyperhomocysteinemia as a causal risk factor of cardiovascular disease are unclear. Data upon a possible relationship between homocysteine and the extent of atherosclerotic lesions in coronary artery disease are not unequivocal. Some investigators found a graded effect of homocysteine levels on coronary artery disease [4,5,6] while other investigators found no relationship[7]. This may be explained by the fact that the investigators used different methods to assess the extent of coronary artery disease; none of them based on quantitative coronary angiography. It is well known that visual estimation of the degree of coronary stenosis is subject to significant operator variability[8]. We found no relationship between total plasma homocysteine levels and the extent and progression of atherosclerosis using quantitative coronary angiography (chapter 8). On the other hand, evidence is accumulating that elevated homocysteine causes endothelial dysfunction. It has been shown that homocysteine may reduce the bioavailability of NO and enhances smooth muscle cell proliferation, both of which are important markers of atherothrombotic disease [9]. Recently, it was hypothesized that hyperhomocysteinemia may stimulate the formation of asymmetrical dimethylarginine, an endogenous inhibitor of NO synthase [10] . Nitric oxide (NO) and prostacyclin are the best characterized vasodilatators and are largely responsible for endothelium dependant vasodilatation in epicardial coronary vessels. However, not all endothelium-dependant relaxation can be explained by nitric oxide or prostacyclin. Some studies have provided compelling evidence

for the existence of another factor, the endothelium-derived hyperpolarizing factor (EDHF) which is especially important in resistance vessels [11]. As for humans with coronary atherosclerosis, endothelial vasodilatator function is not confined only to epicardial conductance vessels but extends also to the coronary resistance vessels. Coronary blood flow in the absence of obstructive lesions is regulated mainly by the coronary resistance vessel [12]. The results of our study (chapter 9), using non-stenotic coronary vessels, suggests that EDHF may be involved in endothelial dysfunction in hyperhomocysteinemic patients.

Most but not all human studies show a relationship between increased total plasma homocysteine levels and impaired endothelial function using a fore arm model [9]. Several investigators have demonstrated a beneficial effect of folic acid on endothelial function using the forearm model [13,14,15]. In some of these studies the results were independent from the patients' plasma homocysteine level [16,17]. But Title et al. demonstrated that the effect of folic acid on endothelial function correlated with plasma homocysteine suggesting that folic acid is more effective in patients with high homocysteine levels[18]. However, the relationship between endothelial dysfunction using the forearm model and cardiovascular prognosis is unclear. In contrast, coronary endothelial dysfunction, demonstrated by an abnormal change in coronary blood flow following the administration of acetylcholine, is an important surrogate endpoint in the progression of vascular disease and is associated with increased cardiovascular events[19,20,21]. In our study (chapter 9) we could demonstrate that changes in coronary blood flow are strongly and gradually related to total plasma homocysteine levels in patients with coronary artery disease. In the intervention study (chapter 10) we demonstrated a beneficial effect of long term treatment with folic acid and cobalamin on coronary endothelial function in hyperhomocysteinemic patients. As for patients with normal homocysteine levels, we could not find an instantl improvement of coronary endothelial function using 5-methyltetrahydrofolate (5-MTHF) compared to placebo (chapter 11). The question remains whether folic acid or 5-MTHF can

immediately restore coronary endothelial dysfunction or that therapy must be continued for a longer period.

