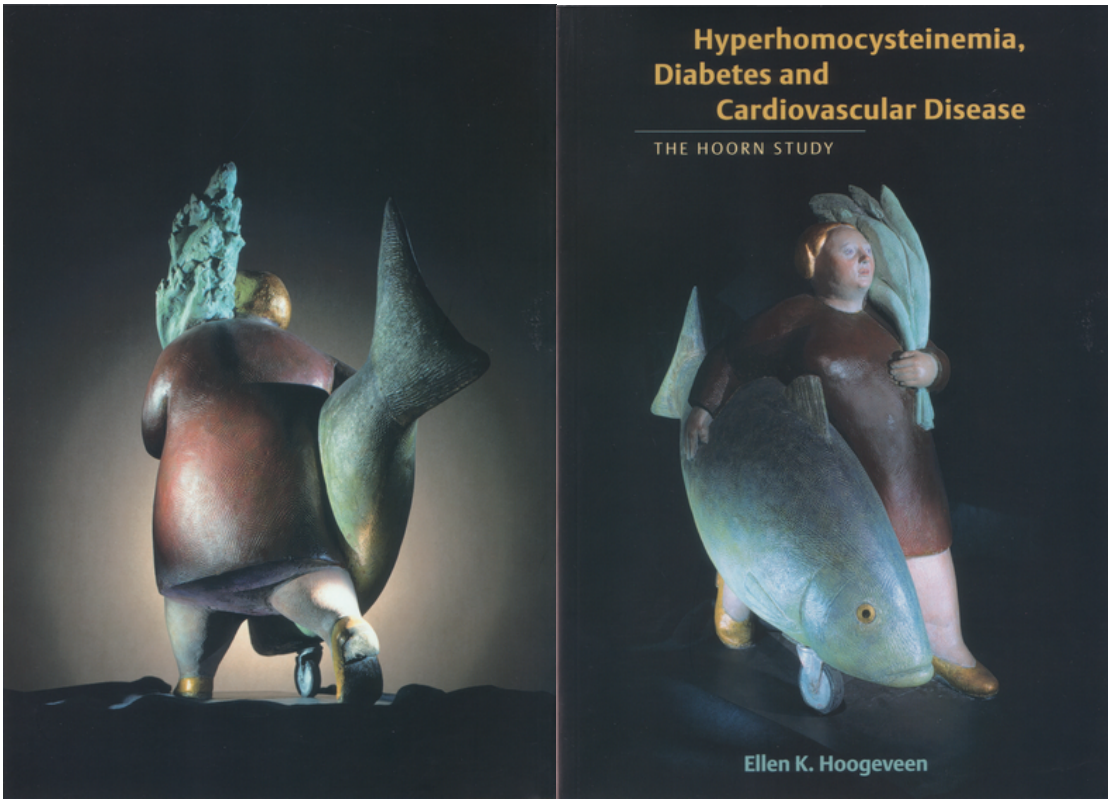
HYPERHOMOCYSTEINEMIA, DIABETES AND CARDIOVASCULAR DISEASE

THE HOORN STUDY



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HYPERHOMOCYSTEINEMIA, DIABETES AND CARDIOVASCULAR DISEASE

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You can observe a lot by just watching.
Yogi Berra

aan mijn ouders
aan Eric en Alarik

Contents

1

General introduction

Introduction

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease independent of established risk factors such as hypertension, hypercholesterolemia, smoking and, probably, diabetes.[1] Little is known about the impact of hyperhomocysteinemia on cardiovascular disease among type 2 diabetic patients. This thesis is focused mainly on the relation between hyperhomocysteinemia on the one hand, and macro- and microangiopathy on the other hand in type 2 diabetic and non-diabetic subjects of a 50- to 75-year old general Caucasian population.

In this chapter a condensed overview is given about type 2 diabetes and its relation with cardiovascular disease (including microangiopathy), and the metabolism of homocysteine, its regulation, and its measurement. Next, studies are reviewed that provide information on the relation between hyperhomocysteinemia and cardiovascular disease and the mechanisms through which hyperhomocysteinemia may cause atherothrombotic disease. Finally, the main objectives of the present thesis are described, in conjunction with a brief outline of The Hoorn Study, which is the basis of the observations presented in this thesis.

Type 2 diabetes and cardiovascular disease

Cardiovascular disease is the most common complication of Type 2 (non-insulin-dependent) diabetes mellitus and accounts for 75 to 80% of the mortality among diabetic subjects.[2] Cardiovascular mortality and morbidity rates are two to four times higher in diabetic patients than in non-diabetic subjects.[3,4] Generally, the etiology of cardiovascular disease (coronary artery, cerebrovascular, and peripheral arterial disease) is thought to be multifactorial. Combined occurrence of various risk factors and/or interaction (synergistic effect of risk factors) may lead to atherosclerosis.[5] The underlying mechanisms for the accelerated atherosclerosis in diabetes are poorly understood. Type 2 diabetes is known to be associated with several adverse cardiovascular risk factors, including hypertension and dyslipidemia, the latter characterized by elevated serum triglycerides and low serum HDL cholesterol.[6] The high prevalence of cardiovascular risk factors in diabetic patients, however, can only partly explain the excess risk of cardiovascular morbidity and mortality.[7] Although accelerated development of atherosclerosis is the main explanation for the excessive morbidity and mortality in type 2 diabetes, microangiopathy may also play some role in the pathogenesis of cardiovascular disease.[2] Clinically, diabetic microangiopathy

leads to microalbuminuria and retinopathy, and is thought to contribute to neuropathy.[8] The prevalence of microalbuminuria varies from 5 to 20% in the 25- to 75-year-old general non-diabetic population to between 20 to 40% among type 2 diabetic patients.[9,10] The prevalence of retinopathy is about 25% among type 2 diabetic patients after 3 to 4 years of diabetes and rises to about 60% after 20 years.[11] The estimates of the prevalence of peripheral polyneuropathy vary due to various definitions of neuropathy, but clinically neuropathy is found in approximately 30% of patients with type 2 diabetes.[12]

The relation between microangiopathy and cardiovascular disease is emphasized by the higher cardiovascular morbidity and mortality rate among subjects with than among those without microalbuminuria and/or retinopathy.[13-16] Although the concept of a single pathogenic mechanism for all diabetes-specific complications is appealing, the discordance in the development of different complications does not support it. At the very least, the risk of various complications may be modified by different risk factors. On the other hand, type 2 diabetic patients with micro- or macroalbuminuria, as compared to those with normoalbuminuria, have a greatly increased risk of cardiovascular morbidity and mortality.[10] This suggests that (micro)albuminuria is accompanied by, or is a marker of, generalized vascular, possibly endothelial dysfunction, and/or that (micro)albuminuria and atherothrombotic disease share certain pathogenic mechanisms.[17,18]

Elevated serum total homocysteine (tHcy) level is a recently recognized risk factor for cardiovascular disease independent of major cardiovascular risk factors such as hypercholesterolemia, hypertension, smoking and, probably, diabetes.[1,19,20] Conceivably, hyperhomocysteinemia may be a risk factor that can partly explain the increased risk of cardiovascular disease among type 2 diabetic patients, because of a high prevalence of hyperhomocysteinemia among type 2 diabetic patients and/or due to biological interaction between hyperhomocysteinemia and diabetes with regard to cardiovascular disease.

Homocysteine metabolism

Homocysteine (Hcy) is a sulfur-containing amino acid derived from dietary methionine by demethylation, whose metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration (Figure 1). In remethylation, Hcy acquires a methyl group from methyltetrahydrofolate (methyl-THF) or from betaine (trimethylglycine), to form

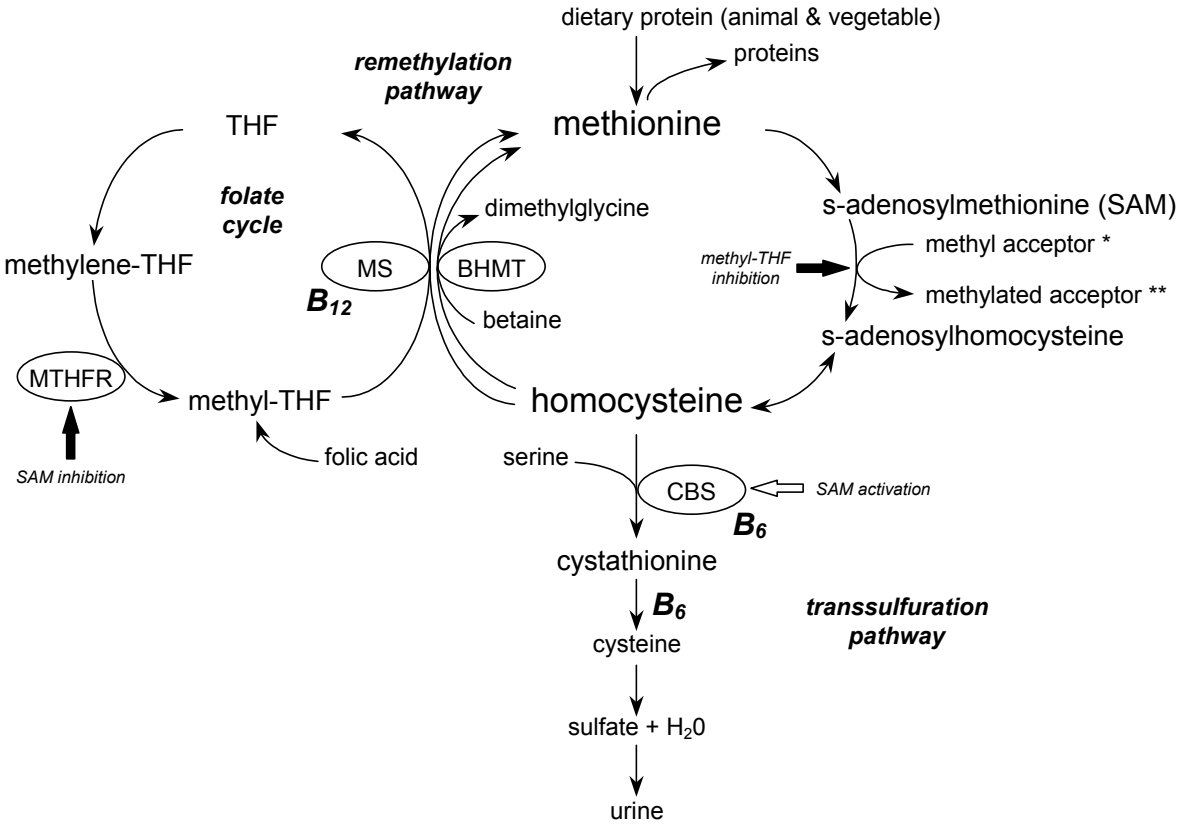

Figure 1. Homocysteine metabolism, modified from ref. 19 & 21.

Large arrows	Indicate enzyme reactions that are regulated by s-adenosylmethionine (SAM) or 5-methyltretrahydrofolate (methyl-THF)
open	indicates activation
closed	indicates inhibition
BHMT	Betaine-homocysteine methyltransferase
B_6	Vitamin B_6
B_{12}	Vitamin B_{12}
CBS	Cystathionine ß-synthase
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
SAM	S-adenosylmethionine
THF	Tetrahydrofolate
*	Methyl acceptor: phosphatidylethanolamine, guanidinoacetate, neurotransmitters (such as dopamine), proteins (such as myelin), DNA, RNA
**	Methylated acceptor: phosphatidylcholine, creatine, methylated neurotransmitters, methylated proteins, methylated DNA, methylated RNA

methionine. In most tissues the remethylation of Hcy is catalyzed by methionine synthase (MS), which uses vitamin B_{12} as a cofactor and methyl-THF as a substrate. The reaction with betaine is confined mainly to the liver and is vitamin B_{12}-independent. A considerable proportion of methionine is then activated to form s-adenosylmethionine (SAM). SAM serves as a universal methyldonor to a variety of acceptors including guanidinoacetate, neurotransmitters, nucleic acids, and hormones. In the transsulfuration

pathway, Hcy condenses with serine to form cystathionine in an irreversible reaction catalyzed by the vitamin B_6-dependent enzyme cystathionine-ß-synthase (CBS). When methionine is in excess, Hcy is directed towards the transsulfuration pathway; under conditions of negative methionine balance, Hcy is primarily remethylated, thus conserving methionine. Selhub et al.[21] have suggested that the regulation of Hcy metabolism is coordinated by the level of SAM and methyl-THF (Figure 1). According to Finkelstein,[22] this switch function of SAM is an oversimplification, as there is evidence that Hcy metabolism is regulated by changes of the abundance of tissue-specific enzymes and their intrinsic kinetic properties.

Because Hcy is not a normal dietary constituent, the sole source of Hcy is methionine. The methionine content of animal proteins is generally two to three times higher than that of plant proteins.[23]

The intracellular concentration of Hcy is kept within narrow bounds, and any increase in production is finally met by export from cells.[20] The concentration of Hcy in blood is therefore an important reflection of its intracellular concentration and of the integrity of the various pathways responsible for its metabolism. Values of serum tHcy in adult populations vary, but usually lie in the range of 5 to 15 μmol/L in the fasting state, a higher level often being referred to as hyperhomocysteinemia.[19] Hyperhomocysteinemia may be caused by inherited enzyme defects, acquired deficiencies of vitamin B_6, B_{12} or folate, by renal failure, and by certain drugs[19] (see below).

Every tissue possesses the methionine cycle. However, transsulfuration, the pathway to catabolize Hcy, occurs only in the liver, kidney, small intestine and pancreas. In addition, hepatocytes have the unique ability to increase the Hcy export in response to extracellular methionine. Finally, some cells have the ability to use extracellular Hcy as a methionine source. Conceivably, tissue-specific pathologies are the consequence of the tissue-specific patterns of metabolism.[22]

Measurement of serum total homocysteine

Approximately 70% of Hcy in blood is bound to proteins, mainly albumin. The remaining unbound Hcy fraction combines by oxidation either with itself to form a dimer or with cysteine to form a mixed disulfide. Only a small proportion (about 1%) circulates as free Hcy. The sum of all these Hcy forms is termed total homocysteine, abbreviated as tHcy. It is not known which form(s) of homocysteine is (are) directly involved in pathological processes. In serum or plasma, free Hcy becomes protein bound, even when

samples are frozen immediately. Therefore, free Hcy may be variable, but tHcy remains constant. In 1985, Refsum et al.[24] developed an assay for the determination of tHcy. However, in the presence of blood cells, there is a time- and temperature-dependent increase of serum tHcy; at room temperature tHcy increases by 5 to 15% per hour[25] due to the continuous production and release of Hcy from the erythrocytes.[26] Therefore, it is important to centrifuge the blood sample within one hour after collection.

Methionine loading

Methionine loading involves the intake of a high dose of methionine (0.1 g/kg), and the tHcy level is measured immediately before methionine loading and usually after 4 to 6 hours. A protein-rich meal may increase serum tHcy levels for at least 8 hours (mean increase 13.5% $\pm$ SD 7.5%), and may therefore represent the physiologic corollary of the methionine load.[27] Fasting and post-methionine load tHcy levels are strongly correlated. The former may reflect vitamin B_{12}- and folate-dependent remethylation, and the latter, vitamin B_6-dependent transsulfuration. Reliance on fasting tHcy level alone results in about 25%[28] fewer subjects classified as hyperhomocysteinemic, and thus fails to identify a substantial proportion of subjects who have normal fasting tHcy but elevated post-methionine load tHcy. Both fasting and post-methionine serum tHcy level are related to risk of cardiovascular disease.[28] However, the inconvenience for the subject makes the methionine loading test less suitable for epidemiological studies.

Determinants of the total homocysteine level

Genetic determinants

Homocystinuria and severe hyperhomocysteinemia (>100 μmol/L) are usually caused by rare inborn errors of Hcy metabolism resulting in marked elevations of serum and urine Hcy concentrations. CBS deficiency is the most common genetic cause of severe hyperhomocysteinemia, with an estimated world-wide incidence of 1:300,000 living births.[29] Heterozygotes (<1% of the general population[19]) have fasting tHcy concentrations in the range of 20 to 40 μmol/L. A homozygous deficiency of MTHFR may also lead to severe hyperhomocysteinemia.[30]

In addition, Kang et al. have reported a thermolabile variant of MTHFR that is caused by a point mutation (C677T) in the coding region for the methylene-THF binding site.[31] This mutation was found in 5 to 15% of

the general Canadian population, virtually identical to that observed in the Dutch population.[32] Persons who are homozygous for this mutation appear to have an exaggerated hyperhomocysteinemic response to the depletion of folic acid.

Other abnormalities of the remethylation cycle that are associated with hyperhomocysteinemia include MS deficiency and disorders of vitamin B_{12} metabolism that impair MS activity.

Nutritional determinants

Both plasma concentration and dietary intake of vitamin B_6, B_{12} and folate show a non-linear inverse correlation with serum tHcy concentration.[33] Individuals with low levels of each of these vitamins have high tHcy concentrations, while those with moderate vitamin levels have substantially lower tHcy concentrations. The strongest association has been reported between folate and tHcy. Inadequate plasma concentration of one or more B vitamins were contributing factors in approximately two thirds of all cases of hyperhomocysteinemia (>14 μmol/L) in an elderly population.[33] Fasting hyperhomocysteinemia in vitamin B_6 deficiency may only occur if the deficiency is severe and sustained over a long period of time.[34]

Other determinants

Women have lower tHcy concentrations than men, and tHcy increases with age. This may partly be due to differences in vitamin status,[33] but also to the influence of sex hormones. Serum tHcy levels increase after menopause,[35,36] and therefore results in a steeper age-related increase in women compared to men. Further evidence for the influence of sex hormones on tHcy level is provided through estrogen and androgen administration, which decreases and increases, respectively, tHcy levels.[37,38] The sex difference may also be related to the stoichiometric formation of Hcy in connection with the creatine/creatinine synthesis that is proportional to muscle mass, and therefore higher in men than in women.[39]

Creatinine clearance and tHcy are strongly inversely correlated.[40] An impaired renal function causes a substantial increase in the half-life of tHcy explained by a reduction in total body clearance, rather than urinary excretion, which is minor (<1%).[41] The mechanism behind this relation is unclear,[42] but the marked hyperhomocysteinemia and the 70% reduction of tHcy clearance in subjects with renal failure[41] emphasize the importance of kidney function for Hcy homeostasis.

Finally, serum cholesterol, blood pressure, smoking, coffee and chronic high alcohol consumption are associated with high, whereas physical activity is associated with low serum tHcy levels.[43-45] With regard to smoking, coffee and alcohol consumption, the association with tHcy is possibly mediated through effects on B vitamin status.

Hyperhomocysteinemia and atherothrombosis

In 1969, McCully noted that arterial and venous thromboembolic disease is a characteristic feature of homocystinuria independent of the site of the metabolic defect; this points to Hcy as the causal agent.[46] Approximately 50% of untreated patients with homocystinuria will have a thromboembolic event before the age of 30 years.[47] On autopsy, the macroscopic findings included arterial and venous thrombosis, and arteriosclerotic lesions in arteries. This is the basis for McCully's theory that elevated tHcy concentrations cause atherosclerosis and therefore could be a risk factor for cardiovascular disease in the general population.

In 1991, Clarke et al. showed that moderate hyperhomocysteinemia is a risk factor for cardiovascular disease, independent of hypertension, hypercholesterolemia and smoking.[48] In 1995, Boushey et al. published a meta-analysis based on 27 studies, both cross-sectional and prospective, on tHcy and cardiovascular disease including about 4000 subjects that provided considerable evidence that elevated tHcy levels are associated with atherosclerotic vascular disease.[1] For this meta-analysis, a linear relation between tHcy levels and risk of cardiovascular disease was assumed. Per 5 μmol/L (about 1 SD) increment of serum tHcy level, the odds ratio [OR; 95% confidence interval (95% CI)] was 1.8 (1.6 to 2.0) for coronary artery disease, 1.5 (1.3 to 1.9) for cerebrovascular disease and 6.8 (2.9 to 15.8) for peripheral vascular disease. Since then, about 40 additional studies have been published about this issue, the majority supporting the conclusions of Boushey et al.[19]

The strongest evidence for a causal relation between hyperhomocysteinemia and cardiovascular disease can be derived from prospective studies. Perry et al.[49] showed a graded positive relation between tHcy and (non)fatal stroke that was stronger, though not significant, in hypertensive than in normotensive subjects. Two studies[50,51] showed a relation between tHcy and (non)fatal stroke in normotensive, but not in hypertensive subjects. Eight prospective studies[52-59] reported a positive relation between hyperhomocysteinemia and (non)fatal myocardial infarction. It remains intriguing that extension, by 2.5 years, of the Physicians' Health Study initial 5-year

follow-up[52] resulted in a much weaker, and no longer significant, relation between hyperhomocysteinemia and (non)fatal myocardial infarction.[60] A possible explanation for this discrepancy might be that with increasing follow-up time the tHcy level at baseline becomes an increasingly inaccurate approximation of the tHcy level over the follow-up period. Another study[57] reported a relation only among women, but not in men. Three population-based studies[51,61,62] with a long follow-up duration of 10 to 20 years could not establish a relation between hyperhomocysteinemia and (non)fatal cardiovascular disease.

The strongest evidence in favor of a thrombotic effect of homocysteine can be derived from studies that estimated the risk for venous thrombosis.[63,64] In a meta-analysis including eight studies, the pooled odds ratio for venous thrombosis was 2.8 (1.9 to 4.2) in subjects with hyperhomocysteinemia.[65]

The European Concerted Action Project on homocysteine and vascular disease is a case-control study of 750 relatively young vascular disease patients (coronary artery, cerebrovascular, and peripheral arterial disease) and 800 controls. In this study, the interactions[28] between tHcy and three major cardiovascular risk factors (hypercholesterolemia, smoking and hypertension) were systematically investigated. Subjects with diabetes were excluded. An elevated tHcy level interacted strongly with hypertension and smoking with regard to risk of atherothrombotic disease.

Interaction

Interaction, or effect modification as it is alternatively called, implies that the combined effect of two risk factors or determinants of disease is different from what we would expect, knowing the effect of each risk factor separately.

A distinction is made between biological interaction, where the underlying biological mechanisms of cause and effect are understood, and statistical interaction, where we merely observe deviations from the expected, without claiming that we understand the underlying mechanisms.

A subtle point in the assessment of statistical interaction is that the presence or absence of interaction depends on the choice of the effect measure. For instance, under a multiplicative statistical model, a relative risk of 2 combined with a relative risk of 3 would make for a relative risk of 6, in the absence of interaction. In an additive model, on the other hand, a risk difference of 2% combined with a risk difference of 3% would make for a risk difference of 5%, again in the absence of interaction. Absence of

interaction on one scale implies interaction on the other scale. To emphasize this, it has been proposed that one should use the term 'effect-measure modification', instead of 'effect modification'.[66]

Pathophysiological mechanisms

There is no unifying hypothesis explaining the atherogenic and thrombogenic effects of circulating Hcy. Studies in humans and animals suggest that the atherogenic propensity associated with hyperhomocysteinemia results from endothelial dysfunction and injury followed by smooth muscle cell proliferation, leukocyte and platelet activation, and thrombus formation (Figure 2). Although the exact mechanism of endothelial dysfunction is unknown, there is growing evidence that Hcy exerts its effects by promoting oxidative damage. It may initiate lipid peroxidation and oxidation of low-density lipoprotein. Hcy also alters the normal antithrombotic phenotype of the endothelium by enhancing the activities of factor XII and factor V, and depressing the activation of protein C. Furthermore, Hcy inhibits the expression of thrombomodulin and heparan sulfate, and induces the expression of tissue factor by the endothelium.[67] Finally, studies in humans revealed a positive association between hyperhomocysteinemia and impaired endothelium-dependent vasodilatation,[68] and between hyperhomocysteinemia and von Willebrand factor (a marker of endothelial dysfunction),[69,70] thus providing some in vivo evidence that hyperhomocysteinemia may induce endothelial dysfunction.

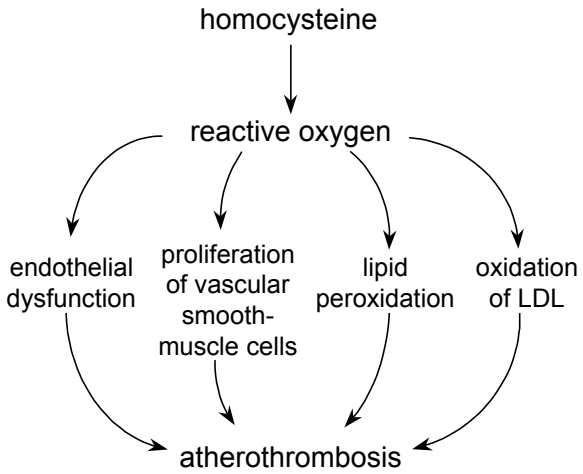

Figure 2. Postulated adverse vascular effects of homocysteine, modified from ref. 67.

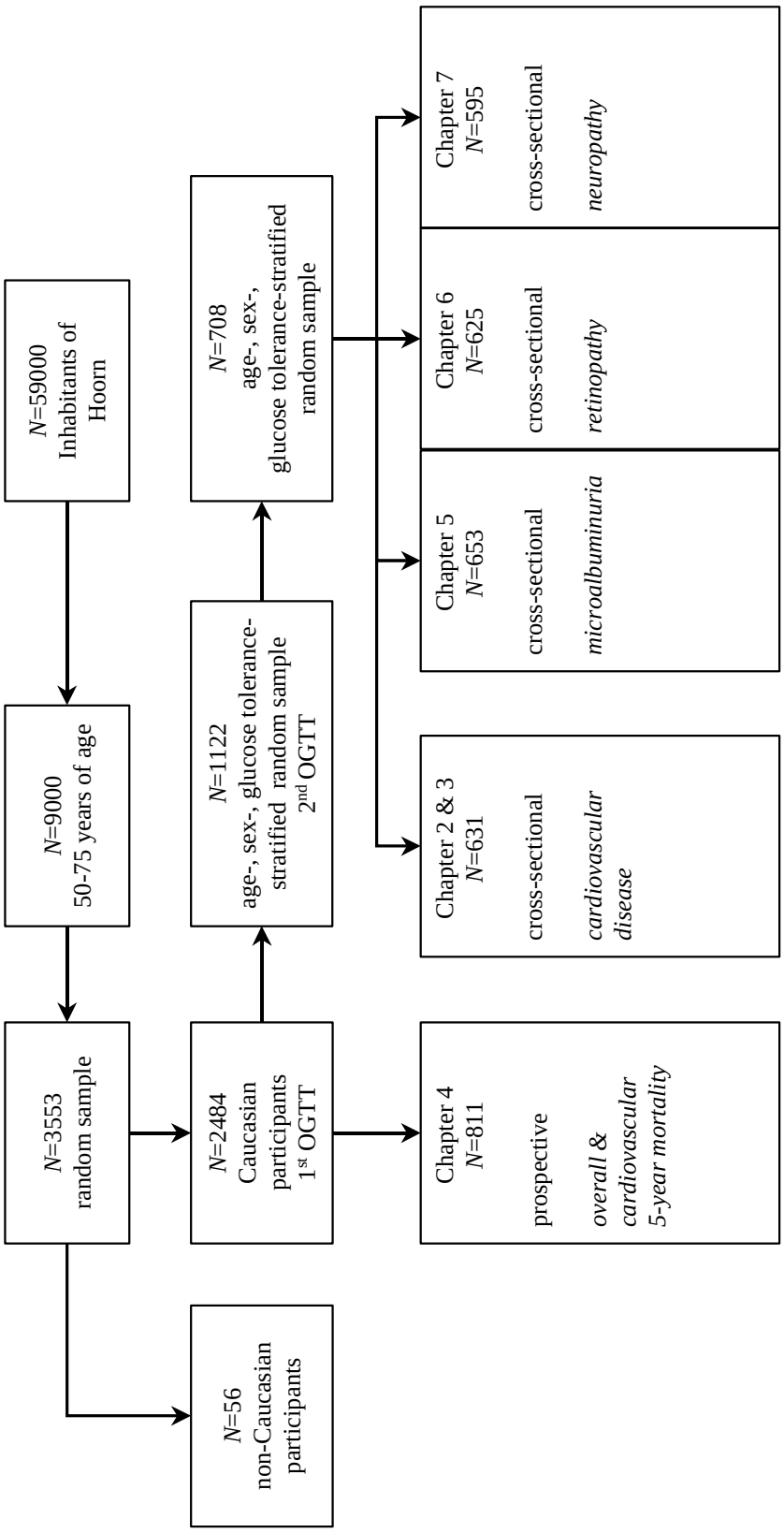

Figure 3. Diagram of the study design of The Hoorn Study. The bottom row boxes refer to the different studies, their study populations, shows the design (prospective or cross-sectional) and the main endpoints. The lines indicate where the populations originate from.

Table 1. Diagnostic criteria for the oral glucose tolerance test according to WHO criteria (1985)[71]

	Diabetes mellitus	Impaired glucose tolerance	Normal glucose tolerance
Fasting	>= 7.8 mmol/L	< 7.8 mmol/L	< 7.8 mmol/L
2 hours post-load	>= 11.1 mmol/L	7.8 - 11.1 mmol/L	< 7.8 mmol/L

Values are venous plasma glucose concentrations.

The Hoorn Study

The Hoorn Study is a prospective study of glucose tolerance, cardiovascular risk factors, and cardiovascular complications in a 50- to 75-year-old general Caucasian population. The baseline examination was conducted from October 1, 1989 until December 31, 1991, and was carried out in the town of Hoorn in the Netherlands. The design of the Hoorn Study is depicted in Figure 3. From the registry office of Hoorn, a middle-sized town in the Netherlands (59,000 inhabitants), a random sample of all inhabitants aged 50 to 75 years was selected. Of the eligible subjects, 71% agreed to participate, resulting in a cohort of 2484 subjects. In all participants, an Oral Glucose Tolerance Test (OGTT: 75 gram glucose load, according to the 1985 WHO criteria[71]) was performed, except in type 2 diabetic patients treated with oral glucose-lowering agents or insulin, of whom a fasting blood sample was taken only. The criteria for the diagnosis of diabetes mellitus and impaired glucose tolerance are given in Table 1. An OGTT is a sensitive method to detect diabetes mellitus, adding to the known diabetic population a group of subjects of about equal size with undiagnosed diabetes. To make a more reliable assessment of glucose tolerance, a second OGTT (participation rate 93%) was performed within 2 to 6 weeks on all subjects with 2-hour post-load plasma glucose levels >=7.5 mmol/L at the first test. For reasons of efficiency, an age- and sex-stratified random sample was taken, with five strata for both sexes (<55, 55-59, 60-64, 65-69, and >70 years) from subjects with 2-hour glucose levels <7.5 mmol/L. Finally, a second age-, sex- and glucose tolerance-stratified random sample (N=708) was drawn to study cardiovascular disease, microalbuminuria, retinopathy and neuropathy (Figure 3).

Outline of the thesis

It is not known whether hyperhomocysteinemia is a risk factor for cardiovascular disease among type 2 diabetic subjects, nor whether hyperhomocysteinemia and type 2 diabetes interact with regard to cardiovascular

disease, including microangiopathy. Therefore, the main aim of the present thesis is to study whether hyperhomocysteinemia is a risk factor for cardiovascular disease among type 2 diabetic subjects. In addition, we investigate whether the strength of the relation between homocysteine and cardiovascular disease is modified by diabetes. Figure 3 schematically depicts the (sub)populations studied, the endpoints investigated, the time relation (cross-sectional or prospective) between tHcy and the endpoint under study, and the chapters in which these relations are discussed.

Because it is not known whether hyperhomocysteinemia is a risk factor for cardiovascular disease independent of type 2 diabetes, we investigate this issue in an age-, sex- and glucose tolerance-stratified random sample of a 50- to 75-year-old Caucasian population. We also assess whether the presence of type 2 diabetes itself is associated with higher tHcy levels. In addition, we compare the three separate risk estimates of peripheral arterial, coronary artery, and cerebrovascular disease, because it is not known whether the strength of the relation of each of these outcomes with hyperhomocysteinemia is similar. Finally, we investigate the presence of interaction between diabetes and hyperhomocysteinemia (that is, a stronger effect of tHcy among diabetic than among non-diabetic subjects) with regard to cardiovascular disease, because such an interaction appears biologically plausible (Chapter 2). In an additional analysis we compare the strength of the relations between hyperhomocysteinemia and different localizations of peripheral arterial disease (i.e., crural, femoropopliteal and aortoiliac arterial obstruction), because risk factors for proximal versus distal peripheral arterial disease are probably different (Chapter 3).

In search for evidence for a causal relation between hyperhomocysteinemia and (cardiovascular) mortality, we prospectively investigate the relation between hyperhomocysteinemia and 5-year mortality. In addition, we assess the presence of interaction between hyperhomocysteinemia and diabetes with regard to 5-year mortality (Chapter 4).

Since hyperhomocysteinemia might induce, for example through oxidative stress, microangiopathy, we investigate the relation between hyperhomocysteinemia and microalbuminuria (Chapter 5), retinopathy (Chapter 6) and neuropathy (Chapter 7).

In Chapter 8, we evaluate the effect of metformin on serum tHcy level. Metformin is an oral glucose-lowering agent that decreases hepatic glucose output, largely by inhibiting gluconeogenesis. Since metformin may lower serum vitamin B_{12}, we investigate the effect of metformin on serum tHcy.

Finally, in Chapter 9, we give an overview of the main findings of the thesis, make suggestions for future research and evaluate the possible consequences of our findings for the treatment of type 2 diabetic patients.

Note

During the writing of this thesis the nomenclature changed from non-insulin-dependent diabetes mellitus (NIDDM) into type 2 diabetes.[72] The choice of nomenclature follows that of the published articles.

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2

Hyperhomocysteinemia is associated with an increased risk of cardiovascular disease, especially in non-insulin-dependent diabetes mellitus

A population-based study

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Abstract

Background A high serum total homocysteine (tHcy) level is an independent risk factor for cardiovascular disease. It is not known whether the strength of the association between hyperhomocysteinemia and cardiovascular disease is similar for peripheral arterial, coronary artery and cerebrovascular disease.

Methods We compared the three separate risk estimates in an age-, sex- and glucose tolerance-stratified random sample ($N=631$) from a 50- to 75-year-old general Caucasian population. Furthermore, we investigated the combined effect of hyperhomocysteinemia and diabetes mellitus with regard to cardiovascular disease.

Results The prevalence of fasting hyperhomocysteinemia (>14.0 μmol/L) was 25.8%. After adjustment for age, sex, hypertension, hypercholesterolemia, diabetes and smoking, the odds ratios (ORs; 95% confidence intervals) per 5 μmol/L tHcy increment were 1.44 (1.10 to 1.87) for peripheral arterial, 1.25 (1.03 to 1.51) for coronary artery, 1.24 (0.97 to 1.58) for cerebrovascular and 1.39 (1.15 to 1.68) for any cardiovascular disease. After stratification by glucose tolerance category and adjustment for the classical risk factors and serum creatinine, the ORs per 5 μmol/L tHcy increment for any cardiovascular disease were 1.38 (1.03 to 1.85) in normal glucose tolerance, 1.55 (1.01 to 2.38) in impaired glucose tolerance, and 2.33 (1.11 to 4.90) in non-insulin-dependent diabetes mellitus ($P=0.07$ for interaction).

Conclusion We conclude that the magnitude of the association between hyperhomocysteinemia and cardiovascular disease is similar for peripheral arterial, coronary artery and cerebrovascular disease in a 50- to 75-year-old general population. High serum total homocysteine may be a stronger (1.6-fold) risk factor for cardiovascular disease in subjects with non-insulin-dependent diabetes mellitus than in non-diabetic subjects.

Introduction

Retrospective and prospective studies have demonstrated that hyperhomocysteinemia is a risk factor for cardiovascular disease that is independent of classic risk factors such as smoking, hypercholesterolemia, diabetes mellitus and hypertension.[1-4] In a recent meta-analysis,[1] the association between hyperhomocysteinemia and peripheral arterial disease [summary odds ratio (OR), 6.8] was considerably stronger than with coronary artery and cerebrovascular disease (ORs, 1.8 and 1.5). The summary estimate of the association between hyperhomocysteinemia and peripheral arterial disease, however, was inferred from one population-based study,[5] which consisted of only men, and two hospital-based studies.[6,7] Therefore, to further investigate this issue, we compared the risk estimates of peripheral arterial, coronary artery and cerebrovascular disease in a random sample of a 50- to 75-year-old general Caucasian population.

A recent large study showed that the risk of cardiovascular disease was especially high among subjects with hyperhomocysteinemia who also smoked or had hypertension, i.e., there was evidence of interaction with these risk factors.[2] However, this study excluded diabetic subjects. Our study was specifically designed to examine glucose tolerance as a cardiovascular risk factor,[8] and therefore we investigated the combined effect of hyperhomocysteinemia and diabetes mellitus with regard to relative risk of cardiovascular disease.

Finally, there is increasing evidence that hyperhomocysteinemia is common in the elderly population.[9,10] A large part of the prevalence of hyperhomocysteinemia in the elderly population is attributable to a low intake of the B vitamins, folate, vitamin B_6 and vitamin B_{12}.[10] Therefore, it has been suggested that lowering serum total homocysteine (tHcy) levels by increasing the intake of folate, probably the most important dietary determinant of serum tHcy levels, may be an effective means of decreasing cardiovascular risk.[11] To estimate the potential maximum benefit of such a strategy, we estimated the proportion of preventable cardiovascular disease caused by hyperhomocysteinemia.

Methods

Design and study population

The Hoorn Study is a cross-sectional survey of glucose tolerance and other cardiovascular risk factors in a 50- to 75-year-old general Caucasian population conducted from 1989 to 1992. A random sample of all men and women aged 50 to 75 years was drawn from the municipal population registry office of Hoorn (The Netherlands); 2484 subjects participated (response rate 71%). An extensive cardiovascular investigation (detailed below) was performed in an age-, sex- and glucose tolerance-stratified random subsample ($N=631$; response rate 89.1%).[8] The Hoorn Study was approved by the Ethical Review Committee of the University Hospital Vrije Universiteit. Informed consent was obtained from all participants.

Cardiovascular disease

Cardiovascular disease was defined as coronary artery, cerebrovascular and/or peripheral arterial disease. Coronary artery disease was defined as a history of myocardial infarction, coronary artery bypass grafting and/or Minnesota codes 1-1 or 1-2 on the ECG ($N=625$).[12] Cerebrovascular disease was defined as a history of transient ischemic attack (TIA)/stroke and/or a carotid artery stenosis of >80%. (A carotid artery stenosis in excess of 80% is associated with a high risk of stroke within 2 years: more than 25% for symptomatic and 10% for asymptomatic carotid stenosis.[13,14]) Peripheral arterial disease was defined as a peripheral arterial reconstruction or limb amputation and/or an ankle brachial pressure index (ABPI) <0.50. (A low ABPI is related to both more extensive peripheral arterial disease and a higher risk of cardiovascular mortality.[15-18]) The cardiovascular history was obtained by means of a self-administered questionnaire and, if positive, accepted only when confirmed by written information from the participant's general practitioner. Ultrasonographic examination of both common, internal and external carotid arteries ($N=628$) was performed by means of a color-coded Duplex scanner as previously described in detail.[19] We classified subjects into two categories on the basis of the maximal percentage of stenosis of the more diseased of the two carotid arteries: 0% to 80% or 81% to 100%.[20] The ABPI was obtained by means of Doppler-assisted systolic blood pressure measurements taken from the brachial and the three crural arteries on both sides as previously described in more detail.[8] The lowest ABPI of either limb was used for statistical analysis.

Measurement of serum total homocysteine

Fasting blood samples were centrifuged within one hour after collection. Serum was stored at -20°C for 4 to 6 years. There is good evidence that serum tHcy levels are stable for 10 years or more.[9,21] Serum total (free plus protein bound) homocysteine level was measured by using tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by high-performance liquid chromatography with fluorescence detection.[22] The intra- and interassay coefficients are 2.1% and 5.1%.

Other cardiovascular risk factors

We measured levels of fasting serum total cholesterol, HDL cholesterol and triglycerides (enzymatic techniques, Boehringer Mannheim) and creatinine (modified Jaffé method). Glycosylated hemoglobin (Hba_{1c}) was determined by ion-exchange high-performance liquid chromatography. Hypercholesterolemia was defined as total cholesterol >=6.5 mmol/L and/or the current use of cholesterol-lowering medication. Hypertension was defined as a blood pressure >=160 mmHg systolic and/or >=95 mmHg diastolic and/or the current use of antihypertensive medication. Impaired glucose tolerance (IGT) and non-insulin-dependent diabetes mellitus (NIDDM) were defined according to the WHO criteria[23] applied to the mean of two oral glucose tolerance tests. Subjects were classified as either nonsmokers or ever smokers. Body mass index and waist-hip ratio were calculated as described elsewhere.[8] All laboratory and vascular measurements and codings of the ECGs were carried out in a blinded fashion with respect to history of cardiovascular disease and glucose tolerance status.

Statistical analysis

Variables are presented as mean $\pm$ standard deviation (SD), number (percentage of the total) or, in case of skewed distribution, median and interquartile range (IQR). Associations of cardiovascular risk factors with serum tHcy level (logarithmically transformed) were studied by calculating Pearson correlation coefficients. All reported probability values are two-tailed. We assessed sex-specific prevalences of hyperhomocysteinemia for different cutoff values (>12, 13, 14, 15 and 16 μmol/L), standardized for age and glucose tolerance as described previously in detail.[8] Briefly, the frequency of hyperhomocysteinemia was determined in 24 strata [age (3), sex (2) and glucose tolerance (4)] of the subsample. To assess the prevalence

of hyperhomocysteinemia in the original population-based sample (standard, $N=2484$), the prevalence of hyperhomocysteinemia was back-calculated from the magnitude of each age, sex, and glucose tolerance category stratum.

We performed logistic regression analyses to study the association of serum tHcy with peripheral arterial, coronary artery, and cerebrovascular disease separately and combined (i.e., total cardiovascular disease). We calculated odds ratios (ORs) and 95% confidence intervals (CIs) per 5 μmol/L (about 1 SD) increment of serum tHcy (assuming a linear logistic relation between homocysteine and risk of cardiovascular disease) and by tertiles with the lowest tertile as a reference category. We used multiple logistic regression analysis to control for age, sex, hypertension, hypercholesterolemia, smoking, and diabetes mellitus. We also tested models that in addition included serum creatinine, total cholesterol, triglycerides, HDL and LDL cholesterol, systolic blood pressure, BMI and/or waist-hip ratio. To evaluate a possible modifying role of other risk factors, we repeated the previous analyses in strata of sex, glucose tolerance categories, smoking, hypertension, and hypercholesterolemia.

We calculated the population-attributable risk (PAR), i.e., the percentage of excess cardiovascular disease in the population attributable to elevated serum total homocysteine levels, as $[P_e(RR-1)x100]/[P_e(RR-1)+1]$, where RR is the relative risk estimated as the OR, and P_e is the proportion of the population liable to benefit from a reduction of serum tHcy levels. The potential benefit of a distribution shift of 5 μmol/L was calculated because we assumed that this is within attainable limits.[1] To calculate the PAR, we conservatively assumed that reduced serum tHcy level would benefit only individuals with levels higher than 12 μmol/L, although the epidemiological evidence more strongly supports a graded than a threshold association between serum tHcy and cardiovascular disease. The cutoff of 12 μmol/L is based on homocysteine levels of vitamin B_{12}- and folate-replete subjects[10,24,25] and thus on nutritional status, not on an estimate of the association with cardiovascular disease. This calculation of the PAR assumes that there is no important risk gradient up to a serum tHcy level of 12 μmol/L. To investigate whether this assumption is reasonable, we also calculated the ORs for cardiovascular disease for several ranges of homocysteine concentrations with 9 to 12 μmol/L serum tHcy as the reference category. We chose the boundaries as small as possible to evaluate the dose-response relation between homocysteine and cardiovascular disease as accurately as possible. All analyses were performed with SPSS for Windows 6.1.