Mildly elevated homocysteine levels are caused by genetic as well as environmental factors. Several lifestyle determinants like folate intake, smoking and coffee intake modulate homocysteine levels causing elevation of plasma homocysteine levels[22]. It is therefore inaccurate to assume that the increased risk for premature vascular disease associated with hyperhomocysteinemia can always be attributed to a single genetic mutation.
Several investigators have confirmed lowering of elevated homocysteine levels using pharmacological amounts of folic acid[23]. The effect of lower, more physiological amounts of folic acid on plasma homocysteine levels has been investigated demonstrating that even at a dose of 400 µg folic acid, homocysteine could be lowered effectively in most patients[24]. In our dose-finding study (chapter 7) there was no difference in homocysteine-lowering effect between 200 µg folic acid and 1 mg folic acid in a twelve-week period. These findings suggest that low-dose folic acid treatment in hyperhomocysteinemia is sufficient. In 1998 manufacturers in the United States fortified flour with folic acid 140 µg/100 g. Jacques et al. determined the effect of this intervention in the Framingham Offspring Cohort demonstrating a decline of plasma homocysteine of 10 to 50% depending on baseline plasma homocysteine levels[25]. However, as has been described in chapter 2, especially for patients with severe hyperhomocysteinemia, therapy should be tailored in these patients to achieve optimal homocysteine-lowering effect. Secondly, considering the patients with the 677 C→T mutation in the MTHFR enzyme the results of homocysteine lowering therapy are less unequivocal than described above. In a study of Nelen et al. a daily dose of 500 µg was effective even in homozygotes for the mutation[26]. Guttormsen et al. however found that the response to 200 µg/day was effective in some subjects but not all [27]. Thirdly, as in our study (chapter 10) all intervention studies, demonstrating a beneficial

effect of folic acid on endothelial function, used folic acid in higher dosages[13,14,15,16,17,18, 28, 29].

About 10% of the North-West European populations are homozygous for the 677 C→T mutation of the MTHFR enzyme. In their meta-analysis, Brattström et al[30] found that the total plasma homocysteine concentration was 2.6-µmol/L higher in those with the MTHFR 677 TT genotype than in those with MTHFR 677 CC genotype. With use of data from prospective studies, a 5 µmol/L total plasma homocysteine increment can be shown to be associated with an odds ratio (OR) of 1.20–1.30. Translated for a difference of 2.6 µmol/L, these odds ratios can be estimated as 1.10 to 1.15, respectively. Standard sample size calculations show that to detect a relative risk in the range of 1.10–1.15 with a power of 80% and a significance level of 5%, 7800–16300 cases and an equal number of controls are required. Thus, the insignificant relation between the C677T MTHFR polymorphism and cardiovascular disease in our study, concerning 735 patients and 1250 controls (chapter 6) does not contradict the homocysteine theory. The relative risk of 1.12 associated with the TT genotype that was reported by Brattström et al. and our report of a relative risk of 1.21 (chapter 6) agrees well with the relative risk reported by Klerk et al. in 11.162 patients and 12.758 controls. They demonstrated that individuals with the MTHFR 677 TT genotype had a 16% higher odds ratio for coronary artery disease compared with individuals with the CC genotype. [31]. This difference was statistically significant, (OR 1.16, 95% CI 1.05-1.28).
In chapter 5 we investigated the pharmacokinetic effects of orally administered 6[R,S] 5-MTHF versus folic acid in patients homozygous for the 677 C→T mutation in the MTHFR gene compared to patients with wild type gene. Our data demonstrate that there are no differences with respect to the pharmacokinetic properties of folic acid and 6[R,S]5-MTHF in relation to the patients' genotype. These results suggest that the activity of MTHFR in vivo is not the rate-limiting step in the conversion of folic acid to 5-MTHF because the TT genotype did not influence the results. A remarkable finding was the high

levels of the non-natural 6[R] 5-MTHF isomer, one week after a single dosage of the racemic mixture 6[R,S] 5-MTHF. Our results suggest that the unnatural 6[R]5-MTHF isomer is stored until it is exchanged with folic acid. The biological effects of 6[R] 5-MTHF storage are however unclear. Since 6[R] 5-MTHF is not a natural isomer it may be not beneficial that 6[R] 5-MTHF is stored in the body although Mader et al. demonstrated there are no serious short-term side effects following high dosages of 6[R,S] 5-MTHF[32]. The bioavailabity of 6[S] 5-MTHF may be reduced due to competition with the 6[R] 5-MTHF diastereoisomer which may have detrimental consequences when used as therapy in hyperhomocysteinemia.