Table 1. General characteristics of the subjects

N	631	
Men *%*	48	
Age *years*	64.3	(7.2)
Body mass index kg/m^2	27.3	(4.0)
Waist-hip ratio	0.92	(0.09)
Ever smoker *%*	66	
Systolic blood pressure *mmHg*	139	(19)
Diastolic blood pressure *mmHg*	83	(10)
Hypertension *%*	39.1	
Impaired glucose tolerance *%*	26.9	
Diabetes mellitus *%*	27.4	
Fasting glucose *mmol/L*	6.7	(2.6)
HbA_{1c} *% of hemoglobin*	5.9	(1.3)
Total cholesterol *mmol/L*	6.6	(1.2)
HDL cholesterol *mmol/L*	1.3	(0.4)
LDL cholesterol *mmol/L*	4.5	(1.1)
Triglycerides *mmol/L*	1.6	(1.1-2.2)*
Creatinine *μmol/L*	92	(19)
Total homocysteine *μmol/L*	11.4	(9.3-14.1)*
Cardiovascular disease	67	(10.6)†
Peripheral arterial disease	17	(2.7)†
Coronary artery disease	40	(6.3)†
Cerebrovascular disease	19	(3.0)†

Data are presented as:
mean (SD)
* median (interquartile range) or
† number (percentage).

Cardiovascular disease indicates peripheral arterial disease, coronary artery disease and/or cerebrovascular disease.

Peripheral arterial disease indicates history of vascular surgery or major amputation (*N*=10) and/or ankle brachial pressure index <0.50 (*N*=9).

Coronary artery disease indicates history of coronary artery bypass grafting (*N*=8), myocardial infarction (*N*=24) and/or Minnesota codes 1-1 or 1-2 on the ECG (*N*=19).

Cerebrovascular disease indicates history of TIA/stroke (*N*=12) and/or carotid artery stenosis >80% (*N*=8).

Results

Table 1 shows the main characteristics of the study population. The median serum tHcy level was 12.2 μmol/L (IQR: 10.0 to 15.3) in men and 10.7 μmol/L (IQR: 9.0 to 13.3) in women. Figure 1 shows the standardized sex-specific prevalences of hyperhomocysteinemia according to different cutoff values. The medians (IQR) for serum tHcy were 11.2 μmol/L (9.2 to 14.4) in normal glucose tolerance (NGT), 12.2 μmol/L (9.7 to 14.5) in IGT and 11.2 μmol/L (9.2 to 13.6) in NIDDM. Serum tHcy levels correlated with age (r=0.17; P<0.001), serum creatinine (r=0.41; P<0.001), systolic blood pressure (r=0.10; P=0.01), waist-hip ratio (r=0.12; P=0.004), and

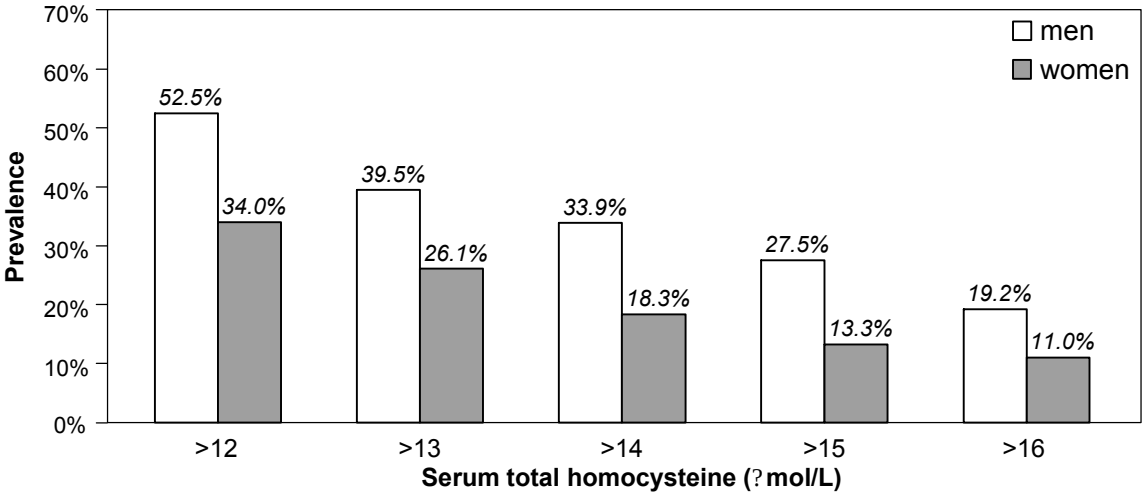

Figure 1. Sex-specific prevalence of hyperhomocysteinemia in Hoorn, The Netherlands, between 1989 and 1992. Prevalences are presented for men and women separately for different cutoff values of serum total homocysteine (μmol/L).

inversely with HDL cholesterol (r=-0.09; P=0.03), but not with BMI (r=-0.02; P=0.6), fasting glucose (r=-0.07; P=0.08), HbA_{1c} (r=-0.02; P=0.7) or duration of NIDDM (r=-0.06; P=0.6).

The mean ± SD HbA_{1c} was 5.3 ± 0.5% in NGT, 5.6 ± 0.5% in IGT, and 7.2 ± 1.8% in NIDDM. Of all NIDDM subjects, 96 (55.5%) were newly diagnosed. Ten (5.8%) were treated with diet alone and 67 (38.7%) with glucose-lowering agents; 15 (8.7%) with insulin, 51 (29.5%) with sulfonylureas and 3 (1.7%) with metformin (2 of whom also used sulfonylureas). The median (IQR) duration of NIDDM of those subjects treated with diet or glucose-lowering agents was 6.1 (2.5 to 11.2) years. The prevalence of cardiovascular disease was 7.3% in NGT, 11.2% in IGT and 15.6% in NIDDM.

A 5 μmol/L increment of serum tHcy was associated with an increased risk of cardiovascular disease, which was of similar magnitude in each of the vascular territories examined (Table 2). Additional adjustment for serum creatinine did not materially change the ORs, nor did inclusion of total cholesterol, triglycerides, HDL and LDL cholesterol, systolic blood pressure, BMI and/or waist-hip ratio in the model. There was no evidence for a threshold if risks were calculated by tertiles of serum tHcy (data not shown). Risk of total cardiovascular disease increased with increasing serum tHcy levels (Figure 2).

We evaluated possible effect modification and did not observe substantial differences among the strata of the following risk factors: male

Table 2. Odds ratios (95% confidence intervals) of cardiovascular disease per 5 μmol/L increment of serum total homocysteine

	Number of subjects	Crude OR	Age- & sex-adjusted OR	Multivariate* adjusted OR
Cardiovascular disease	67†	1.39‡ (1.16 - 1.67)	1.34§ (1.12 - 1.60)	1.39‡ (1.15 - 1.68)
Peripheral arterial disease	17	1.38§ (1.13 - 1.68)	1.38§ (1.12 - 1.69)	1.44§ (1.10 - 1.87)
Coronary artery disease	40	1.26§ (1.06 - 1.51)	1.23¶ (1.02 - 1.47)	1.25¶ (1.03 - 1.51)
Cerebrovascular disease	19	1.24¶ (1.01 - 1.54)	1.24 (0.98 - 1.56)	1.24 (0.97 - 1.58)

* Adjusted for age, sex, hypertension (yes/no), ever smoking (yes/no), hypercholesterolemia (yes/no), and non-insulin-dependent diabetes mellitus (yes/no).
† Subjects were counted once with regard to cardiovascular disease endpoint.
‡ *P*<0.001
§ *P*<0.01
¶ *P*<0.05

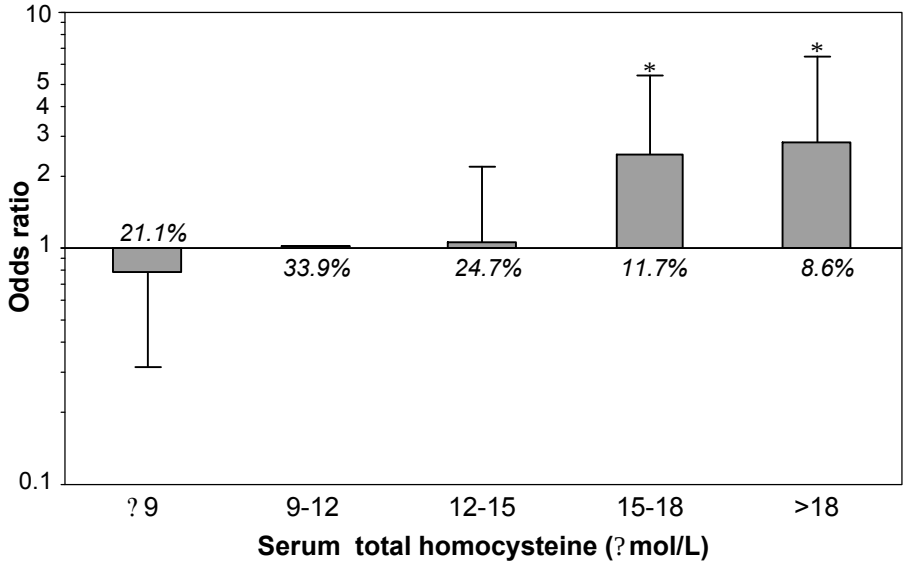

Figure 2. Odds ratio for cardiovascular disease according to serum total homocysteine level adjusted for age and sex. The reference category was serum total homocysteine values of 9 to 12 μmol/L. Percentages of the subsample for each serum total homocysteine range are presented. The error bars represent the lower or upper half of the 95% confidence intervals. * *P*<0.05, significantly different from the reference category. *P*=0.001 for trend.
A logarithmic scale was used since the odds ratio is a multiplicative measure of association; equal differences on the logarithmic scale correspond to equal ratios between odds ratios.

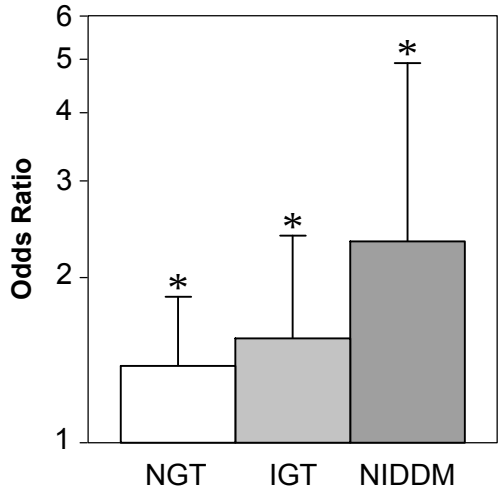

Figure 3. Odds ratio for cardiovascular disease after stratification by glucose tolerance category.
The error bars represent the upper half of the 95% confidence intervals.
Odds ratios are calculated per 5 μmol/L increment of serum total homocysteine, adjusted for age, sex, hypertension, ever smoking, hypercholesterolemia and serum creatinine.

* $P < 0.05$
$P = 0.07$ for interaction

NGT = normal glucose tolerance
IGT = impaired glucose tolerance
NIDDM = non-insulin-dependent diabetes mellitus

sex, hypertension, hypercholesterolemia, and smoking (data not shown). However, after stratification by glucose tolerance category, exclusion of one outlier, and adjustment for age, sex, hypertension, hypercholesterolemia, smoking, and serum creatinine, the ORs (95% CI) per 5 μmol/L increment in serum tHcy of cardiovascular disease were 1.38 (1.03 to 1.85) in NGT, 1.55 (1.01 to 2.38) in IGT, and 2.33 (1.11 to 4.90) in NIDDM (P=0.07 for interaction; Figure 3). These results indicate that high serum tHcy is a stronger (1.6-fold) risk factor for cardiovascular disease in NIDDM than in subjects with normal or impaired glucose tolerance.

The standardized prevalence of cardiovascular disease was 8.0%. From the incremental PAR percent values for serum tHcy levels >12 μmol/L, we calculated that the proportion of preventable cardiovascular disease caused by a 5 μmol/L decrease was 10.6% for a 50- to 75-year-old general Caucasian population.

Discussion

There are four main findings in this study. First, the magnitude of the association between hyperhomocysteinemia and cardiovascular disease was similar with respect to peripheral, coronary, and cerebral arterial disease. Second, hyperhomocysteinemia appeared to be a stronger (1.6-fold) risk factor for cardiovascular disease in subjects with NIDDM than in subjects with normal or impaired glucose tolerance. Third, the prevalence of hyperhomocysteinemia was high in this 50- to 75-year-old general popu-

lation. Finally, we estimated a potential reduction of approximately 10% of the total burden of cardiovascular disease by a distribution shift of 5 μmol/L serum tHcy level.

In a recent meta-analysis,[1] the summary ORs per 5 μmol/L increment of fasting serum tHcy were 1.7 for coronary artery disease, 1.5 for cerebrovascular disease, and 6.8 for peripheral arterial disease. Compared with the ORs we found, these ORs were somewhat higher for coronary and cerebrovascular disease but much higher for peripheral disease. For coronary and peripheral arterial disease, it is unlikely that we underestimated the relative risk because of misclassification of disease because the diagnostic criteria we used are quite specific.[8,14] In contrast, the diagnostic category 'TIA/stroke', which was part of our definition of cerebrovascular disease, included self-reported TIAs, a diagnosis that is liable to nondifferential misclassification. Thus, we may have underestimated the OR for cerebrovascular disease to some extent. Nevertheless, the results of the present study clearly do not support the hypothesis that hyperhomocysteinemia is a stronger risk factor for peripheral arterial than for coronary and cerebrovascular disease, at least among 50- to 75-year-olds. A recent study among younger subjects (mean age, 45 years) reached a similar conclusion.[2]

Because the previously mentioned meta-analysis[1] was based mostly on studies that comprised to a large extent persons younger than 55 years, another explanation for the weaker association we found might be that the relative risk of hyperhomocysteinemia with regard to cardiovascular disease is weaker among older persons. However, in a recent study[26] that comprised subjects aged 25 to 65 years, an OR of 1.3 per 5 μmol/L increment of tHcy for severe coronary artery disease was found, which is of similar magnitude to the OR in the present study.

Little is known about the impact of NIDDM on serum tHcy levels. As in previous studies,[27,28] we found no important difference in fasting serum tHcy level between diabetic and non-diabetic subjects. Although Araki et al.[29] and Munshi et al.[27] have demonstrated that diabetic subjects who also had macrovascular disease had a higher fasting and post-methionine load tHcy level, respectively, than non-diabetic controls who were free of cardiovascular disease, it is not clear from their studies that the higher tHcy levels were due to the diabetic state *per se*. In addition, we found no relation between serum tHcy and fasting glucose, HbA_{1c} or duration of NIDDM. Although more than 55% of the diabetic subjects were newly diagnosed, we cannot rule out that changes of dietary habits of the 45% of diabetic patients who were aware of their disease may have improved their B vitamin status

and thereby lowered the tHcy level. There is no indication that insulin or sulfonylureas alter tHcy metabolism.[29] In contrast, metformin may induce vitamin B_{12} malabsorption and thereby increase the serum tHcy level. However, we did not find an important effect on serum tHcy levels in subjects with NIDDM.[30] Taken together, there is no clear evidence that the diabetic state influences tHcy levels, but more detailed studies of this issue are needed.

The design of the study, with oversampling of diabetic subjects, provided an opportunity to investigate the combined role of diabetes and hyperhomocysteinemia with regard to cardiovascular disease. Because the oversampling was performed before identification of cardiovascular disease, there was no introduction of bias. Hyperhomocysteinemia appeared to be a stronger risk factor for cardiovascular disease in patients with NIDDM than in subjects with normal or impaired glucose tolerance. The biological mechanism for the interaction between diabetes and hyperhomocysteinemia with regard to cardiovascular disease is not known. However, both smoking and hypertension interact with hyperhomocysteinemia.[2] Taken together, these data suggest that hyperhomocysteinemia can enhance atherogenic and/or thrombogenic pathways common to classic risk factors such as smoking, hypertension and diabetes mellitus. Because NIDDM is associated with a high risk of cardiovascular disease, interaction with hyperhomocysteinemia may have important implications with regard to risk management. The substantial difference we found therefore merits further examination in a larger number of subjects than were available in this study. In contrast to the findings of a recent study,[2] we found no interactions between serum tHcy and other classic risk factors, but our study had limited power to do so.

Because the ORs for the three arterial territories did not differ significantly, we calculated a summary OR for cardiovascular disease by pooling all subjects with coronary, peripheral and/or cerebrovascular disease. We estimated a proportion of preventable cardiovascular disease of 10% for a distribution shift of 5 μmol/L serum tHcy level. A similar result was obtained in a recent meta-analysis:[1] 10% of the proportion of death caused by coronary heart disease was estimated to be attributable to hyperhomocysteinemia. The present study illustrates that although the OR of hyperhomocysteinemia for cardiovascular disease is relatively modest, hyperhomocysteinemia is an important risk factor because the frequency is high in the general population. An increased risk of cardiovascular disease has been observed if homocysteine levels exceed 14 μmol/L.[2-4] The prevalences of

hyperhomocysteinemia (>14.0 μmol/L) we found was 34% for men and 18% for women, which are somewhat higher than the 25% and 20% observed in a 67- to 74-year-old population of the Framingham Study (based on plasma tHcy).[10] Part of this difference could be related to the fact that levels of serum compared to plasma tHcy levels are slightly higher.[31]

A limitation of the present study is the absence of assessment of serum folate, which would have provided important information about the relation between serum tHcy and folate in the general population. However, additional measurements of serum folate would not have altered the conclusions of the present study with regard to the association between serum tHcy and cardiovascular disease, because the associations between hyperhomocysteinemia and cardiovascular disease exist regardless of the underlying cause of hyperhomocysteinemia.

Obviously, this cross-sectional study cannot resolve the temporal relation between homocysteine concentration and cardiovascular disease. However, there is evidence that the relation between tHcy and cardiovascular disease is causal because prospective studies have shown a positive association between hyperhomocysteinemia and cardiovascular disease.[3,4,32,33]

In conclusion, hyperhomocysteinemia is positively associated with cardiovascular disease in a 50- to 75-year-old general population, independent of classic risk factors. The magnitude of the relative risk of hyperhomocysteinemia is similar with regard to peripheral arterial, coronary artery and cerebrovascular disease. With regard to cardiovascular disease, hyperhomocysteinemia appeared to be a stronger risk factor in patients with NIDDM than in subjects with normal or impaired glucose tolerance.

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3

Hyperhomocysteinemia is not associated with isolated crural arterial occlusive disease
The Hoorn Study

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Abstract

Background Hyperhomocysteinemia is an independent risk factor for peripheral arterial disease (PAD). The localization of PAD is clinically relevant, because proximal (aortoiliac and femoropopliteal) disease is associated with a particularly poor overall prognosis, whereas isolated distal (i.e., crural) disease is associated with a better overall prognosis. Since it is not known whether the strength of the association between hyperhomocysteinemia and PAD differs according to the localization of the anatomical obstruction, we studied this in an age-, sex- and glucose-tolerance stratified random sample (N=631) of a 50- to 75-year-old general Caucasian population.

Methods Fasting serum total homocysteine (tHcy) was measured in all subjects. History of a peripheral arterial reconstruction was recorded. Aortoiliac, femoropopliteal and crural arterial obstructions were registered by means of Doppler flow velocity curves.

Results The median serum tHcy level was 12.2 μmol/L [interquartile range (IQR): 10.0 to 15.3] in men and 10.7 μmol/L (IQR: 9.0 to 13.3) in women. The prevalences of aortoiliac, femoropopliteal and crural obstructions were 2.1%, 2.7% and 11.9%, respectively. After adjustment for age, sex, systolic blood pressure, current smoking, serum cholesterol and diabetes mellitus, the odds ratios (95% confidence interval) per 5 μmol/L tHcy increment were 1.41 (1.05 to 1.89) for aortoiliac, 1.03 (0.70 to 1.52) for femoropopliteal and 0.82 (0.59 to 1.15) for crural obstructions. Finally, diabetes mellitus, HbA_{1c} and current smoking were significantly associated with crural and femoropopliteal disease, whereas systolic blood pressure was significantly associated with aortoiliac obstructions.

Conclusion We conclude that hyperhomocysteinemia is associated with aortoiliac but not with isolated crural arterial occlusive disease. This finding may have clinical relevance since aortoiliac occlusive disease is associated with a greatly increased overall mortality and hyperhomocysteinemia can effectively be lowered through an increased intake of B vitamins.

Introduction

Peripheral arterial disease (PAD) has two important clinical consequences: it is associated with an increased risk of overall mortality, presumably because it is a marker of generalized atherosclerosis, and it impairs local blood flow, which may result in ischemic symptoms, such as intermittent claudication and critical limb ischemia.[1-3]

PAD can occur from the aortoiliac to the crural territories. The localization of PAD is clinically relevant, because proximal (aortoiliac and femoropopliteal) disease is often accessible to local treatment, but is thought to be associated with a particularly poor overall prognosis, whereas isolated distal (i.e., crural) disease is often difficult to treat locally, but is associated with a better overall prognosis than is proximal arterial disease.[4]

It is therefore of interest to investigate whether risk factors for proximal vs distal PAD are different. In this regard, it is well-established that diabetes mellitus is more strongly associated with distal PAD, whereas smoking, hypertension and hypercholesterolemia are more closely associated with proximal PAD.[5-8]

Hyperhomocysteinemia is a novel risk factor for cardiovascular disease, which is independent of established risk factors such as smoking, hypercholesterolemia, diabetes mellitus and hypertension.[9-12] Although the mechanisms by which homocysteine promotes atherothrombosis are unknown, the epidemiologic evidence of the association of hyperhomocysteinemia with atherothrombotic disease is strong.[9,13] A recent meta-analysis[14] showed that treatment with 0.5 to 5.0 mg folic acid daily can lower serum total homocysteine (tHcy) levels by 15 to 40% within approximately six weeks. Studies which specifically investigated the relation between hyperhomocysteinemia and PAD are relatively scarce.[15-19] It is not known whether the strength of the association between hyperhomocysteinemia and PAD is similar for proximal and distal disease.

In order to further explore this issue, we compared the strength of the association between serum total homocysteine (tHcy) and other risk factors on the one hand and the level of peripheral arterial obstruction (i.e., aortoiliacal vs femoropopliteal vs crural), on the other hand in a 50- to 75-year-old general Caucasian population.

Methods

Design and study population

The Hoorn Study is a cross-sectional survey of glucose tolerance and other cardiovascular risk factors in a 50- to 75-year-old general Caucasian population conducted from 1989 to 1992. A random sample of all men and women aged 50 to 75 years was drawn from the municipal population registry office of Hoorn (The Netherlands); 2484 subjects participated (response rate 71%). An extensive peripheral arterial investigation (detailed below) was performed in an age-, sex- and glucose tolerance-stratified random subsample ($N=631$; response rate 89%).[20] The Hoorn Study was approved by the Ethical Review Committee of the University Hospital Vrije Universiteit. Informed consent was obtained from all participants.

The peripheral arterial history was obtained by means of a self-administered questionnaire and, if positive, accepted only when confirmed by written information of the participant's general practitioner. Flow velocity curves were recorded from the femoral, popliteal, posterior tibial and dorsalis pedis arteries by means of a 5 or 8 MHz bi-directional continuous wave Doppler connected to a real-time frequency analyzer. Tri- or biphasic curves indicate a normal arterial inflow to that level. Monophasic or absent curves are considered abnormal, signifying the presence of an obstruction of 50% or more proximal to the examination site.[21-23] An aortoiliac obstruction was defined as an abnormal Doppler flow velocity curve from the femoral, the popliteal and the three crural arteries, or having received a bifurcation prosthesis; a femoropopliteal obstruction as a normal flow velocity curve from the femoral artery in combination with abnormal curves from the popliteal artery and the crural arteries, or having received a femoropopliteal reconstruction; and a crural obstruction as normal curves from the femoral and the popliteal arteries in combination with an abnormal curve from one or more of the crural arteries. The absence of, or a monophasic Doppler flow velocity curve from only the peroneal artery in combination with normal curves from the other two crural arteries and an ankle brachial pressure index (ABPI) >0.9 in the same limb was considered a technical failure ($N=18$). The ABPI, an estimate of the overall severity of occlusive disease, was obtained by means of Doppler-assisted systolic blood pressure measurements taken from the brachial and the three crural arteries on both sides as previously described in more detail.[20]

Measurement of serum total homocysteine

Fasting blood samples were centrifuged within one hour following collection. Serum was stored at -20°C for 4 to 6 years. There is good evidence that serum tHcy levels in frozen samples are stable for 10 years or more.[24] Serum total (free plus protein bound) homocysteine level was measured by using tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by high-performance liquid chromatography with fluorescence detection.[25] The intra- and interassay coefficients are 2.1% and 5.1%.

Other cardiovascular risk factors

We measured fasting serum total cholesterol, HDL cholesterol and triglycerides by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany). The Friedewald formula was used to calculate the LDL cholesterol concentration, except in subjects with serum triglyceride levels >8.0 mmol/L ($N=3$).[26] Blood pressure was measured on the right arm of seated subjects, after at least 5 minutes of rest, using a random zero sphygmomanometer (Hawksley-Gelman Ltd., Lancing, Sussex, UK). The average of duplicate measurements on two occasions was used for analysis. Hypertension was defined as a blood pressure >=160 mmHg systolic and/or >=95 mmHg diastolic and/or the current use of antihypertensive medication. Normal glucose tolerance, impaired glucose tolerance (IGT) and diabetes mellitus were defined according to the WHO criteria (1985)[27] applied to the mean of two oral glucose tolerance tests, except in patients with drug-treated diabetes mellitus, as previously described in detail.[20] Glycated hemoglobin (HbA_{1c}) was determined by an ion-exchange high-performance liquid chromatography, using a Modular Diabetes Monitoring System (Bio-Rad, Veenendaal, the Netherlands). Immunospecific insulin was measured in serum by a double-antibody radioimmunoassay (lot SP21, Linco Research, St. Louis, U.S.A.). The interassay coefficient of variation was 6%. The lower limit of sensitivity was 12 pmol/L. Subjects were classified as either nonsmoker or current smoker. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Waist and hip circumferences were measured and the waist-hip ratio (WHR) was calculated as described elsewhere.[20] Central adiposity was defined as a WHR >1.00 in men and >0.90 in women. All laboratory and vascular measurements were carried out

by technicians unaware of the subjects' history of peripheral arterial disease and glucose tolerance status.

Statistical analysis

Variables are presented as mean ± standard deviation (SD), number (percentage of the total) or, in case of skewed distribution, median and interquartile range (IQR). The most proximal obstruction of either limb was used for statistical analysis. We performed logistic regression analyses to study the associations of serum tHcy and of other cardiovascular risk factors with each level of PAD. As the dependent variable we took aortoiliac, femoropoliteal or crural obstruction and contrasted each group with subjects without any PAD. We chose this procedure because, in subjects with aortoiliac or femoropopliteal disease, more distal (i.e. femoropopliteal or crural, respectively) disease cannot be excluded. For tHcy, we calculated odds ratios (ORs) and 95% confidence intervals (CIs) per 5 μmol/L (about 1 SD) increment of serum tHcy. We used multiple logistic regression analysis, after adjustment for age and sex, to investigate the associations between level of PAD and systolic blood pressure, serum total cholesterol, current smoking, diabetes mellitus and tHcy. We also tested models that also included triglycerides, HDL and LDL cholesterol, glucose, HbA_{1c}, insulin, BMI and/or WHR. The small number of cases with aortoiliac or femoropopliteal obstruction did not allow for extensive multivariate adjustment, nor for analyses of hypertension as dichotomous variable. We repeated all analyses without taking peripheral arterial reconstructions into account. All reported *P*-values are two-tailed. All analyses were performed with SPSS for Windows 7.5.2.

Results

Table 1 shows the characteristics of the study population. Ten subjects had previously had a peripheral arterial reconstruction: three had had an aortoiliacal bifurcation prosthesis and seven a femoropopliteal bypass. Two subjects had previously undergone a limb amputation, so data apply to only one leg. One of these subjects had had a traumatic limb amputation, which was not considered as peripheral arterial disease. Table 2 shows the prevalences of peripheral arterial disease according to localization of PAD. After exclusion of subjects who had undergone reconstructive surgery, an ABPI <0.5 (a proxy measure of multi-level disease[28]) was present in 50% (5 of 10) of the subjects with an aortoiliac obstruction and 28.6% (4 of 14)

Table 1. Characteristics of the study population

N	631	
Men *%*	48	
Age *years*	64.3	(7.2)
Body mass index kg/m^2	27.3	(4.0)
Waist-hip ratio	0.92	(0.09)
Current smoker *%*	29	
Systolic blood pressure *mmHg*	139	(19)
Diastolic blood pressure *mmHg*	83	(10)
Hypertension *%*	39.1	
Impaired glucose tolerance *%*	26.9	
Diabetes mellitus *%*	27.4	
HbA_{1c} *% of hemoglobin*	5.9	(1.3)
Fasting insulin *pmol*/L	84	(63-119)*
Total cholesterol *mmol/L*	6.6	(1.2)
HDL cholesterol *mmol/L*	1.3	(0.4)
LDL cholesterol *mmol/L*	4.5	(1.1)
Triglycerides *mmol/L*	1.6	(1.1-2.2)*
Total homocysteine μ*mol/L*	11.4	(9.3-14.1)*

Data are presented as:
mean (SD), or as
* median (interquartile range).

Table 2. Odds ratios (95% confidence intervals) for proximal and distal peripheral arterial disease per 5 μmol/L increase of serum total homocysteine

	Cases	Prevalence (%) (95% CI)	Crude OR	Age- & sex-adjusted OR	Multivariate* adjusted OR
Any obstruction	105	16.6 (13.7 - 19.5)	1.08 (0.92 - 1.27)	1.04 (0.87 - 1.24)	1.03 (0.85 - 1.23)
Aortoiliac obstruction†	13	2.1 (1.1 - 3.5)	1.37¶ (1.11 - 1.68)	1.36¶ (1.11 - 1.68)	1.41§ (1.05 - 1.89)
Femoropopliteal obstruction‡	17	2.7 (1.6 - 4.3)	1.11 (0.82 - 1.50)	1.06 (0.71 - 1.57)	1.03 (0.70 - 1.52)
Crural obstruction	75	11.9 (9.4 - 14.4)	0.87 (0.67 - 1.14)	0.81 (0.59 - 1.11)	0.82 (0.59 - 1.15)

Each level of peripheral arterial obstruction was contrasted with subjects without any obstruction.

* Adjusted for age, sex, diabetes mellitus (yes/no), systolic blood pressure, current smoking (yes/no) and serum cholesterol.
† Aortoiliac obstruction (*N*=10) and/or bifurcation prosthesis (*N*=3).
‡ Femoropopliteal obstruction (*N*=14) and/or femoropopliteal reconstruction (*N*=7).
§ P<0.05
¶ P<0.01
OR Odds ratio
CI Confidence interval

of the subjects with a femoropopliteal obstruction. The small number of subjects did not allow further analysis of the determinants of proximal PAD as defined here, i.e. without or with multi-level disease, versus the determinants of isolated proximal PAD, i.e. proximal PAD without multi-level disease.

The median serum tHcy level was 12.2 μmol/L (IQR: 10.0 to 15.3) in men and 10.7 μmol/L (IQR: 9.0 to 13.3) in women. We found a positive association between the serum tHcy level and more proximally located lower limb arterial obstruction (Table 2). The ORs did not change materially when subjects with a history of vascular reconstruction were excluded (data not shown).

After adjustment for age and sex, we found that diabetes mellitus, HbA_{1c} and current smoking were significantly associated with crural and femoropopliteal obstruction, whereas systolic blood pressure was significantly associated with aortoiliac obstruction (Table 3). Additional adjustment for use of antihypertensives (yes/no) did not materially change the association between blood pressure and aortoiliac obstruction OR (95% CI) 1.26 (1.10 to 1.46). Total cholesterol was more strongly associated with aortoiliac than distal peripheral obstruction (Table 3), although the association with aortoiliac obstruction was not statistically significant ($P=0.3$). After adjustment for HbA_{1c}, the strength of the association between diabetes and aortoiliac disease was not attenuated, whereas that with femoropopliteal obstruction was. IGT was not significantly associated with any obstruction (data not shown). We therefore pooled subjects with normal and impaired glucose tolerance in all analyses. HDL cholesterol, triglycerides, fasting insulin, BMI and WHR were not significantly associated with a specific level of PAD and adjusting for each of theses variables did not materially affect the association between diabetes and crural, femoropopliteal or aortoiliac obstruction (data not shown).

We considered subjects with a monophasic Doppler flow velocity curve from only the peroneal artery and an ABPI >0.9 in the same limb not to have crural disease ($N=18$; see methods). Categorization of these subjects as having crural disease did not materially affect the results (data not shown).

Finally, the small number of premenopausal women ($N=13$; defined as those women who had had a menstruation within the last year) was too small to allow for a sub-analysis of the association between tHcy and localization of PAD before and after menopause. Exclusion of premenopausal women did not materially affect the results (data not shown).

Table 3. Odds ratios (95% confidence intervals) for aortoiliac, femoropopliteal and crural arterial obstructions of selected cardiovascular risk factors

	Aortoiliac obstruction† *N*=13	Femoropopliteal obstruction‡ *N*=17	Crural obstruction *N*=75	
	Age- & sex-adjusted OR	Age- & sex-adjusted OR	Age- & sex-adjusted OR	Multivariate* adjusted OR
Diabetes mellitus *yes/no*	2.77 (0.90 - 8.51)	4.33§ (1.58 - 11.87)	1.98§ (1.19 - 3.28)	1.11 (0.54 - 2.25)
HbA_{1c} *per %*	1.22 (0.80 - 1.84)	1.65¶ (1.26 - 2.16)	1.42¶ (1.21 - 1.67)	1.42¶ (1.14 - 1.76)
Current smoking *yes/no*	1.34 (0.39 - 4.53)	4.19¶ (1.53 - 11.51)	2.49¶ (1.47 - 4.21)	2.65¶ (1.54 - 4.57)
Total cholesterol *per mmol/L*	1.28 (0.83 - 1.99)	1.15 (0.77 - 1.71)	1.00 (0.82 - 1.23)	0.95 (0.77 - 1.17)
Systolic blood pressure *per 5 mmHg*	1.29¶ (1.13 - 1.46)	1.09 (0.97 - 1.24)	0.94 (0.88 - 1.01)	0.93 (0.87 - 1.00)

After adjustment for age, sex and HbA_{1c}, the ORs (95% CIs) for the association between diabetes mellitus and aortoiliac and femoropopliteal obstruction were 2.91 (0.77-11.00) and 2.06 (0.55-7.63).

* Adjusted for age, sex and diabetes mellitus (yes/no), current smoking (yes/no), and HbA1c if this was not the variable under consideration.
† Aortoiliac obstruction and/or bifurcation prosthesis.
‡ Femoropopliteal obstruction and/or femoropopliteal reconstruction.
§ P<0.01
¶ P<0.001

Discussion

There are two main findings of this study. First, hyperhomocysteinemia is more strongly associated with proximal than with distal peripheral arterial disease. Second, diabetes, level of glycemia (estimated from HbA_{1c}) and current smoking were associated with crural and femoropopliteal disease, whereas systolic blood pressure was associated with aortoiliac obstructions.

We previously reported[19] that hyperhomocysteinemia is associated with severe peripheral arterial disease. The current study brings localization of peripheral arterial disease into focus rather than severity and shows that hyperhomocysteinemia is more strongly associated with aortoiliac than with distal peripheral arterial obstructions. Although there are no previous studies that have addressed this issue, several observations from studies in which serum total homocysteine (tHcy) was not measured may support this finding. First, premature peripheral atherosclerosis (i.e. disease affecting those under 50) is often located in the aortoiliac bed, i.e. proximally,[5] and is known to be associated with hyperhomocysteinemia.[15,16,18] Second, aortoiliac obstruction

without distal occlusions occurs twice as often in (premature) postmenopausal as compared with premenopausal women,[29,30] and postmenopausal women have higher serum tHcy levels than premenopausal women.[31] Several pathophysiological mechanisms have been proposed through which hyperhomocysteinemia may induce atherosclerosis. Hyperhomocysteinemia may induce dysfunction of the vascular endothelium,[32-34] a critical initiating event in the development of atherosclerosis. In addition, hyperhomocysteinemia may stimulate proliferation of vascular smooth muscle cells and elastinolytic processes in the arterial wall.[34-37] Atherosclerosis occurs at definite sites of predilection within the vascular tree, such as bifurcations, angulations or fixed points. The abdominal aorta is at high risk of atherosclerosis due to the thickness of the avascular zone of the arterial wall, which increases the risk of ischemia. However, it is not known how hyperhomocysteinemia affects the mechanisms leading to aortoiliac, as opposed to more distal, atherosclerosis, and further studies are needed to clarify this.

This study confirms that diabetes is associated with distal peripheral arterial disease.[4,8] After adjustment for HbA_{1c} the association between diabetes and crural or femoropopliteal obstruction disappeared. Adjustment for other factors of the insulin resistance syndrome did not affect the associations. This result may indicate that hyperglycemia itself is more important with regard to the development of crural obstruction than other factors of the insulin resistance syndrome.[38] Finally, the present study confirms that systolic blood pressure is more closely associated with proximal arterial disease, and, in addition, suggests that systolic blood pressure is probably not a risk factor for crural disease.

We have to consider several limitations of the present study. First, we relied on a non-invasive technique to assess peripheral arterial disease which detects the most proximal obstruction rather than the degree or extent of the obstruction. Although angiography is not suitable for a population-based study, duplex scanning would have provided more precise information on the extent and severity of obstruction. As a consequence, we could not distinguish whether hyperhomocysteinemia is especially associated with obstructions confined to the aortoiliac region or with multi-level disease.[15] The small number of subjects with an ABPI <0.5, a proxy of multi-level disease,[28] did not allow further analysis of this issue. Second, since the present study is cross-sectional, we cannot rule out that hyperhomocysteinemia is a consequence of the disease rather than a cause. However, there is increasing evidence that the relation between tHcy and cardiovascular disease is causal.[9,11,12,32-37] Finally, the small number of subjects with

a proximal arterial obstruction warrants careful interpretation of the results of the present study. Specifically, we cannot exclude that diabetes, smoking and hypercholesterolemia are also important risk factors for proximal disease.[4,6,7,38]

Proximal aortoiliac disease is associated with a particularly poor overall prognosis. Therefore, the results of the present study may have clinical relevance because hyperhomocysteinemia can effectively be lowered through an increased intake of B vitamins, particularly folate.[14]

In conclusion, hyperhomocysteinemia and systolic blood pressure are more strongly associated with proximal than with distal peripheral arterial disease, whereas diabetes and level of glycemia are associated with distal and possibly with proximal peripheral arterial disease.

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4

Hyperhomocysteinemia increases risk of death, especially in type 2 diabetes: 5-year follow-up of the Hoorn Study

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Abstract

Background A high serum total homocysteine (tHcy) level is a risk factor for mortality, but the strength of the relation in type 2 diabetic patients is unknown. A recent cross-sectional study suggests that the association between tHcy and cardiovascular disease is stronger in diabetic than in non-diabetic subjects. We therefore prospectively investigated the combined effect of hyperhomocysteinemia and type 2 diabetes on mortality.

Methods Between October 1, 1989 and December 31, 1991 serum was saved from 2484 men and women, aged 50 to 75 years, randomly selected from the town of Hoorn, in the Netherlands. Fasting serum tHcy concentration was measured in 171 subjects who died (cases; 76 of cardiovascular disease) and in a stratified random sample of 640 survivors (controls). Mortality risks were calculated over 5 years of follow-up by means of logistic regression. Hyperhomocysteinemia was defined as serum tHcy >14 μmol/L. Causes of death were coded according to the International Classification of Diseases (ICD-9).

Results The prevalence of hyperhomocysteinemia was 25.8%. After adjustment for major cardiovascular risk factors, serum albumin and HbA_{1c}, the odds ratio (95% CI) for 5-year mortality was 1.56 (1.07 to 2.30) for hyperhomocysteinemia and 1.26 (1.02 to 1.55) per 5 μmol/L (about 1 SD) increment of serum tHcy. The odds ratio for 5-year mortality for hyperhomocysteinemia was 1.34 (0.87 to 2.06) in non-diabetic and 2.51 (1.07 to 5.91) in diabetic subjects (P=0.08 for interaction).

Conclusion Hyperhomocysteinemia, independently of other major risk factors, is related to 5-year mortality and appears to be a stronger (1.9-fold) risk factor for mortality in type 2 diabetic patients than in non-diabetic subjects. The effects on mortality risk of homocysteine-lowering treatment have yet to be assessed, but the present study suggests that this may be especially effective in type 2 diabetes.

Introduction

Cardiovascular disease is the major cause of mortality in diabetic and non-diabetic subjects. The overall and cardiovascular mortality rates are two to four times higher in type 2 diabetic patients than in non-diabetic subjects.[1-5] Type 2 diabetes is known to be associated with several other cardiovascular risk factors, including dyslipidemia and hypertension, but these do not fully explain the excess mortality in type 2 diabetes. Therefore, increased risk must be due, at least in part, to diabetes itself, poor metabolic control, or other factors.

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease that is independent of major risk factors such as diabetes, hypertension, hypercholesterolemia and smoking.[6-8] The prevalence estimates of hyperhomocysteinemia (>14 μmol/L) vary between 5% and 30% in the general population.[9-12] Although the mechanisms by which homocysteine promotes atherothrombosis are unknown, the epidemiologic evidence for the association of hyperhomocysteinemia with atherothrombotic disease is strong.[6,7,13] A recent meta-analysis[14] showed that treatment with 0.5 to 5.0 mg folic acid daily can lower serum total homocysteine (tHcy) by 15 to 40% within approximately six weeks. In addition, it has been estimated that lowering tHcy by 5 μmol/L (about 1 SD) may reduce risk of cardiovascular death by about 10%.[7] Taken together, hyperhomocysteinemia may be an important modifiable risk factor, although this has to be confirmed in randomized studies of homocysteine-lowering treatment.

In a cross-sectional analysis, hyperhomocysteinemia appeared to be a stronger risk factor for cardiovascular disease in type 2 diabetic than in non-diabetic subjects.[12] Such an interaction between hyperhomocysteinemia and type 2 diabetes with regard to cardiovascular risk may be clinically important, as it implies that homocysteine-lowering treatment may be especially effective in type 2 diabetes. In view of these considerations, we investigated the combined effect of hyperhomocysteinemia and diabetes with respect to 5-year risk of mortality in a population-based study.

Methods

Design and study population

The Hoorn Study is a prospective study of glucose tolerance and other cardiovascular risk factors in a 50- to 75-year-old general Caucasian population. The baseline examination was conducted from October 1, 1989 until

December 31, 1991, as previously described in detail.[15] Briefly, a random sample of all men and women aged 50 to 75 years was drawn from the municipal population registry office of Hoorn, in the Netherlands; 2484 subjects were enrolled in this cohort (response rate 71%). All subjects, except previously diagnosed diabetic subjects treated with oral glucose-lowering agents or insulin, underwent a 75 g oral glucose tolerance test (OGTT) and were classified according to the WHO (1985) criteria.[16] A second OGTT (participation rate 93%) was performed, for reasons of efficiency, in a random subsample (N=1122) stratified by 2-hour glucose values of the first test, age and sex. Finally, from this subsample an age-, sex-, and glucose tolerance-stratified random subsample (N=715), the 'subcohort', was drawn. Glucose tolerance was divided into three categories on the basis of the mean of the two OGTTs: normal glucose tolerance (NGT, N=334), impaired glucose tolerance (IGT, N=197) and type 2 diabetes mellitus (N=184).

A case-control study nested within the cohort was performed. The survivors of the subcohort (as defined above) served as controls. Every case (a subject who died within five years of follow-up) of the entire cohort was ascertained and selected for the present study. Information on subjects' vital status on January 1, 1997 was collected from the mortality registry of the municipality of Hoorn. Of 137 subjects who moved out of town, information on vital status was obtained from the new local municipalities. For each subject, we determined whether or not death had occurred during the first five years of follow-up. Causes of death were extracted from medical records of the general practitioner and the hospital of Hoorn, verified by a physician and classified according to the 9th revision of the International Classification of Diseases (ICD).[17] Death from cardiovascular disease was defined by ICD codes 390-459.