Conclusions and future perspectives:

This thesis contributes to the concept that hyperhomocysteinemia is not just a consequence of atherosclerosis but is a moderate risk factor for cardiovascular disease.

Since folic acid, in the absence of vitamin B12 deficiency, has no important side effects a daily therapeutic dosage of at least 1 mg seems imperative. Studies with clinical endpoints investigating an optimal dosage of folic acid are an important field for future research.

The 677 C→T mutation in the MTHFR genotype is a moderate genetic risk factor for cardiovascular disease. Pharmacokinetic properties of orally administered folates are however not influenced by the genotype of the patient. Future research should focus on the use and safety of racemic mixtures of folates in patients with vascular disease. Especially the question whether the unnatural isomers may have detrimental effects on cardiovascular prognosis is unclear.

We could demonstrate that hyperhomocysteinemia is associated with coronary endothelial dysfunction and that homocysteine-lowering therapy restores coronary endothelial function in patients with coronary artery disease. These

findings may have important prognostic consequences for patients with cardiovascular disease. Future studies, investigating the role of the endothelium, should reveal the pathophysiological mechanism responsible for this increased risk. Especially the possible role of EDHF in hyperhomocysteinemia is, from the perspective of our results in chapter nine, an interesting target for future research. Whether folates are able to restore coronary endothelial function instantly is an important question since endothelial dysfunction is an important mechanism in acute coronary syndromes. Clinical studies are needed to reveal this question.

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Chapter

Samenvatting

Hart-en vaatziekten zijn de belangrijkste doodsoorzaak in Nederland. Bekende risicofactoren als een verhoogd cholesterolgehalte in het bloed, roken, hypertensie en diabetes mellitus kunnen slechts een deel van het grote aantal patiënten, die lijden aan deze aandoeningen, verklaren. Een verhoogd plasma homocysteine gehalte is eveneens geassocieerd met een verhoogd cardiovasculair risico.

Op basis van gegevens uit prospectieve en retrospectieve studies blijkt dat ongeveer 10 tot 20% van de Nederlandse bevolking een verhoogd plasma homocysteine heeft. Een verhoogd plasma homocysteine is in de meeste gevallen te behandelen met foliumzuur.

Hoofdstuk 1 is een algemene inleiding waarin de vraagstelling van dit proefschrift wordt besproken.

Hoofdstuk 2 : Hierin wordt de relatie vaatlijden en hyperhomocysteinemie besproken.

Hoofdstuk 3 gaat in op het homocysteine metabolisme en de genetische basis die leidt tot hyperhomocysteinemie.

Hoofdstuk 4 gaat in op het pathofysiologie van hyperhomocysteinemie in relatie tot de ontwikkeling van atherosclerose en endotheel dysfunctie.

Hoofdstuk 5 beschrijft de resultaten van een onderzoek waarbij de farmacokinetische eigenschappen van oraal toegediend 6[R,S] 5-MTHF, de biologisch actieve diastereoisomeer van folaat, worden vergeleken met foliumzuur in patienten die homozygoot zijn voor de methyleen tetrahydrofolaat reductase(MTHFR) mutatie 677 C→T . De resultaten tonen aan dat 6[R,S] 5-MTHF een ander farmacokinetisch profiel heeft dan foliumzuur met een hogere

biologische beschikbaarheid. De resultaten zijn niet afhankelijk van het genotype van de patiënt.

Hoofdstuk 6: In dit hoofdstuk wordt het belang van de MTHFR 677 C→T mutatie als risicofactor voor hart-en vaatziekten onderzocht . De prevalentie van deze mutatie is onderzocht in patiënten met aanwijzingen voor coronarialijden die deelnamen aan de regression growth evaluation study (REGRESS). De resultaten tonen aan dat patiënten, homozygoot voor deze mutatie, vaker cardiovasculaire ziekten hebben dan patienten met een normale enzym activiteit. (9.5% vergeleken met 8.5 %, Odds ratio 1.21 (95% C.I. 0.87-1.68)). De resultaten van dit onderzoek zijn echter niet statistisch significant. De meta-analyse die verricht werd bij 2476 patiënten en 2481 controle patienten toont een Odds ratio van 1.22 (95% C.I.: 1.01-1.47). Daaruit blijkt dat patiënten homozygoot voor deze mutatie een matig maar significant (22%) verhoogd risico lopen op het ontwikkelen van coronarialijden.