During the 5-year follow-up, 172 participants died, 75 of whom were included in the subcohort (N=715). Of one of the subjects who died, no serum was available for the measurement of tHcy. Thus analyses were performed on 811 subjects. The cause of death could be retrieved in 93.6% (160 out of 171); 76 of 160 (47.5%) died of cardiovascular disease, of whom 34 belonged to the subcohort. The Hoorn Study was approved by the Ethical Review Committee of the University Hospital Vrije Universiteit Amsterdam. Informed consent was obtained from all participants.

Measurement of serum total homocysteine

Fasting blood samples were centrifuged within one hour following collection. Serum was stored at –20°C for 4 to 7 years. There is good evidence that serum total homocysteine (tHcy) levels in frozen samples are stable for 10 years or more.[18] Serum total (free plus protein bound) homocysteine level was measured by using tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by HPLC with fluorescence detection.[19] The intra- and interassay coefficients are 2.1% and 5.1%.

Other variables

Subjects were classified as current smokers or nonsmokers of cigarettes. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Blood pressure was measured as the mean of, in total, four measurements, performed on two different occasions, using a random-zero sphygmomanometer under standardized conditions. Hypertension was defined as a blood pressure >=160 mmHg systolic and/or >=95 mmHg diastolic and/or the current use of antihypertensive medication. Fasting and 2-hour post-load venous plasma glucose levels were measured with a glucose dehydrogenase method (Merck, Darmstadt, Germany). Fasting serum total cholesterol, HDL cholesterol and triglycerides were measured by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany). Hypercholesterolemia was defined as total cholesterol >=6.5 mmol/L and/or the current use of cholesterol-lowering medication. Dyslipidemia was defined as levels of triglycerides >2.3 mmol/L and/or levels of HDL cholesterol <1.0 mmol/L in men and <1.1 mmol/L in women.[20] Serum albumin was assessed using the bromcresol purple method. Hypoalbuminemia was defined as albumin <=34 g/L.[21] All laboratory measurements were carried out in a blinded fashion with respect to mortality, glucose tolerance status, and other clinical data.

Statistical analysis

Data are presented as mean (SD) or median (interquartile range) unless stated otherwise. Prevalence of hyperhomocysteinemia, defined as serum tHcy level >14 μmol/L,[11] in the entire cohort was back-calculated by means of direct standardization as described in detail elsewhere.[12] Association of fasting glucose and HbA_{1c} with serum tHcy level (logarithmically transformed because of the skewed distribution) was studied by calculation of

Pearson correlation coefficients. We assessed the relation between tHcy and 5-year overall mortality in the nested case-control study with logistic regression analyses. We chose two different approaches to investigate the nature of the relation between tHcy and mortality, because it is unclear whether this relation is linear or has a certain threshold. We calculated odds ratios plus 95% confidence intervals (CI) for serum tHcy both as a continuous variable, expressed per 5 μmol/L (about 1 SD) increment of serum tHcy, and as a categorical variable (to allow for a non-linear dose-response relation and reduce the influence of outliers). For the analyses with serum tHcy as a categorical variable, we calculated odds ratios for tHcy divided in two (>14 μmol/L versus <=14 μmol/L) and in four categories (<=9.0 μmol/L, 9.1 to 14.0 μmol/L, 14.1 to 19.0 μmol/L and >19.0 μmol/L). To test for trend, the four categories were entered in the model as an ordinal variable. Odds ratios of mortality were adjusted for the stratifying variables (i.e., age, sex and glucose tolerance) and potentially confounding major cardiovascular risk factors (i.e., hypertension, hypercholesterolemia and current smoking[10,20]). We also tested models that included dyslipidemia, HbA_{1c} and BMI. Possible interactions between tHcy and cardiovascular risk factors were assessed in stratified analyses and with interaction terms.

To assess whether the observations were distorted by underlying disease that might cause both high values of serum tHcy and increased mortality,[22] we performed two additional analyses. First, we adjusted for serum albumin, a putative marker of health and nutrition status.[23] Second, we adjusted for the presence of cardiovascular disease at baseline, as defined elsewhere,[12] although the latter analysis might obscure a true effect because cardiovascular disease may well be an intermediate factor in the causal pathway linking tHcy to mortality.[24]

Finally, we assessed the relation between tHcy and 5-year cardiovascular mortality. This analysis was restricted to the subcohort, because it required the Cox proportional hazards model. All analyses were performed with SPSS for Windows 7.5.2. A 95% CI not including 1.0 was considered to indicate statistical significance.

Results

The baseline characteristics of cases and controls are presented in Table 1. Median (range) serum tHcy levels were higher in cases than in controls [12.9 μmol/L (4.0 - 56.8) versus 11.5 μmol/L (4.9 - 77.5)]. The back-calculated prevalence of hyperhomocysteinemia (>14 μmol/L) in the cohort was 25.8%. Of all type 2 diabetic subjects, 115 (62.5%) were newly

Table 1. Baseline characteristics of the study population

	Cases†	Controls‡
N	171	640
Men* *%*	58.5	46.4
Age* *years*	66.6 (7.1)	63.9 (7.0)
Body mass index *kg/m²*	26.7 (4.2)	27.2 (4.0)
Cigarette smokers, *current %*	41.4	27.9
Systolic blood pressure *mmHg*	141 (23)	139 (19)
Diastolic blood pressure *mmHg*	82 (12)	82 (10)
Hypertension§ *%*	50.3	37.3
Impaired glucose tolerance* *%*	10.5	28.1
Diabetes mellitus* *%*	22.2	22.8
Plasma fasting glucose *mmol/L*	6.6 (2.9)	6.5 (2.2)
HbA_{1c} *% of hemoglobin*	6.0 (1.4)	5.8 (1.2)
Serum total cholesterol *mmol/L*	6.7 (1.1)	6.7 (1.2)
Hypercholesterolemia¶ *%*	61.4	54.1
Serum HDL cholesterol *mmol/L*	1.2 (0.4)	1.3 (0.4)
Total: HDL cholesterol ratio	5.9 (1.8)	5.5 (1.7)
Serum triglycerides *mmol/L*	1.6 (1.2-2.2)	1.5 (1.1-2.1)
Dyslipidemia** *%*	42.7	33.0
Serum albumin *g/L*	38 (3.2)	39 (2.9)
Serum total homocysteine *μmol/L*	12.9 (9.9-16.2)	11.5 (9.4-14.1)

Data are presented as mean (SD), percentage of the total or median (interquartile range).

* Stratifying variable.

† Cases are subjects who died during the five years of follow-up.

‡ Controls are survivors taken from an age-, sex- and glucose tolerance-stratified random sample of the cohort (see Methods).

§ Hypertension was defined as a blood pressure >=160 mmHg systolic and/or >=95 mmHg diastolic and/or the current use of antihypertensive medication.

¶ Hypercholesterolemia was defined as total cholesterol >=6.5 mmol/L and/or the current use of cholesterol-lowering medication.

** Dyslipidemia was defined as levels of triglycerides >2.3 mmol/L and/or levels of HDL cholesterol <1.0 mmol/L in men and <1.1 mmol/L in women.

diagnosed and 69 (37.5%) were known with diabetes and were treated with glucose-lowering agents; 16 (8.7%) with insulin, 52 (28.3%) with sulfonylureas, and 3 (1.6%) with metformin (of whom 2 also used sulfonylureas). The median known duration of diabetes of subjects in whom type 2 diabetes had previously been diagnosed was 6.4 (interquartile range: 2.7 to 12.0) years. Serum tHcy levels did not correlate with fasting glucose (r=0.001; P=1.0) or HbA_{1c} (r=-0.03; P=0.4).

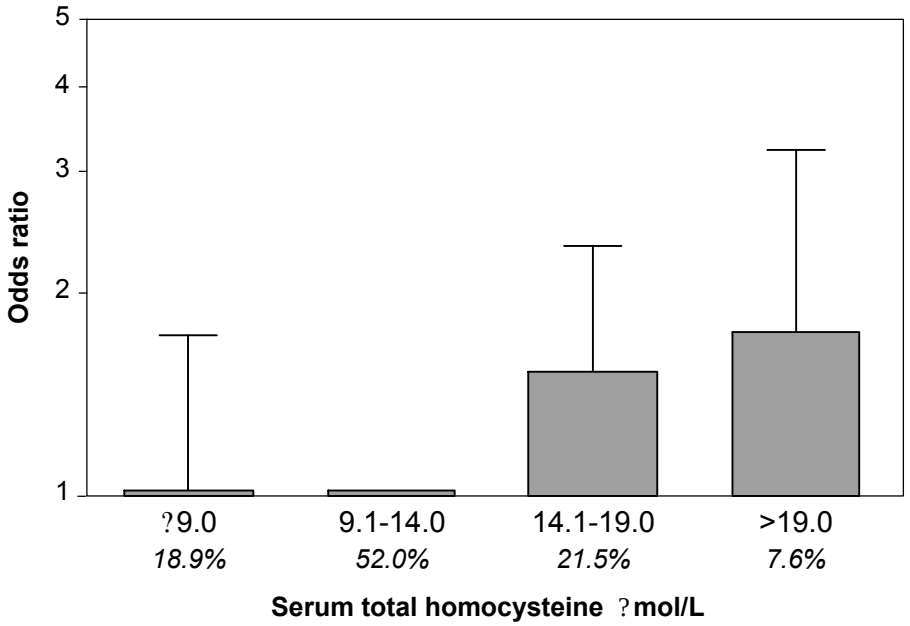

Figure 1. Odds ratios for 5-year overall mortality according to serum total homocysteine level adjusted for age, sex, diabetes, hypertension, hypercholesterolemia, current smoking, HbA_{1c} and serum albumin.
The reference category was serum total homocysteine values 9.1 to 14.0 μmol/L. The percentages of the population under study for each serum total homocysteine range are presented. Error bars represent the upper half of the 95% confidence intervals. *P*=0.04 for trend.

Overall mortality

In the entire cohort, the 5-year mortality risk was 5.7% in subjects with NGT, 7.1% in subjects with IGT and 18.5% in subjects with diabetes; it was 5.5% in subjects with serum tHcy <=14 μmol/L and 10.8% in subjects with serum tHcy >14 μmol/L.

Risk of 5-year overall mortality increased markedly above a serum tHcy level of 14 μmol/L (Figure 1), which may suggest the presence of a threshold. Table 2 shows the odds ratios of overall mortality in the presence versus the absence of other major cardiovascular risk factors. Additional adjustment for dyslipidemia or BMI did not attenuate the strength of the association between serum tHcy and mortality, nor did additional adjustment for serum albumin (Table 2). Eight outliers with serum tHcy levels >35 μmol/L markedly influenced the analysis if homocysteine was entered as a continuous variable (Table 2). Mortality risk for these 8 subjects was 12.5% (95% CI: 2.0 to 47.0%). The wide confidence interval indicates that in the present study, due to the limited number of subjects with high serum tHcy values, we can calculate the odds ratio only with fair precision up to a

Table 2. Odds ratios (95% confidence intervals) for 5-year overall mortality

Risk factors	Adjusted for age, sex and diabetes	* Adjusted for age, sex, diabetes and other risk factors
Hyperhomocysteinemia (>14 vs <=14 µmol/L)	1.58 (1.09 - 2.28)†	1.56 (1.07 - 2.30)
Total homocysteine (per category increment)‡	1.31 (1.06 - 1.63)	1.27 (1.02 - 1.59)
Total homocysteine (per 5 µmol/L increment)§	1.31 (1.06 - 1.60)	1.26 (1.02 - 1.55)
Hypertension (yes/no)	1.60 (1.12 - 2.28)	1.58 (1.08 - 2.29)
Current smoking (yes/no)	2.01 (1.39 - 2.90)	1.66 (1.13 - 2.45)
Hypercholesterolemia (yes/no)¶	1.49 (1.04 - 2.13)	1.45 (1.00 - 2.11)
Serum albumin (per 2.5 g/L increment)	0.72 (0.62 - 0.84)	0.73 (0.63 - 0.86)
HbA_{1c} (per % increment)	1.24 (1.05 - 1.48)	1.17 (0.98 - 1.40)

* Adjusted for age, sex, diabetes and the other five risk factors mentioned in this table. When these analyses were adjusted for homocysteine, homocysteine was entered as a 4-category variable in the models.
† After additional adjustment for dyslipidemia: 1.58 (1.09 to 2.29), or for BMI: 1.54 (1.06 to 2.23).
‡ Serum total homocysteine was divided in four categories (see Methods).
§ After exclusion of 8 outliers (1 case and 7 controls with serum total homocysteine >35 µmol/L); if outliers were included the odds ratios were 1.10 (0.96 - 1.25) and 1.10 (0.95 - 1.26).
¶ If hypercholesterolemia was replaced by total:HDL cholesterol ratio, the odds ratios were 1.18 (1.07 to1.31) and 1.12 (1.01 to 1.24).

serum tHcy level of approximately 25 μmol/L. Therefore, excluding the 8 outliers from the analysis with tHcy as a continuous variable appears reasonable. There was a graded, inverse, relation between serum albumin and mortality which was not altered by adjustment for potential confounders (Table 2). Subjects with hypoalbuminemia had a 2.2-fold (95% CI 1.2 to 3.9) greater mortality risk compared to subjects with serum albumin levels higher than 34 g/L.

We evaluated possible interaction (effect modification) and did not observe substantial differences among the strata of the following risk factors: male sex, hypertension, hypercholesterolemia and current smoking (data not shown). However, after stratification by diabetes and adjustment for age, sex, hypertension, current smoking, hypercholesterolemia and serum albumin, the odds ratio of 5-year mortality associated with hyperhomocysteinemia (>14.0 μmol/L) was 1.34 (0.87 to 2.06) in non-diabetic and 2.51 (1.07 to 5.91) in diabetic subjects (P=0.08 for interaction; Figures 2 and 3). This indicates that hyperhomocysteinemia is a stronger (1.9-fold, 95% CI 0.7 to 4.9) risk factor for mortality in diabetic than in non-diabetic subjects. Per 5 μmol/L increment of serum tHcy the odds ratio was 1.17 (0.92 to 1.50) in non-diabetic and 1.60 (1.02 to 2.51) in diabetic subjects.

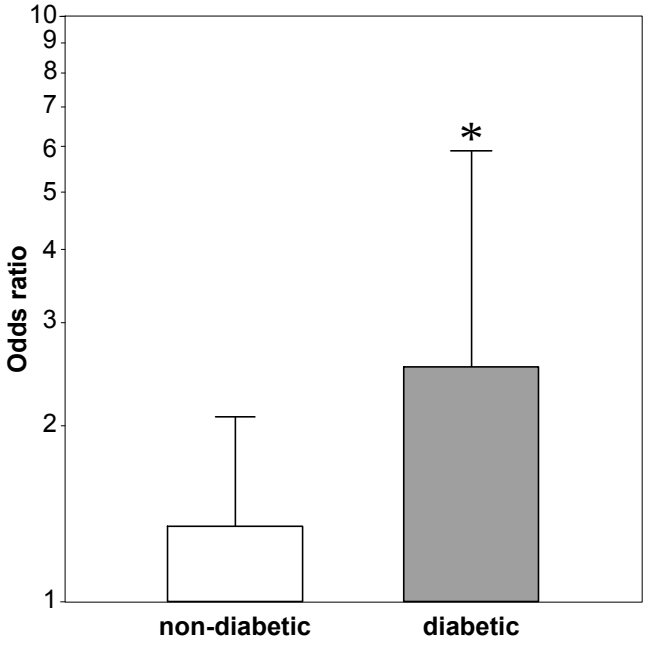

Figure 2. Odds ratios for 5-year overall mortality associated with hyperhomocysteinemia (>14 μmol/L) after stratification by diabetes (yes/no).
The error bars represent the upper half of the 95% confidence intervals.
Odds ratios are adjusted for age, sex, hypertension, hypercholesterolemia, current smoking and serum albumin. * *P*<0.05; *P*=0.08 for interaction.

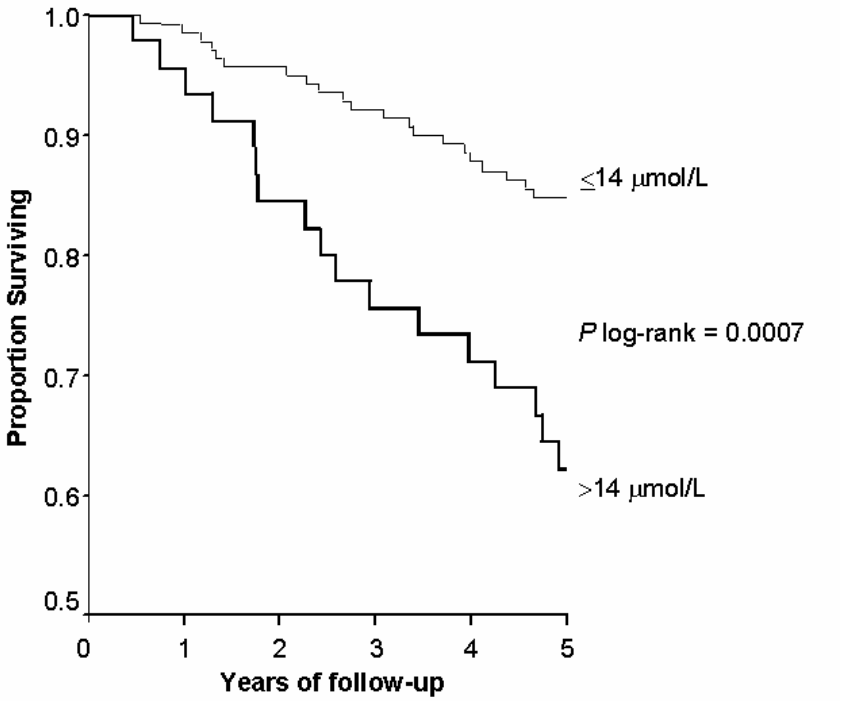

Figure 3. This figure illustrates estimated survival among type 2 diabetic subjects in the subcohort (see Methods), according to presence of hyperhomocysteinemia (>14 µmol/L, yes/no). Survival was estimated with Kaplan-Meier product-limit method and compared with the log-rank test.

(Subjects with NGT and IGT were pooled because the odds ratio of 5-year mortality associated with hyperhomocysteinemia did not differ substantially between these categories and the odds ratio remained similar if NGT and IGT were pooled; data not shown.) An additional analysis revealed that among diabetic subjects with hyperhomocysteinemia, those with known diabetes had the highest odds ratio of mortality. After adjustment for age and sex, the odds ratio of 5-year mortality associated with hyperhomocysteinemia was 2.58 (0.90 to 7.40) for subjects with newly diagnosed diabetes and 3.18 (0.74 to 13.74) for subjects with known diabetes. This interaction showed a significant trend ($P=0.04$): the odds ratio increased gradually over the three subgroups: non-diabetic, newly diagnosed diabetic and known diabetic subjects. Finally, in a stratified analysis and after additional adjustment for the presence of cardiovascular disease at baseline, we again found interaction (data not shown).

Cardiovascular mortality

After adjustment for the stratifying variables, the hazard ratio (95% CI) of cardiovascular mortality was 1.65 (0.81 to 3.31) for hyperhomocysteinemia, 1.58 (1.04 to 2.42) per category increment of serum tHcy and 1.55 (1.08 to 2.23) per 5 μmol/L increment of serum tHcy. After additional adjustment for hypertension, hypercholesterolemia and current smoking, these hazard ratios were 1.60 (0.65 to 3.01), 1.51 (0.98 to 2.32) and 1.45 (1.01 to 2.08), respectively. Due to the limited number of cases in the subcohort, we could not investigate the issue of interaction of hyperhomocysteinemia and diabetes with regard to cardiovascular mortality.

Discussion

This prospective population-based study shows that hyperhomocysteinemia is a risk factor for overall mortality in type 2 diabetic patients, independently of major cardiovascular risk factors and serum albumin, a putative general marker of health. Moreover, hyperhomocysteinemia appeared to be a stronger (about 2-fold) risk factor for mortality in diabetic than in non-diabetic subjects. We found a clear dose-response relation between serum tHcy and mortality for tHcy levels above 14 μmol/L. For each 5 μmol/L (about 1 SD) increment of serum tHcy, the risk of 5-year mortality rose by 17% in the non-diabetic and by 60% in the diabetic subjects.

There are few prospective studies that have investigated the relation between tHcy and risk of cardiovascular disease. Most,[25-31] but not all,[22,32,33] found a positive relation. None of the previous studies, however, investigated the possibility of interaction between hyperhomocysteinemia and diabetes with regard to risk of mortality. The design of the present study, with a high prevalence and an accurate diagnosis of type 2 diabetes, provided an opportunity to do so. Hyperhomocysteinemia was a stronger risk factor for mortality in patients with type 2 diabetes (odds ratio 2.51) than in non-diabetic subjects (odds ratio 1.34), i.e. there was evidence for interaction. An interaction of hyperhomocysteinemia with diabetes is biologically plausible. High homocysteine levels may exert an atherothrombotic effect through increasing oxidative stress, which may induce endothelial dysfunction.[13,34] Homocysteine can also affect the properties of the extracellular matrix and increase smooth muscle cell proliferation.[13] Oxidative stress is thought to be increased in type 2 diabetes,[35] and matrix alterations are a prominent feature of diabetes in general, both of which might make diabetes patients more susceptible to the adverse affect of hyperhomocysteinemia. The interaction with hyperhomocysteinemia observed in the present study, if confirmed, may have important implications with regard to risk management in type 2 diabetes.

Little is known about the impact of diabetes *per se* on tHcy metabolism.[12] In the present study we found no relation between tHcy and fasting glucose or HbA_{1c}. However, approximately 40% of the diabetic subjects had previously been diagnosed, and we therefore cannot rule out the possibility that changes of dietary habits may have resulted in an increase of vitamin B-intake. (A low fat diet normally has a higher folate content compared to a high-fat diet[36]). Finally, there is no indication that insulin or sulfonylureas alter tHcy metabolism. In contrast, metformin may induce vitamin B_{12} malabsorption and thereby increase serum tHcy level, but, in a case-control study, we found at most a weak effect.[37] Taken together, there is no clear evidence that the diabetic state or its treatment influences tHcy levels.

Overall mortality is an unequivocal endpoint, ascertainment was unbiased and complete. This endpoint was sufficiently frequent to estimate the relation of tHcy with mortality with fair precision. In contrast, cardiovascular mortality was too infrequent to adequately assess the effect of hyperhomocysteinemia as precisely as that for overall mortality. In addition, the relation between hyperhomocysteinemia and death from a specific cause, such as cardiovascular mortality, might be affected by nondifferential

misclassification, which in general results in effect attenuation,[24] and by competing mortality risks.

In conclusion, hyperhomocysteinemia is a risk factor for 5-year overall and cardiovascular mortality independently of major cardiovascular risk factors and is a stronger risk factor for overall mortality in diabetic as compared to non-diabetic subjects.

Acknowledgments

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Serum homocysteine level and protein intake are related to risk of microalbuminuria
The Hoorn Study

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Kidney Int 1998;54:203-209

Abstract

Background Microalbuminuria (MA) is a strong predictor of cardiovascular disease, but its causes are incompletely understood. Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease independent of established risk factors. It is not known whether hyperhomocysteinemia is associated with MA, and thus could be a possible cause of microalbuminuria.

Methods We studied an age-, sex- and glucose tolerance-stratified random sample of a 50- to 75-year-old general Caucasian population (N=653). The urinary albumin-to-creatinine ratio (ACR) was measured in an early morning spot urine sample. MA was defined as ACR >3.0 mg/mmol.

Results The prevalence of MA was 4.3% (13 of 304) in subjects with normal glucose tolerance, 9.2% (17 of 185) in impaired glucose tolerance and 18.3% (30 of 164) in non-insulin-dependent diabetes mellitus (NIDDM); it was 3.7% (15 of 402) in subjects without hypertension and 17.9% (45 of 251) in those with hypertension. After adjusting for age, sex, glucose tolerance category, hypertension, dyslipidemia and smoking, the odds ratio [OR; 95% confidence interval (95% CI)] for MA per 5 μmol/L total homocysteine increment was 1.33 (1.08 to 1.63). Additional adjustment for HbA_{1c}, waist-hip ratio, protein intake and serum creatinine did not attenuate the association between MA and total homocysteine. A 0.1 g/kg.day increment of protein intake was also associated with an increased risk for MA after adjustment for age, sex, classical risk factors and serum total homocysteine [OR (95% CI); 1.20 (1.08 to 1.32)].

Conclusion Both hyperhomocysteinemia and protein intake are related to microalbuminuria independent of NIDDM and hypertension. Hyperhomocysteinemia may partly explain the link between MA and increased risk of cardiovascular disease.

Introduction

A slightly elevated urinary albumin excretion rate, so-called microalbuminuria, is a strong predictor of cardiovascular morbidity and mortality in both non-insulin-dependent diabetes mellitus (NIDDM) and non-diabetic individuals.[1,2] The increased risk of cardiovascular disease in individuals with microalbuminuria is only partly due to a higher prevalence of established risk factors such as diabetes, hypertension, smoking and dyslipidemia. The pathophysiological basis of the association between microalbuminuria and cardiovascular disease remains to be elucidated. The most widely held view is that microalbuminuria indicates underlying generalized vascular, possibly endothelial, dysfunction.[3,4] Therefore, microalbuminuria and atherothrombotic disease may have certain pathogenetic mechanisms in common.

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease independent of established risk factors.[5,6] It is not known whether hyperhomocysteinemia is associated with and thus a possible cause of microalbuminuria. Therefore, we investigated this issue in a 50- to 75-year-old general population, an age range in which both hyperhomocysteinemia[7,8] and microalbuminuria[2] are known to be relatively common.

A high protein intake may increase the risk of developing microalbuminuria[9,10] and conceivably may also increase serum total homocysteine (tHcy) levels.[11] It is thus a potential confounder of the relation, if any, between hyperhomocysteinemia and microalbuminuria. We therefore specifically examined the relations between protein intake, serum total homocysteine, and presence of microalbuminuria.

Methods

Design and Study Population

The Hoorn Study is a cross-sectional survey of glucose tolerance and other cardiovascular risk factors in a 50- to 75-year-old general Caucasian population conducted from 1989 to 1992. A random sample of all men and women aged 50 to 75 years was drawn from the municipal population registry office of Hoorn (The Netherlands); 2484 subjects participated (response rate 71%). The presence of microalbuminuria was investigated (as detailed below) in an age-, sex- and glucose tolerance-stratified random subsample (N=680, response rate 96%), which was constructed by means of a second sampling, as described previously in detail.[12] The Hoorn Study was approved by the Ethical Review Committee of the University Hospital Vrije

Universiteit Amsterdam. Informed consent was obtained from all participants.

An early morning first voided spot urine sample was collected. Urinary albumin was measured by rate nephelometry (Array Protein System; Beckman, Ireland) with a threshold of 6.2 mg/L and intra- and interassay coefficients of variation of <=5% and <=8%, respectively. Urinary creatinine was measured by a modified Jaffé method. The urinary albumin-to-creatinine ratio (ACR) was calculated. For those urine samples with an albumin concentration less than the threshold, the value 6.2 was taken to calculate the ACR. An ACR <=3.0 mg/mmol was defined as normoalbuminuric, an ACR >3.0 mg/mmol as (micro)albuminuric and an ACR >30.0 mg/mmol as macroalbuminuric. [An ACR of 3 to 30 mg/mmol is approximately equivalent to an albumin excretion rate (AER) of 30 to 300 mg/24h].[13,14] We also considered a lower cutoff value (ACR >2.0 mg/mmol) to define (micro)albuminuria. To investigate the reproducibility of the ACR, we collected a second urine sample from a representative sample of 185 subjects in similar fashion. Of all subjects (N=680), 24 urine samples were missing. In addition, we excluded three subjects from further analyses because no serum was available for homocysteine measurement. Thus, analyses were performed on 653 subjects, of whom 185 had an ACR on the basis of the mean of two measurements. By utilizing the available duplicate measurements we reduced the effect of possible misclassification of (micro)albuminuric subjects. Finally, the presence of leukocytes was evaluated by microscopy and scored as positive (>5 leukocytes per high-power field) or negative; a microscopic report was missing in 60 of the available urine samples.

Measurement of serum total homocysteine (tHcy)

Fasting blood samples were centrifuged within one hour following collection. Serum was stored at -20°C for 6 years. There is good evidence that serum tHcy levels are stable for 10 years or more.[15] Serum total (free plus protein bound) homocysteine level was measured by using tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by HPLC with fluorescence detection.[16] The intra- and interassay coefficients are 2.1% and 5.1%.

Other procedures

We measured fasting serum total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany). The Friedewald formula was used to calculate the low density lipoprotein (LDL) cholesterol concentration, except in subjects with serum triglyceride levels >8.0 mmol/L ($N=3$).[17] Serum creatinine was measured by the modified Jaffé method. The creatinine clearance was calculated from serum creatinine, using the Cockcroft and Gault formula.[18] Normal renal function and mild and moderate renal failure were defined as creatinine clearance >80, 51–80 and 24–50 mL/min, respectively. (There were no subjects with creatinine clearance <24 mL/min.) Immunospecific insulin was measured in serum by a double-antibody radioimmunoassay (lot SP21, Linco Research, St. Louis, MO, U.S.A.). The interassay coefficient of variation was 6%. The lower limit of sensitivity was 12 pmol/L. Hypercholesterolemia was defined as total cholesterol >=6.5 mmol/L and/or the current use of cholesterol-lowering medication. Dyslipidemia was defined as levels of HDL cholesterol in the lowest (<=1.02 mmol/L) and/or levels of triglycerides in the highest quartile (>=2.10 mmol/L). Hypertension was defined as a blood pressure >=160 mmHg systolic and/or >=95 mmHg diastolic and/or the current use of antihypertensive medication. Impaired glucose tolerance (IGT) and NIDDM were defined according to the WHO criteria (1985).[19] Subjects were classified as either nonsmokers or current smokers. Waist-hip ratio was measured as previously described.[12] Body mass index was calculated as weight divided by height squared (kg/m^2). Information on protein (animal and vegetable) intake was obtained by a self-administered validated semi-quantitative food frequency questionnaire ($N=638$).[20] All laboratory measurements were carried out by technicians unaware of the subjects' history of cardiovascular disease and glucose tolerance status.

Statistical analysis

Variables are presented as mean ± standard deviation (SD), number (percentage of the total) or, in case of skewed distribution, median and interquartile range (IQR) or geometric mean. Differences between subjects with normo- and those with (micro)albuminuria were tested with Student's *t* or Wilcoxon's rank sum test for continuous variables and Pearson's chi-square test for frequency measures. Associations of cardiovascular risk factors with serum tHcy level (logarithmically transformed) were studied by

calculating Pearson's correlation coefficients. The kappa coefficient was calculated to estimate the agreement of the ACR >3.0 mg/mmol between two measurements.

We performed logistic regression analyses to study the association of serum tHcy with (micro)albuminuria. We calculated odds ratios (ORs) and 95% confidence intervals (CIs) per 5 μmol/L (about 1 SD) increment of serum tHcy, assuming a linear logistic relation between homocysteine and risk of (micro)albuminuria. We used multiple logistic regression analysis to control for age, sex, glucose tolerance category, hypertension, current smoking and dyslipidemia. To control for confounding as thoroughly as possible, we used, in all analyses, four categories of glucose tolerance: normal glucose tolerance (NGT), IGT, newly detected and known NIDDM. We also tested models that in addition included HbA_{1c}, waist-hip ratio (or BMI), protein intake (g/kg.day), serum creatinine or creatinine clearance and/or fasting insulin level. To evaluate a possible modifying role of other risk factors, we repeated the previous analyses in strata of sex, diabetes, hypertension, dyslipidemia, hypercholesterolemia and current smoking. In addition, we investigated product terms in the logistic regression models. We also evaluated a possible dose-response relation by calculating ORs for (micro)albuminuria for several ranges of homocysteine concentrations with values of serum tHcy equal to or less than 10 μmol/L as the reference category. By using this procedure we evaded assumptions about linearity.

Finally, the associations between animal and vegetable protein intake and (micro)albuminuria were investigated. We evaluated a possible modifying role of hypertension and glycemic control (HbA_{1c}). A possible dose-response relation was investigated by calculating ORs for (micro)albuminuria for several ranges of daily protein intake with values of total protein intake equal to or less than 0.75 g/kg.day as the reference category. All reported *P*-values are two-tailed. All analyses were performed with SPSS for Windows 7.5.2.

Results

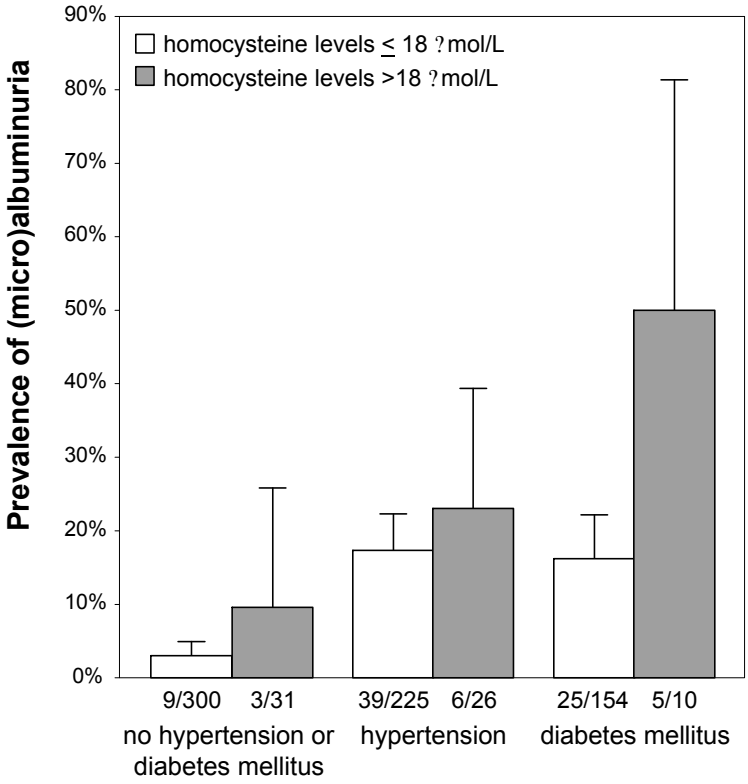
The prevalence of (micro)albuminuria was 9.2% (60 of 653). The kappa coefficient (95% CI) of two urine sample measurements was 0.53 (0.28 to 0.77) for ACR >3.0 mg/mmol, indicating a moderate agreement. As compared to subjects with normoalbuminuria, those with (micro)albuminuria were older, had a higher waist-hip ratio and systolic and diastolic blood pressures, more often had NIDDM and moderate renal failure, and had higher serum levels of creatinine and tHcy (Table 1). The

Table 1. Characteristics of the subjects

	Normoalbuminuria ACR <= 3.0 mg/mmol	(Micro)albuminuria ACR > 3.0 mg/mmol	*P*-value*
N	593	60	
Men *%*	48.1	45.0	0.7
Age *years*	63.9 (7.1)	67.3 (6.7)	<0.001
Body mass index kg/m^2	27.1 (3.7)	28.2 (6.0)	0.2
Waist-hip ratio	0.91 (0.09)	0.94 (0.07)	0.003
Systolic blood pressure *mmHg*	138 (19)	154 (21)	<0.001
Diastolic blood pressure *mmHg*	82 (10)	87 (13)	<0.001
Hypertension *%*	34.7	75.0	<0.001
Use of antihypertensives *%*			
Angiotensin-converting enzyme inhibitor	4.7	10.0	0.09
Other	19.7	41.7	<0.001
Impaired glucose tolerance *%*	28.3	28.3	1.0
Non-insulin-dependent diabetes mellitus *%*	22.6	50.0	<0.001
Current smoker *%*	28.9	25.4	0.6
Total protein intake *g/kg.day*	0.97 (0.27)	1.04 (0.39)	0.2
Animal protein intake *g/kg.day*	0.68 (0.22)	0.73 (0.29)	0.1
Vegetable protein intake *g/kg.day*	0.30 (0.11)	0.31 (0.17)	0.3
HbA_{1c} *% of hemoglobin*	5.8 (1.2)	6.5 (1.7)	0.002
Fasting insulin *pmol/L*	84 (63-114)	90 (70-143)	0.007
Total cholesterol *mmol/L*	6.7 (1.2)	6.5 (1.2)	0.3
LDL cholesterol *mmol/L*	4.6 (1.1)	4.3 (1.0)	0.06
HDL cholesterol *mmol/L*	1.3 (0.4)	1.2 (0.3)	0.1
Triglycerides *mmol/L*	1.5 (1.1-2.1)	1.5 (1.1-2.8)	0.1
Dyslipidemia *%*	38.0	48.3	0.1
Creatinine *µmol/L*	91 (16)	104 (35)	0.005
Creatinine clearance† *mL/min*	75 (18)	67 (20)	0.001
Mild renal failure‡ *%*	58.7	56.7	0.8
Moderate renal failure§ *%*	5.6	18.3	<0.001
Total homocysteine *µmol/L*	11.4 (9.4-14.2)	12.7 (10.0-15.5)	0.05

Data are presented as mean (SD), percentage of the total or median (interquartile range).
Dyslipidemia was defined as levels of HDL cholesterol in the lowest (<=1.02 mmol/L) and/or levels of triglycerides in the highest quartile (>=2.10 mmol/L).

* Tested with Student's *t*-test or Wilcoxon's rank sum test for continuous variables and Pearson's chi-square test for frequencies.
† Calculated from serum creatinine using the Cockcroft and Gault formula.
‡ Creatinine clearance 51 to 80 mL/min.
§ Creatinine clearance 24 to 50 mL/min.
ACR = albumin-to-creatinine ratio.



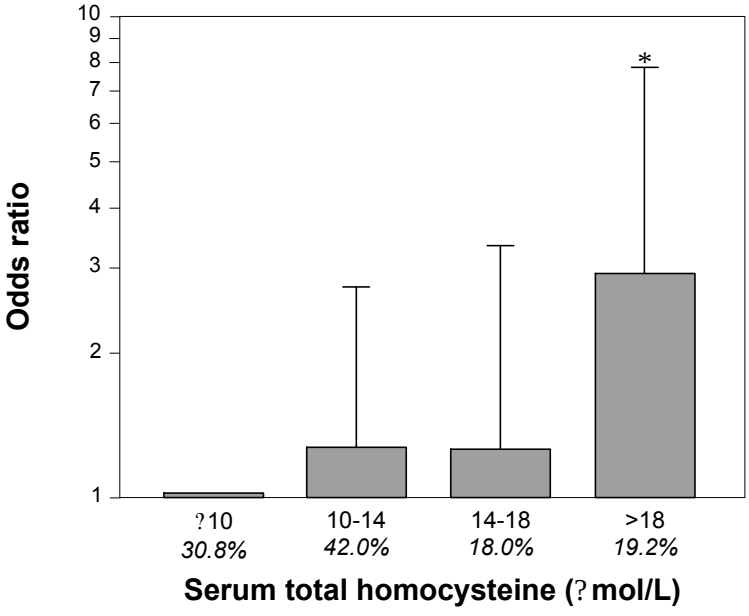
White and grey bars represent subjects with homocysteine levels equal to or less than 18 µmol/L and higher than 18 µmol/L, respectively.

Risk of (micro)albuminuria increases markedly above this cutoff value (see Figure 2).

The error bars represent the upper half of the 95% confidence intervals.

Number of subjects with (micro)albuminuria of each subgroup are presented below the bars.

Figure 1. Prevalence of (micro)albuminuria according to absence or presence of hyperhomocysteinemia (>18.0 µmol/L) in subjects without or with hypertension and/or diabetes mellitus.



The reference category was serum total homocysteine values equal or lower than 10 µmol/L.

Percentages of the population under study for each serum total homocysteine range are presented.

The error bars represent the upper half of the 95% confidence intervals.

A logarithmic scale was used since the odds ratio is a multiplicative measure of association: equal differences on the logarithmic scale correspond to equal ratios between odds ratios.

P=0.06 for trend

* *P*<0.05, significantly different from the reference category.

Figure 2. Odds ratio for (micro)albuminuria according to serum total homocysteine level adjusted for age, sex, impaired glucose tolerance/diabetes mellitus, hypertension and protein intake.

Table 2. Odds ratios (ORs; 95% confidence intervals) for (micro)albuminuria per 5 µmol/L increment of serum total homocysteine

(Micro)albuminuria ACR mg/mmol	Cases	Crude OR	Age- & sex-adjusted OR	Model 1 OR	Model 2 OR
>3.0	60	1.30 (1.09 - 1.54)	1.30 (1.08 - 1.56)	1.33 (1.08 - 1.63)	1.28 (1.03 - 1.59)

Model 1 Adjusted for age, sex, glucose tolerance category, hypertension (yes/no), current smoking (yes/no) and dyslipidemia (yes/no).
Model 2 Model 1 + additional adjustment for protein intake and serum creatinine.
ACR = Albumin-to-creatinine ratio.

prevalence of (micro)albuminuria was 4.3% (13 of 304) in subjects with NGT, 9.2% (17 of 185) in IGT and 18.3% (30 of 164) in NIDDM; it was 3.7% (15 of 402) in subjects without hypertension and 17.9% (45 of 251) in those with hypertension. Figure 1 shows the prevalence of (micro)-albuminuria according to absence or presence of hyperhomocysteinemia (>18 μmol/L) in subjects without or with hypertension and/or diabetes mellitus. We chose this cutoff value since risk of (micro)albuminuria increased markedly above this value (Figure 2).

The median serum tHcy level was 12.2 μmol/L (IQR: 10.0 to 15.4) in men and 11.0 μmol/L (IQR: 9.1 to 13.6) in women. Serum tHcy levels correlated with age (r=0.20; P<0.0001), serum creatinine (r=0.41; P<0.0001), systolic blood pressure (r=0.11; P=0.004), waist-hip ratio (r=0.10; P=0.008), fasting insulin (r=0.08; P<0.05), and inversely with creatinine clearance (r=-0.3; P=0.01) and animal protein intake (r=-0.16; P<0.0001), but not with vegetable protein intake (r=-0.1; P=0.1), BMI (r=-0.05; P=0.3), diastolic blood pressure (r=0.04; P=0.3), serum total cholesterol (r=0.01; P=0.8), HDL cholesterol (r=-0.07; P=0.06) or HbA_{1c} (r=-0.02; P=0.6). The geometric mean serum tHcy level in subjects with moderate and mild renal failure and those with normal renal function were 14.7, 12.4 and 10.6 μmol/L (P for trend <0.0001).

A 5 μmol/L increment of serum tHcy was associated with an increased risk of (micro)albuminuria independent of classical risk factors (Table 2, model 1; Figure 1). Additional adjustment for serum creatinine (Table 2, model 2) or creatinine clearance did not materially change the ORs, nor did inclusion of total cholesterol, triglycerides, HDL and LDL cholesterol, fasting insulin, systolic blood pressure and/or BMI in the model (data not shown). Exclusion of subjects (N=7) who had an ACR >3.0 mg/mmol based on an albumin concentration of 6.2 mg/L (=threshold) yielded similar results (data not shown). If (micro)albuminuria was defined as an ACR >2.0 mg/mmol, the ORs (95% CIs) for model 1 and 2 were 1.27 (1.04 to 1.54) and 1.25 (1.02 to 1.55).