Hoofdstuk 7: Dit hoofdstuk beschrijft de resultaten van een onderzoek naar de lange termijn effecten van verschillende doseringen foliumzuur met of zonder vitamine B12 en/of vitamine B6 op de plasma homocysteine spiegel. Bij 130 patiënten met bekend coronarialijden die behandeld werden met 200 μg foliumzuur gedurende 12 weken kon een even grote reductie in het plasma homocysteine worden bereikt als met 1 mg foliumzuur. Het toevoegen van vitamine B12 had daarbij slechts gering effect terwijl vitamine B6 in het geheel geen effect had. De effecten die bereikt worden zijn groter naarmate de uitgangs plasma homocysteine spiegel hoger is.

Hoofdstuk 8: In dit hoofdstuk wordt ingegaan op de effecten van homocysteine op de progressie van atherosclerose in de coronair vaten. Daartoe werd in de patiënten populatie die had deelgenomen aan het REGRESS onderzoek een nuchter plasma homocysteine spiegel bepaald. Met behulp van kwantitatieve coronair angiografie werd aan het begin van de studie en na een follow up van

twee jaar gekeken naar minimale obstruction diameter (MOD) en mean segment diameter (MSD) van de coronair vaten. Eindpunten van de studie waren de verandering van MOD en MSD in relatie tot de plasma homocysteine spiegel. Deze studie kon geen relatie aantonen tussen de mate van progressie van het atherosclerotisch proces en de plasma homocysteine spiegels. Ook bleek er geen relatie te bestaan tussen de ernst van het pre-existente coronarialijden en de plasma homocysteine spiegels.

Hoofdstuk 9: Dit hoofdstuk beschrijft het onderzoek naar de relatie tussen plasma homocysteine spiegels en de functie van het endotheel in de coronair vaten. In dit onderzoek werd een plasma homocysteine spiegel van ≥15 µmol/l als abnormaal beschouwd. Coronair endotheel functie wordt uitgedrukt als een verandering in de coronair flow gemeten met behulp van flow doppler techniek en kwantitatieve coronair angiografie. Het onderzoek toont aan dat bij patienten met een verhoogd plasma homocysteine er sprake is van endotheel dysfunctie in tegenstelling tot patienten met een normaal plasma homocysteine. Belangrijk is dat deze resultaten onafhankelijk bleken van de foliumzuur spiegels en vitamine B12 spiegels.

Hoofdstuk 10: Dit hoofdstuk beschrijft het effect van foliumzuur 5 mg en vitamine B12 400 µg gedurende 6 maanden op coronair endotheel bij patiënten met een hyperhomocysteinemie. De functie van coronair endotheel werd bepaald met behulp van flow doppler techniek en kwantitatieve coronair angiografie bij de start van de studie en na 6 maanden therapie. Er was sprake van een significante verbetering van de functie van het coronair endotheel vergeleken met placebo.

Hoofdstuk 11: Dit hoofdstuk beschrijft een pilot studie naar de directe effecten van 5-MTHF i.v. op coronair endotheel functie bij patiënten met een normaal plasma homocysteine. Hoewel er een verbetering kon worden gedemonstreerd was deze in deze kleine populatie niet significant.

Conclusies en aanbevelingen

De resultaten van dit proefschrift dragen bij aan het concept dat hyperhomocysteinemie niet slechts de consequentie kan zijn van het atherosclerotisch proces maar een risicofactor voor de ontwikkeling van hart-en vaatziekten is.