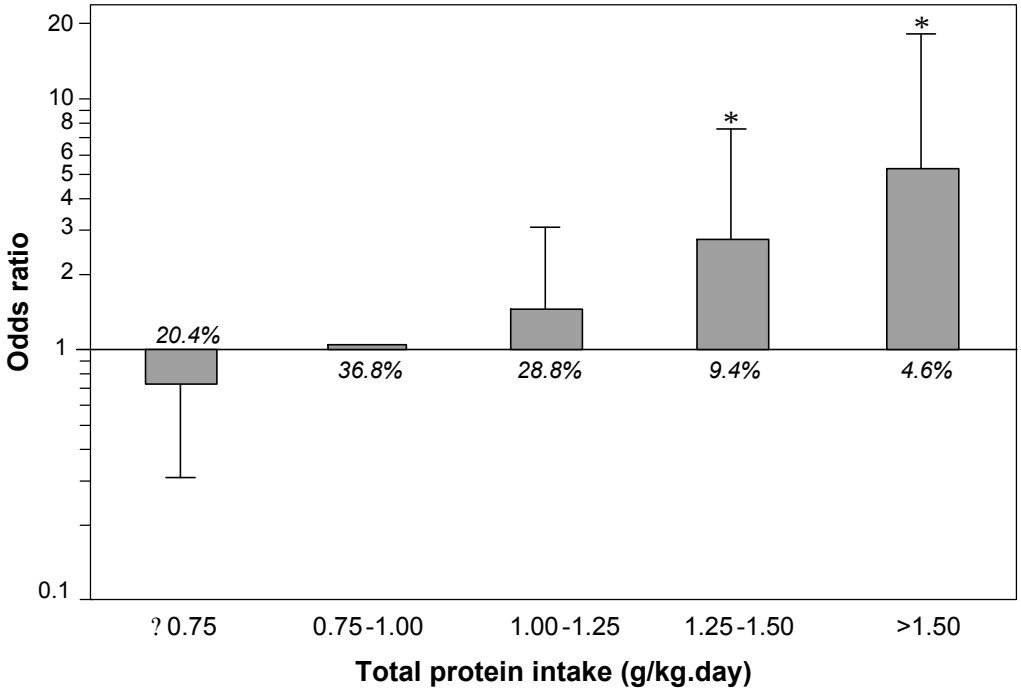


Figure 3. Odds ratio for (micro)albuminuria according to total protein intake adjusted for age, sex, impaired glucose tolerance/diabetes mellitus, hypertension and total homocysteine.

The reference category was total protein intake 0.75-1.00 g/kg.day.
Percentages of the population under study for each total protein intake range are presented.
The error bars represent the lower or upper half of the 95% confidence intervals.

P = 0.001 for trend.
* *P*<0.05, significantly different from the reference category.

Macroalbuminuria was present in seven subjects. After adjustment for age and sex, the OR (95% CI) for macroalbuminuria per 5 μmol/L increment serum tHcy was 1.33 (1.02 to 1.72). After exclusion of the seven macroalbuminuric subjects and adjustment for age, sex and classical risk factors, the OR for microalbuminuria (ACR: 3 to 30 mg/mmol) per 5 μmol/L increment serum tHcy was 1.28 (1.01 to 1.61). Since the strength of the association between micro- or macroalbuminuria and serum tHcy did not differ substantially, we pooled all subjects with an ACR >3.0 mg/mmol.

Angiotensin-converting enzyme inhibitors and other antihypertensives can reduce the urinary albumin excretion. The geometric means of tHcy of subjects who did not or did use an angiotensin-converting enzyme inhibitor were 11.8 and 12.5 μmol/L, respectively (P=0.5). Adjustment for use of angiotensin-converting enzyme inhibitors (yes/no) in addition to classical risk factors (model 1 in Table 2) did not materially change the association between tHcy and (micro)albuminuria [OR (95% CI); 1.32 (1.07 to 1.62)].

Adjustment for use of antihypertensives (yes/no) yielded similar results. The geometric means of tHcy of subjects without or with leukocyturia were 11.9 and 12.4 μmol/L, respectively ($P=0.2$). Additional adjustment for leukocyturia [that is, yes/no; present in 15.7% (93 of 594) of the subjects] also yielded similar results [OR (95% CI); 1.28 (1.01 to 1.61)], as did adjustment for the presence of cardiovascular disease (10.7%: 65 of 607), as defined elsewhere[8] OR 1.25 (1.05 to 1.50).

We evaluated possible modification by other risk factors of the effect of tHcy on risk of (micro)albuminuria and did not observe substantial differences among the following strata: male sex, diabetes, hypertension, dyslipidemia, hypercholesterolemia and current smoking (data not shown). In addition, interaction terms between serum tHcy and these risk factors were not significant. Risk of (micro)albuminuria increased with increasing serum tHcy levels (Figure 2). In none of the above analyses did log-transformation of tHcy result in a better fit (data not shown). Finally, although (micro)albuminuria is usually considered as a dichotomous variable, we also performed multiple linear regression analysis with log-transformed ACR as the (continuous) dependent variable. After adjustment for age, sex, diabetes, hypertension and renal function, ACR showed a highly significant association with tHcy ($P<0.0001$).

The present study was too small to adequately investigate whether the relation between microalbuminuria and cardiovascular disease can be explained to some extent by hyperhomocysteinemia (data not shown).

Protein intake correlated with creatinine clearance ($r=0.2$; $P<0.01$). An 0.1 g/kg.day increment of the daily animal or vegetable protein intake was associated, after adjustment for age, sex, classical risk factors and serum tHcy, with an increased risk of (micro)albuminuria [OR (95% CI); 1.22 (1.08 to 1.39) and 1.32 (1.07 to 1.62)]. Since these ORs did not differ substantially, we calculated the strength of the association between total protein intake and (micro)albuminuria to be OR 1.20 (1.08 to 1.32). After exclusion of known diabetic patients the OR was 1.18 (1.04 to 1.33). There was no evidence of interaction between hypertension or glycemic control and protein intake with regard to risk of (micro)albuminuria. Risk of (micro)albuminuria increased with increasing daily total protein intake (Figure 3).

Discussion

This is, to our knowledge, the first population-based study unequivocally showing that serum total homocysteine is positively associated with the

presence of microalbuminuria independent of major determinants, that is, diabetes mellitus, hypertension, protein intake and renal function. For each 5 μmol/L (about 1 SD) increase in serum tHcy level, the risk of microalbuminuria being present increased by about 30%. The result of the present study is in line with a few studies that reported a positive association between albuminuria and tHcy level,[21,22] but contradicts other studies.[23,24] However, none of these studies adjusted for all major determinants of microalbuminuria and some[23,24] used relatively small populations.

We chose two different approaches to investigate the nature of the association between tHcy and microalbuminuria. First, we analyzed the association with tHcy as a continuous variable, since there is evidence that the association of tHcy with risk of cardiovascular disease is graded.[6,25] Second, we evaluated a possible dose-response relation between microalbuminuria and several ranges of tHcy. Inspection of Figure 2 suggests that there might be a threshold (at 18 μmol/L) above which an increased risk of microalbuminuria exists. Obviously the present study is not large enough to solve the question whether the association between microalbuminuria and tHcy is graded or has a certain threshold.

Microalbuminuria is thought to be caused by increased glomerular albumin filtration as a result of decreased glomerular charge selectivity, size selectivity and/or increased intraglomerular pressure,[26,27] which regulation is affected by renal endothelial and mesangial cell function.[28,29] Mesangial cells have some properties in common with vascular smooth muscle cells.[29] Hyperhomocysteinemia may induce dysfunction of the vascular endothelium[30] and increase proliferation of vascular smooth muscle cells,[31] possibly by increasing oxidative stress.[32] Therefore, it is conceivable that hyperhomocysteinemia is causally related to microalbuminuria through changes in renal endothelial and mesangial cell function and might thus be one of the factors that link the presence of microalbuminuria to an increased risk of atherothrombotic disease.[1,2]

We considered three sources of disease misclassification that may have resulted in bias of the association between hyperhomocysteinemia and persistent microalbuminuria. First, of the majority (72%) of subjects we collected only one urine sample to assess microalbuminuria. Repeated measurements would have improved the accuracy of classification of persistent microalbuminuria since there is a considerable day-to-day intra-individual variability of albumin excretion.[33,34] Second, approximately 24% of all subjects classified as normoalbuminuric used antihypertensive medication. Antihypertensives, especially angiotensin-converting enzyme

inhibitors, are likely to decrease urinary albumin excretion.[35] Finally, the presence of leukocyturia, which, insofar as it reflects urinary tract infection, might increase urinary albumin excretion. In all three cases, the possible disease misclassification was nondifferential with regard to serum tHcy level. In general, nondifferential disease misclassification would likely bias the findings toward effect attenuation (that is, underestimation of the strength of the association between hyperhomocysteinemia and microalbuminuria).[36]

The present study confirms and extends previous observations that tHcy and creatinine clearance are strongly associated.[22] An impaired renal function causes a substantial increase in the half-life of tHcy explained by a reduction in total body clearance.[37,38] In addition, an impaired renal function is a risk factor for microalbuminuria. As the association between tHcy and microalbuminuria did not materially change after adjustment for serum creatinine or creatinine clearance (Table 2), we consider it improbable that impaired renal function confounded the association between tHcy and the presence of microalbuminuria. Finally, we cannot fully exclude that proximal tubular dysfunction in the presence of a normal glomerular filtration rate (GFR) results in both decreased albumin reabsorption (and thus microalbuminuria) and impaired tHcy metabolism, but this appears unlikely.

A poor folate, vitamin B_{12} and/or vitamin B_6 status can increase serum tHcy level.[7] Since we did not assess B vitamins and the present study is cross-sectional, we cannot rule out the possibility that low vitamin B levels may increase urinary albumin excretion or that microalbuminuria *per se* can raise serum tHcy levels, although this appears biologically implausible. Even though in an elderly population hyperhomocysteinemia, microalbuminuria and vitamin B deficiency frequently coexist, this does not explain the positive association we found between serum tHcy and microalbuminuria. Only when vitamin B deficiency would be the cause of both microalbuminuria and hyperhomocysteinemia, which seems unlikely with respect to microalbuminuria, could vitamin B deficiency have been a confounder of the association between tHcy and microalbuminuria. Serum tHcy levels can be lowered with an increased intake of B vitamins, particularly folate. A randomized clinical trial could thus support the hypothesis that tHcy might be a causal factor of microalbuminuria. Although the dose-response relation we found between hyperhomocysteinemia and microalbuminuria does not necessarily support a causal relation between tHcy and microalbuminuria, departures from expected biologic gradients do provide evidence against causation.

We investigated whether dietary protein intake confounded the association between tHcy and microalbuminuria. We found that dietary protein intake did not explain the relation between tHcy and microalbuminuria. However, we did observe an increased risk of microalbuminuria with increasing consumption of total protein independent of serum tHcy levels. This observation is consistent with some[9,39] but not all studies.[40] A high protein intake may result in an increased GFR and renal workload and therefore aggravate proteinuria.[41] In addition, it has previously been demonstrated that an animal compared to a vegetable protein diet, independently of the daily amount of protein intake, results in a higher GFR and urinary albumin excretion.[42]

In the present study additional adjustment for animal or total protein intake, if anything, strengthened the association between serum tHcy and risk of microalbuminuria. Taken together, it appears unlikely that hyperhomocysteinemia and high animal protein intake share a common causal pathway with regard to risk of microalbuminuria. Therefore, both appear important determinants of microalbuminuria.

We conclude that both hyperhomocysteinemia and a high protein intake are related to microalbuminuria independent of non-insulin-dependent diabetes mellitus and hypertension. Hyperhomocysteinemia may partly explain the link between microalbuminuria and increased risk of cardiovascular disease.

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6

Hyperhomocysteinemia is associated with presence of retinopathy in type 2 diabetes
The Hoorn Study

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Abstract

Background Retinopathy is the leading cause of blindness among type 2 diabetic patients. The main identified causes so far are hyperglycemia and hypertension. Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease, independent of established risk factors. It is not known whether hyperhomocysteinemia is associated with retinopathy, and thus could be a possible cause of retinopathy.

Methods We studied an age-, sex- and glucose tolerance-stratified random sample of a 50- to 75-year-old general Caucasian population in the Hoorn Study (*N*=625). Retinal vascular changes ('retinopathy') were assessed by means of ophthalmoscopy and/or fundus photography. Hyperhomocysteinemia was defined as serum tHcy >16 μmol/L.

Results The prevalence of retinopathy was 9.8% (28 of 285) in subjects with normal glucose tolerance, 11.8% (20 of 169) in those with impaired glucose tolerance and 18.1% (31 of 171) in those with type 2 diabetes; it was 10.3% (39 of 380) in subjects without hypertension and 16.3% (40 of 245) in subjects with hypertension; it was 12.0% (64 of 534) in subjects with serum tHcy levels <=16 μmol/L and 16.5% (15 of 91) in those with serum tHcy levels >16 μmol/L. After stratification for diabetes and adjustment for age, sex, HbA_{1c} and hypertension, the odds ratio (95% CI) for the relation between retinopathy and hyperhomocysteinemia was 0.97 (0.42 to 2.82) in non-diabetic and 3.44 (1.13 to 10.42) in diabetic subjects (*P*=0.08 for interaction).

Conclusion We conclude that hyperhomocysteinemia may be a risk factor for retinopathy in type 2 diabetic, but probably not in non-diabetic subjects.

Introduction

Diabetic retinopathy is the leading cause of blindness among type 2 diabetic patients.[1,2] About a third of all cases of impaired visual function in type 2 diabetic patients is attributable to retinopathy, the remainder to cataract, macular degeneration, glaucoma, and other causes. Blindness has been estimated to be 25 times more common in people with diabetes than in those without.[3] After 20 years of diabetes, more than 60% of type 2 diabetic patients have some degree of retinopathy.[2] The pathogenetic mechanisms of diabetic retinopathy are incompletely understood.

Diabetic retinopathy appears to be essentially a retinal vascular disorder, probably beginning in the capillary bed. Epidemiological studies have shown that the risk and severity of diabetic retinopathy are strongly related to the duration of diabetes, hyperglycemia, and hypertension, and also, but less consistently, to hypercholesterolemia, and smoking.[2,4-9] Furthermore, there is a close relation between the presence of diabetic retinopathy and risk of microalbuminuria. Type 2 diabetic patients with, as compared to those without diabetic retinopathy, have an about 2-fold increased risk of developing microalbuminuria.[10,11] Conversely, diabetic patients with micro- or macroalbuminuria, as compared to those with normoalbuminuria, have a higher prevalence and an increased severity of diabetic retinopathy, as well as an increased risk of developing this complication.[12-15] Taken together, these findings suggest that diabetic retinopathy and microalbuminuria have certain pathogenetic mechanisms in common.

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease independent of major cardiovascular risk factors.[16-18] In addition, hyperhomocysteinemia appears to be a risk factor for microalbuminuria independent of major determinants, i.e. diabetes, hypertension, protein intake and renal function.[19] It is not known whether hyperhomocysteinemia is also associated with and thus a possible contributing cause of retinopathy. We therefore investigated this issue in the 50- to 75-year-old general population of the Hoorn Study.

Methods

Design and study population

The Hoorn Study is a cross-sectional survey of glucose tolerance and other cardiovascular risk factors in a 50- to 75-year-old general Caucasian

population conducted from 1989 to 1992.[21] A random sample of all men and women aged 50 to 75 years was drawn from the municipal population registry office of Hoorn (The Netherlands); 2484 subjects participated (response rate 71%). All subjects, except previously diagnosed diabetic subjects treated with oral glucose-lowering agents or insulin, underwent a 75 g oral glucose tolerance test (OGTT) and were classified according to the WHO (1985) criteria.[22] A second OGTT (participation rate 93%) was performed, for reasons of efficiency, in a random subsample (N=1122), stratified by 2-hour glucose values of the first test, age and sex. Finally, from this subsample another age-, sex-, and glucose tolerance-stratified random sample (N=708) was drawn. The presence of retinal vasculopathy (as defined below) was investigated (N=625; response rate 88%) by two experienced ophthalmologists. The examination included both ophthalmoscopy and fundus photography (detailed below). Glucose tolerance was divided into three categories on the basis of the mean of the two OGTTs: normal glucose tolerance (NGT, N=285), impaired glucose tolerance (IGT, N=169) and type 2 diabetes mellitus (N=171). The Hoorn Study was approved by the Ethical Review Committee of the University Hospital Vrije Universiteit Amsterdam. Informed consent was obtained from all participants.

Ophthalmologic investigation

Retinopathy was assessed by ophthalmoscopy and/or fundus photography. In each participant, both eyes were dilated with 0.5% tropicamide and 5% phenylephrine eye drops. After an average period of 15 minutes, indirect and direct ophthalmoscopy (N=625) was carried out by one of two ophthalmologists, and findings regarding the retinal status were reported on standard forms. Thereafter, two 45° standard field 35 mm black and white fundus photographs (Kodak Tri-X 400 ASA, Kowa Pro 1 fundus camera, Kowa optical industry, Japan) were taken of each eye. Photographs were taken with a green filter (to improve the contrast), centered on the macular area and the optic disc. Fundus photographs of 148 subjects were inadvertently lost. [The fundus photographs were randomly lost with regard to age, sex, hypertension, glucose tolerance category and serum tHcy level of the subjects (data not shown).] Thus, for the present analysis fundus photographs of 477 subjects were available.

Both ophthalmoscopic and fundus photographic findings were graded according to the modified Airlie House classification.[23,24] The fundus photographs were independently graded by two ophthalmologists. The independent judgment of a third ophthalmologist was taken to be decisive in

case of disagreement with regard to the grading of retinopathy on the fundus photograph. For the present analysis 'the worst eye' of each subject according to ophthalmoscopy or fundus photography was used.[24] Any retinopathy (yes/no) was defined as the presence of one or more hemorrhages, microaneurysms, soft or hard exudates, neovascularization and/or laser coagulation scars in one or both eyes.

Measurement of serum total homocysteine (tHcy)

Fasting blood samples were centrifuged within one hour following collection. Serum was stored at -20°C for 6 years. There is good evidence that serum tHcy levels in frozen samples are stable for 10 years or more.[25] Serum total (free plus protein bound) homocysteine level was measured by using tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by HPLC with fluorescence detection.[26] The intra- and interassay coefficients are 2.1% and 5.1%, respectively.

Other measurements

Subjects were classified as either current smoker or nonsmoker of cigarettes. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Blood pressure was measured as the mean of, in total, four measurements, performed on two different occasions, with a random zero sphygmomanometer under standardized conditions. Hypertension was defined as a blood pressure >=160 mmHg systolic and/or >=95 mmHg diastolic and/or the current use of antihypertensive medication. Fasting and 2-hour post-load venous plasma glucose levels were measured with a glucose dehydrogenase method (Merck, Darmstadt, Germany) and glycated hemoglobin (HbA_{1c}) by ion-exchange high-performance liquid chromatography, using a Modular Diabetes Monitoring System (Bio-Rad, Veenendaal, the Netherlands). Fasting serum total cholesterol was measured by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany). Hypercholesterolemia was defined as total cholesterol >=6.5 mmol/L and/or the current use of cholesterol-lowering medication. Immunospecific insulin was measured in serum by a double-antibody radioimmunoassay (lot SP21, Linco Research, St. Louis, U.S.A.). The interassay coefficient of variation was 6%. The lower limit of sensitivity was 12 pmol/L. Ophthalmologic investigations and laboratory measurements were carried out in a blinded fashion with respect to glucose tolerance status and other clinical data.

Statistical analysis

Variables are presented as mean ± standard deviation (SD), number (percentage of the total) or, in case of skewed distribution, median and interquartile range (IQR). We assessed the back-calculated prevalence of any retinopathy, standardized for age, sex and glucose tolerance category as described previously in detail.[18] Kappa coefficients (**K**) were calculated to assess agreement with regard to presence of retinopathy on fundus photographs between two ophthalmologists, and of presence of retinopathy between fundus photographic and ophthalmoscopic examinations. Differences between subjects with and without any retinopathy were tested with Student's *t* or Wilcoxon's rank sum test for continuous variables and Pearson's chi-square test for frequency measures. Associations of risk factors for retinopathy with serum tHcy level (logarithmically transformed) were studied by calculation of Pearson's correlation coefficients.

We performed logistic regression analyses to study the relation between serum tHcy and presence of any retinopathy. The number of cases in the separate classes of retinopathy was insufficient to allow a more detailed analysis. We chose two different approaches to investigate the nature of the relation between tHcy and retinopathy, because it is unclear whether this relation, if any, is linear or has a certain threshold. We calculated odds ratios and 95% confidence intervals (CI) both for serum tHcy as a categorical (to allow for a non-linear dose-response relation and to reduce the influence of outliers) and as a continuous variable, the latter expressed per 5 μmol/L (about 1 SD) increment of serum tHcy. For the analysis with serum tHcy as a categorical variable, we calculated odds ratios for tHcy divided in two (>16 μmol/L versus <=16 μmol/L) and in three categories (<=9.0 μmol/L, 9.1 to 16.0 μmol/L and >16.0 μmol/L). To test for trend, the three categories were entered in the model as an ordinal variable. We used multiple logistic regression analysis to control for potential confounders, i.e., age, sex, glucose tolerance category, known duration of diabetes of more than 10 years (in the present study a duration effect was seen after 10 years, which is in agreement with other studies[2,5,27]), HbA_{1c} and hypertension. In order to exclude the possibility of residual confounding as thoroughly as possible, we also tested models which in addition included hypercholesterolemia, current smoking, BMI and/or fasting insulin level. Possible interaction between tHcy and diabetes with regard to risk of retinopathy was assessed in a stratified analysis and with an interaction term. Finally, we performed an additional analysis with '*diabetic* retinopathy' as

Table 1. General characteristics of the subjects

	No retinopathy	Retinopathy	*P*-value*
N	546	79	
Men *%*	49.1	41.8	0.3
Age *years*	64.1 (7.3)	65.7 (6.7)	0.06
Body mass index *kg/m²*	27.1 (3.9)	28.0 (4.5)	0.07
Current smoker *%*	28.7	28.2	0.9
Systolic blood pressure *mmHg*	138 (19)	147 (22)	0.001
Diastolic blood pressure *mmHg*	82 (10)	85 (11)	0.04
Hypertension *%*	37.5	50.6	0.03
Normal glucose tolerance *%*	47.1	35.4	
Impaired glucose tolerance *%*	27.3	25.3	
Newly-diagnosed diabetes mellitus *%*	17.6	12.7	
Known diabetes mellitus *%*	8.1	26.6	<0.001†
Duration of diabetes‡ *years*	5.9 (2.0-9.5)	9.4 (4.3-16.2)	0.02
Fasting glucose *mmol/L*	6.5 (2.3)	7.8 (3.6)	0.002
HbA_{1c} *% of hemoglobin*	5.8 (1.2)	6.6 (1.8)	<0.001
Fasting insulin *pmol/L*	83 (62-116)	93 (72-143)	0.01
Total cholesterol *mmol/L*	6.6 (1.2)	6.8 (1.2)	0.08
Hypercholesterolemia *%*	53.5	63.3	0.1
Total homocysteine *µmol/L*	11.5 (9.3-14.1)	11.1 (9.4-14.0)	1.0

Data are presented as mean (SD), percentage of the total or median (interquartile range).

* Tested with Student's *t*-test or Wilcoxon's rank sum test for continuous variables and Pearson's chi-square test for frequencies.