Dit proefschrift toont aan dat een verhoogd plasma homocysteine niet leidt tot een versnelling van het atherosclerotisch proces met anatomische veranderingen in de coronair vaten. Wel blijkt een verhoogd plasma homocysteine te leiden een abnormale endotheel functie in de coronair vaten. Tevens kon worden aangetoond dat een daling van het plasma homocysteine met behulp van foliumzuur en vitamine B12 bij patienten met een milde hyperhomocysteinemie leidt tot een verbetering van de functie van het coronair endotheel. Deze bevindingen kunnen belangrijke prognostische consequenties hebben voor patienten met een verhoogd plasma homocysteine. Of folaten een rol kunnen spelen bij een directe verbetering van de endotheel functie, is nog een belangrijke vraag waarop het antwoord niet gegeven kan worden.

Studies die op basis van klinische eindpunten worden verricht zullen het definitieve antwoord moeten geven op de vraag hoe belangerijk het behandelen van een verhoogd plasma homocysteine is.

Het mechanisme dat leidt tot endotheel dysfunctie is niet duidelijk. Toekomstige studies zouden het pathofysiologisch mechanisme dat leidt tot endotheel dysfunctie moeten ophelderen. Met name de mogelijke rol van EDHF in hyperhomocysteinemie is, vanuit het perspectief van de resulaten van ons onderzoek een interessante basis voor verder onderzoek.

Omdat foliumzuur, indien er geen vitamine B12 deficiëntie is, geen belangrijke bijwerkingen heeft, lijkt een dosering van tenminste 1 mg foliumzuur per dag zinvol. Ook hier zullen studies met klinische eindpunten de vraag wat de

optimale dosering van foliumzuur en eventueel vitamine B12 is verder moeten beantwoorden.

De 677 C→T mutatie in het MTHFR genotype is een milde genetische risicofactor voor hart-en vaatziekten. De farmacokinetische eigenschappen van folaten worden echter niet door het genotype van de patiënt beïnvloed. Toekomstige research zal zich ook moeten richten op het gebruik en veiligheid van racemische mengsels van folaten bij patiënten met vasculaire ziekten. Speciale aandacht zal moeten uitgaan naar de aanwezigheid van niet-natuurlijke isomeren van folaten die een mogelijk ongunstig effect bij de patiënt kunnen oproepen.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 21 Mei 1958 te Ede. Na het voltooien van het atheneum-B aan het Marnix College te Ede werd in 1977 gestart met de studie Scheikunde aan de Katholieke Universiteit van Nijmegen. Na een korte periode werkzaam geweest te zijn in de weg-en waterbouw waarbij hij behulpzaam was bij de aanleg van een aantal kribben in de Waal bij Nijmegen werd in 1979 gestart met de studie Geneeskunde aan de Katholieke Universiteit van Nijmegen. Tussentijds was hij gedurende een klein jaar werkzaam in het Hôpital d'Enongal, Ebolowa te Kameroen. In 1988 werd het artsexamen afgelegd. Aansluitend was hij werkzaam als arts-assistent cardiologie niet in opleiding in het Slingeland Ziekenhuis te Doetinchem en het Academisch Ziekenhuis St. Radboud te Nijmegen. In 1990 werd gestart met de opleiding tot cardioloog (opleiders Prof. Dr. T van der Werf en Prof. Dr. F.W.A. Verheugt). De vooropleiding Interne Geneeskunde werd gedaan in het St. Maartens Gasthuis te Venlo (opleider Dr. J.J.J. Mattousch). Registratie tot cardioloog volgde op 1 december 1996. Daarna was hij werkzaam als tijdelijk staflid op de afdeling Cardiologie van het Hartcentrum te Nijmegen (Hoofd Prof. Dr. FWA Verheugt). In deze periode werd tevens gestart met het onderzoek dat de basis vormt voor dit proefschrift. Vanaf 1 mei 1998 is hij werkzaam als cardioloog in de maatschap Cardiologie te Arnhem-Velp.
Hij is gehuwd met Franske van Duuren en heeft drie dochters, Femke, Mieneke en Iris.