† Chi-square test for trend.

‡ Diabetes duration since diagnosis of those subjects known with diabetes mellitus.

dependent variable. We defined 'diabetic retinopathy' as presence of >=1 microaneurysm and/or laser coagulation scars in one or both eyes (there were no subjects with neovascularization), regardless of other abnormalities. All analyses were performed with SPSS for Windows 7.5.2. A 95% CI not including 1.0 was considered to indicate statistical significance.

Results

Of five right and eight left eyes, ophthalmoscopic findings were missing. In addition to the photographs of 148 subjects that were lost, photographs of three right and four left eyes were missing. Moreover, because of poor quality, photographs of 18 right and 20 left eyes were ungradable for retinopathy. Thus, ophthalmologic data of 625 subjects were available for

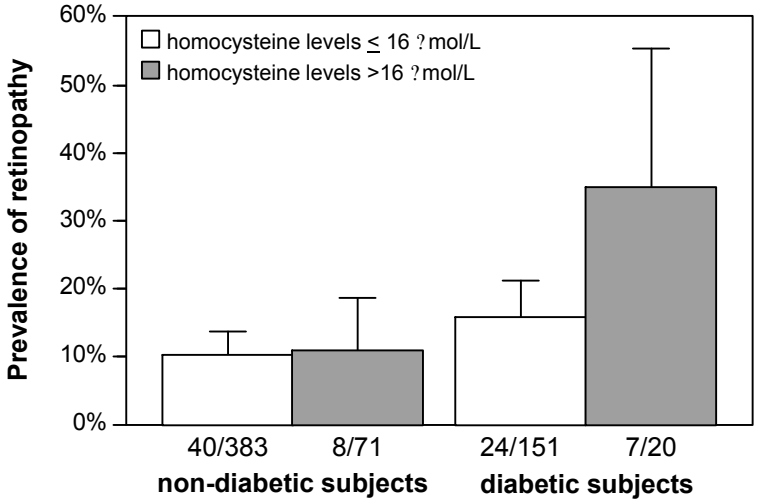

Figure 1. Prevalence of retinopathy according to absence or presence of hyperhomocysteinemia (>16 µmol/L) in non-diabetic and diabetic subjects. Error bars represent the upper half of the 95% confidence intervals.

further analysis; 76% (477 of 625) were based on both ophthalmoscopic and fundus photographic findings.

The baseline characteristics of the study population are presented in Table 1. With regard to the fundus photographs, the **K** (95% CI) between the ophthalmologists was 0.87 (0.78 to 0.96) for the right eyes (*N*=437) and 0.95 (0.89 to 1.00) for the left eyes (*N*=428), indicating a good agreement; between ophthalmoscopic and fundus photographic findings, the **K** was 0.39 (0.22 to 0.56) for the right eyes (*N*=450) and 0.39 (0.21 to 0.58) for the left eyes (*N*=443), indicating moderate agreement.

The standardized back-calculated prevalence of any retinopathy was 10.7%. The prevalence was 9.8% (28 of 285) in subjects with NGT, 11.8% (20 of 169) in IGT, 9.4% (10 of 106) in subjects with newly diagnosed diabetes mellitus (NDM) and 32.3% (21 of 65) in subjects known with diabetes mellitus (KDM); it was 10.3% (39 of 380) in subjects without hypertension and 16.3% (40 of 245) in those with hypertension; it was 12.0% (64 of 534) in subjects with serum tHcy levels **<=**16 µmol/L and 16.5% (15 of 91) in those with serum tHcy levels **>**16 µmol/L. The prevalence of 'diabetic retinopathy' was 4.6% (13 of 285) in subjects with NGT, 5.9% (10 of 169) in IGT, 4.7% (5 of 106) in NDM and 23.1% (15 of 65) in KDM. Figure 1 shows the prevalence of any retinopathy according to absence or presence of hyperhomocysteinemia (**>**16 µmol/L) in non-diabetic and diabetic subjects. We chose this cutoff value since risk of

Table 2. Odds ratios (95% confidence intervals) for retinopathy

Total homocysteine (µmol/L)	Prevalence of retinopathy	Adjusted for age and sex	Adjusted for age, sex, HbA_{1c} and hypertension	Adjusted for age, sex, HbA_{1c}, hypertension and diabetes duration >10 years
Diabetic subjects				
<= 9.0	10.0% (4/40)	0.53 (0.16 - 1.71)	0.56 (0.17 - 1.85)	0.54 (0.15 - 1.90)
9.1 - 16.0*	18.0% (20/111)	1.00	1.00	1.00
> 16.0	35.0% (7/20)	2.68 (0.92 - 7.80)	3.05 (0.99 - 9.42)	3.00 (0.95 - 9.51)
Per category increment		2.28 (1.09 - 4.79)	2.38 (1.10 - 5.17)	2.41 (1.09 - 5.31)
P for trend		0.03	0.03	0.03
Non-diabetic subjects				
<= 9.0	12.1% (11/91)	1.38 (0.64 - 2.98)	1.52 (0.69 - 3.34)	
9.1 - 16.0*	9.9% (29/292)	1.00	1.00	
> 16.0	11.3% (8/71)	1.19 (0.52 - 2.74)	1.05 (0.45 - 2.46)	
Per category increment		0.91 (0.54 - 1.55)	0.82 (0.47 - 1.40)	
P for trend		0.7	0.5	

* Reference category.

retinopathy increased markedly above this value among diabetic subjects (Table 2).

The median serum tHcy level was 12.2 µmol/L (IQR: 10.0 to 15.3) in men and 10.7 µmol/L (IQR: 9.0 to 13.3) in women. Serum tHcy levels correlated with age (r=0.17; P<0.001). After adjustment for age and sex, there was no substantial correlation between serum tHcy levels and the following variables: systolic blood pressure (r=0.06; P=0.1), diastolic blood pressure (r=0.03; P=0.5), BMI (r=-0.02; P=0.7), fasting glucose (r=-0.07; P=0.07), fasting insulin (r=0.05; P=0.2), HbA_{1c} (r=-0.02; P=0.6), duration of diabetes in subjects known with diabetes (r=-0.03; P=0.8) or serum total cholesterol (r=0.04; P=0.3).

After adjustment for age and sex, the odds ratio (95% CI) for any retinopathy was 1.37 (1.18 to 1.59) per % increment HbA_{1c}, 1.76 (1.07 to 2.90) for diabetes (yes/no), 1.57 (0.97 to 2.55) for hypertension (yes/no), 1.46 (0.89 to 2.39) for hypercholesterolemia (yes/no), 1.09 (0.64 to 1.86) for current smoking (yes/no) and, among subjects with KDM, 7.31 (2.69 to 19.85) for diabetes duration more than 10 years.

After stratification by diabetes (yes/no) and adjustment for age, sex, HbA_{1c} and hypertension, we observed a substantial difference between the

two strata with regard to relative risk of retinopathy. The odds ratio of retinopathy associated with hyperhomocysteinemia (>16 μmol/L) was 0.97 (0.42 to 2.82) in non-diabetic and 3.44 (1.13 to 10.42) in diabetic subjects (P=0.08 for interaction). The results per category increment of serum tHcy are shown in Table 2 (P=0.03 for interaction). This indicates that hyperhomocysteinemia is a risk factor for retinopathy in diabetic but not in non-diabetic subjects. After exclusion of 6 outliers (serum tHcy >35.0 μmol/L; five non-diabetic subjects and one diabetic subject) and adjustment for age, sex, HbA_{1c} and hypertension, the odds ratio per 5 μmol/L increment of serum tHcy was 0.89 (0.60 to 1.34) in non-diabetic and 1.50 (0.93 to 2.41) in diabetic subjects. Additional adjustment for hypercholesterolemia, current smoking, BMI and/or fasting insulin level did not markedly change the results (data not shown).

In the above analyses, subjects with NGT and IGT were pooled, as were those with newly diagnosed and known diabetes, because the odds ratios for retinopathy associated with hyperhomocysteinemia did not differ substantially between these categories. In addition, the odds ratios remained similar if categories were pooled (data not shown).

To reduce the effect of possible misclassification, we repeated the analysis after classifying subjects with only hemorrhages in one or both eyes as having no retinopathy in an additional analysis. After adjustment for age, sex and HbA_{1c}, the odds ratio for hyperhomocysteinemia was 1.11 (0.46 to 2.68) in non-diabetic and 5.28 (1.67 to 16.67) in diabetic subjects. Per category increment of serum tHcy, it was 0.93 (0.51 to 1.69) in non-diabetic and 2.62 (1.13 to 6.07) in diabetic subjects, and per 5 μmol/L increment of serum tHcy it was 0.96 (0.62 to 1.48) and 1.64 (1.00 to 2.71), respectively. If 'diabetic retinopathy' (see Methods) was taken as the dependent variable, the odds ratio for hyperhomocysteinemia was 1.11 (0.36 to 3.41) in non-diabetic and 4.45 (1.21 to 16.37) in diabetic subjects. Per category increment of serum tHcy, it was 0.84 (0.40 to 1.79) in non-diabetic and 2.45 (0.95 to 6.32) in diabetic subjects, and per 5 μmol/L increment of serum tHcy it was 0.96 (0.55 to 1.66) and 1.45 (0.84 to 2.53), respectively.

Discussion

This is, to the best of our knowledge, the first population-based study showing that hyperhomocysteinemia is a risk factor for retinopathy in type 2 diabetic subjects, independent of known determinants, i.e., diabetes duration, glycemic level and hypertension. We found a dose-response

relation between serum tHcy and retinopathy among type 2 diabetic subjects (Table 2). For each 5 μmol/L (about 1 SD) increment in serum tHcy level, the risk of retinopathy rose by about 50% (95% CI: -7% to 141%) in the diabetic subjects. Hyperhomocysteinemia, arbitrarily defined as serum tHcy >16 μmol/L, was also related to retinopathy among type 2 diabetic subjects [odds ratio 3.4 (95% CI: 1.1 to 10.6)]. The results of the present study are in line with two studies that reported a higher serum tHcy level in type 1 and type 2 diabetic subjects with diabetic retinopathy than in those without.[28,29] Another study showed an association between the C677T polymorphism of the methylenetetrahydrofolate reductase (MTHFR) and presence of diabetic retinopathy among type 2 diabetic patients.[30] A point mutation in the MTHFR gene results in impaired enzyme activity, leading to an exaggerated hyperhomocysteinemic response to a low intake of folic acid. In contrast, Agardh et al.[31] and Araki et al.[32] found no association between hyperhomocysteinemia and diabetic retinopathy in type 1 and 2 diabetes, respectively. However, none of these studies adjusted for the known determinants of retinal vasculopathy and one[31] was rather small.

Diabetic retinopathy involves both morphological and functional changes of the retinal capillaries.[33,34] Hyperhomocysteinemia may induce endothelial dysfunction and injury followed by platelet activation and thrombus formation, possibly by increasing oxidative stress.[35] Therefore, it is conceivable that hyperhomocysteinemia is causally related to retinal vasculopathy through changes of the retinal vasculature and formation of microthrombi. Since oxidative stress is thought to be increased in type 2 diabetes,[36] this may make them more susceptible to hyperhomocysteinemia-induced oxidative damage.

We can think of three sources of disease misclassification that may have resulted in bias of the relation between hyperhomocysteinemia and retinopathy. Of 24% of all subjects, the fundus photographs were missing, and therefore in these subjects the diagnosis of retinopathy was solely dependent on the ophthalmoscopic examination. There is evidence that the sensitivity to detect retinopathy by ophthalmoscopy, even in the hands of an experienced ophthalmoscopist, is lower than that of fundus photography using color or black/white transparencies.[24,38-40] In addition, the method of detecting any retinopathy with two stereoscopic standard fields compared to seven is slightly less sensitive (sensitivity about 0.85).[39] Finally, a number of early small lesions may be missed on 45° fundus photographs compared with photographs taken with a smaller angle. All three limitations of the present study may have introduced false negative disease misclassification,

which was, in all likelihood, nondifferential with regard to serum tHcy level. This would tend to underestimate the strength of the reported relation between hyperhomocysteinemia and retinopathy.[41]

The Beaver Dam Eye Study is a population-based study among non-diabetic individuals aged 43 through 84 years that reported a prevalence of retinopathy of 7.8% as assessed by means of two standard photographic fields.[42] The Rotterdam Study, a population-based study of the elderly (aged >=55 years) reported a prevalence of retinopathy of 4.8%, as detected by grading one standard photographic field,[7] which is lower than the 10.7% we found. The difference in reported prevalences may partly be explained by less sensitive methods used to detect retinopathy in the Rotterdam Study. In the present study both ophthalmoscopic and photographic findings were used to asses the presence of retinopathy. The low agreement we found between retinal photography and ophthalmoscopy is comparable with other studies.[43,44]

We evaluated a possible dose-response relation between serum tHcy and retinopathy, because it is not known whether this relation is graded or has a certain threshold. The limited number of subjects with retinopathy, however, did not allow for a precise assessment of the presence of a possible threshold, which may be at 16 μmol/L among type 2 diabetic subjects, but this result clearly needs to be confirmed in other studies. The boundaries of the serum tHcy categories were quite broad and chosen post-hoc. Another limitation is that, due to the limited number of subjects with retinopathy, we could not explore the association between tHcy and the separate degrees of diabetic retinopathy. Finally, because we did not assess B vitamins and the present study is cross-sectional, we cannot rule out the possibility that low vitamin B levels may cause diabetic retinopathy or that diabetic retinopathy *per se* can raise serum tHcy levels, although the latter appears biologically implausible.

Treatment with folic acid can lower serum tHcy levels by about 30%.[45] If the finding of the present study, that hyperhomocysteinemia is a risk factor for diabetic retinopathy among type 2 diabetic patients, can be confirmed in a prospective study, this may have important implications with regard to slowing down the progression of diabetic retinopathy and/or preventing its occurrence.

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Hyperhomocysteinemia is not related to risk of distal somatic polyneuropathy
The Hoorn Study

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Abstract

Background Distal somatic polyneuropathy is a major contributing factor in the pathogenesis of chronic foot infections and ulcers, leading to lower limb amputations, but its causes are poorly understood. Both metabolic and vascular abnormalities may contribute to the development of impaired nerve function. Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease. It is not known whether hyperhomocysteinemia is associated with neuropathy, and thus a possible cause of neuropathy.

Methods We studied an age-, sex- and glucose tolerance-stratified random sample of a 50- to 75-year-old general Caucasian population ($N=595$). Any polyneuropathy ($N=95$) was defined as the absence of at least two of the three following sensory modalities or reflexes of either foot: light touch sense, ankle reflex, and vibration sensation. Definite polyneuropathy ($N=25$) was present if, in addition, the vibration perception threshold of the right big toe was abnormal.

Results The prevalence of any polyneuropathy was 12.4% (33 of 266) in subjects with normal glucose tolerance (NGT), 12.6% (21 of 167) in those with impaired glucose tolerance (IGT), and 25.3% (41 of 162) in those with type 2 diabetes. The prevalence of definite polyneuropathy was 2.6% (7 of 266) in subjects with NGT, 2.4% (4 of 167) in those with IGT, and 8.7% (14 of 161) in type 2 diabetic subjects. After adjustment for age, sex, HbA_{1c} and hypertension, the odds ratio (95% CI) for any polyneuropathy per 5 μmol/L (about 1 SD) serum total homocysteine increment was 1.00 (0.72 to 1.39). After adjustment for age and sex, it was 0.62 (0.21 to 1.89) for definite polyneuropathy.

Conclusion Although a weak relation (as judged from the confidence intervals) cannot be excluded, we conclude that hyperhomocysteinemia is probably not related to risk of distal somatic polyneuropathy.

Introduction

Distal somatic polyneuropathy is one of the most common complications of type 2 diabetes. Neuropathy is a major contributing factor in the pathogenesis of chronic foot infections and ulcers, sometimes leading to lower limb amputations.[1] The estimates of the prevalence of distal polyneuropathy vary due to various definitions, but clinically neuropathy is found in approximately 30% of patients with type 2 diabetes.[2] The intensity and extent of the functional and anatomical abnormalities of diabetic neuropathy parallel the degree and duration of hyperglycemia, but the pathogenesis is unknown.[3,4] Chronic hyperglycemia increases oxidative stress which may induce functional and structural changes of the nerve tissue.[5,6] The development of diabetic neuropathy is not only associated with metabolic changes in the nerve, but also with reduced tissue oxygenation and nerve blood flow.[7] Therefore, one mechanism linking oxidative stress to neuropathy is oxygen free-radical-induced injury to the vascular endothelium.

Hyperhomocysteinemia is a recently recognized risk factor for cardiovascular disease independent of major risk factors.[8-10] In a cross-sectional analysis, hyperhomocysteinemia also appeared to be a risk factor for microalbuminuria,[11] i.e. a putative marker of endothelial dysfunction.[12] It is currently thought that hyperhomocysteinemia may induce endothelial dysfunction through increasing oxidative stress.[13] It is not known whether hyperhomocysteinemia is associated with neuropathy and may thus be a contributing factor in the pathogenesis of diabetic neuropathy. In view of these considerations, we investigated this issue in a 50- to 75-year-old general population.

Methods

Design and study population

The Hoorn Study is a cross-sectional survey of glucose tolerance and other cardiovascular risk factors in a 50- to 75-year-old general Caucasian population conducted from 1989 to 1992.[14] A random sample of all men and women aged 50 to 75 years was drawn from the municipal population registry office of Hoorn (The Netherlands); 2484 subjects participated (response rate 71%). All subjects, except previously diagnosed diabetic subjects treated with oral glucose-lowering agents or insulin, underwent a 75 g oral glucose tolerance test (OGTT) and were classified according to the WHO (1985) criteria.[15] A second OGTT (participation rate 93%) was

performed, for reasons of efficiency, in a random subsample (*N*=1122), stratified by 2-hour glucose values of the first test, age and sex. Finally, from this subsample another age-, sex-, and glucose tolerance-stratified random sample (*N*=708) was drawn. The presence of distal polyneuropathy was assessed (*N*=629; response rate 89%) as detailed below. We excluded subjects who used diphantoine (*N*=1), or were known to have a neurological disorder (*N*=33: Parkinson's disease, history of lumbar disc disease, cerebrovascular event, systemic lupus erythematosus or spinal cord lesion) which could influence results of neurological examination. Glucose tolerance was divided into three categories on the basis of the mean of the two OGTTs: normal glucose tolerance (NGT, *N*=266), impaired glucose tolerance (IGT, *N*=167) and type 2 diabetes mellitus (*N*=162). The Hoorn Study was approved by the Ethical Review Committee of the University Hospital Vrije Universiteit Amsterdam. Informed consent was obtained from all participants.

Nerve function

Clinical neurological examination

The clinical neurological examination included testing of sensory functions and tendon reflexes of the lower extremities. A single observer classified, as present or absent, light touch sense (cotton wool) of the dorsum of both feet, ankle reflexes, and vibratory sensation at the medial malleoli (128 Hz tuning fork) in each subject.

Quantitative sensory testing

Vibration perception threshold (VPT) expressed in μm was assessed at the dorsum of the right big toe. The VPT was measured using a Vibrameter (Somedic, Stockholm, Type 4). The amplitude of a probe, vibrating at 100 Hz, was increased from zero and the subject was asked to indicate the moment when he or she started to feel the vibration (method of limits) as previously described in detail.[16] The VPT was measured by one of two observers. An abnormal VPT was defined as a value exceeding the 95% upper limit of the age-, sex-, and height-specific reference value derived from subjects of the present study population with NGT, as described elsewhere.[16]

There is no 'gold standard' available for the assessment of distal somatic polyneuropathy. Therefore, we defined polyneuropathy in two different ways. First, *any polyneuropathy* (*N*=95) was defined as the absence

of at least two of the three following sensory modalities or reflexes of either foot: light touch sense, ankle reflex, and vibration sensation. Second, we defined *definite polyneuropathy* ($N=25$) if, in addition, the VPT of the right big toe was abnormal.

Measurement of serum total homocysteine (tHcy)

Fasting blood samples were centrifuged within one hour following collection. Serum was stored at -20°C for 4 to 6 years. There is good evidence that serum tHcy levels in frozen samples are stable for 10 years or more.[17] Serum total (free plus protein bound) homocysteine level was measured by using tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by high-performance liquid chromatography with fluorescence detection.[18] The intra- and interassay coefficients are 2.1% and 5.1%.

Other measurements

Subjects were classified as either current smoker or nonsmoker of cigarettes. Body height was measured with all subjects barefoot. Waist-hip ratio was measured as previously described.[14] Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Blood pressure was measured as the mean of, in total, four measurements, performed on two different occasions, with a random zero sphygmomanometer under standardized conditions. Fasting and 2-hour post-load venous plasma glucose levels were measured with a glucose dehydrogenase method (Merck, Darmstadt, Germany) and glycated hemoglobin (HbA_{1c}) by ion-exchange high-performance liquid chromatography, using a Modular Diabetes Monitoring System (Bio-Rad, Veenendaal, the Netherlands). Immunospecific insulin was measured in serum by a double-antibody radioimmunoassay (lot SP21, Linco Research, St. Louis, U.S.A.). The interassay coefficient of variation was 6%. The lower limit of sensitivity was 12 pmol/L. Fasting serum total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides were measured by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany). Information on alcohol and vitamin B_6 intake was obtained by a self-administered validated semi-quantitative food frequency questionnaire ($N=586$).[19] The relative difference of the mean intake from the self-administered questionnaire relative to the modified

Burke's dietary history interview[20] was –0.4% for alcohol (g/day) and 9.9% for vitamin B_6 (mg/day).

All laboratory and nerve function measurements were carried out by technicians unaware of the subjects' history of cardiovascular disease and glucose tolerance status.

Statistical analysis

Variables are presented as mean ± standard deviation (SD), number (percentage of the total) or, in case of skewed distribution, median and interquartile range (IQR). Differences between subjects with and without any or definite polyneuropathy were tested with Student's *t* or Wilcoxon's rank sum test for continuous variables and Pearson's chi-square test for frequency measures. Associations of risk factors for polyneuropathy with serum tHcy level (logarithmically transformed) were studied by calculating Pearson's correlation coefficients.

We performed logistic regression analyses to study the relation between serum tHcy and polyneuropathy (any and definite). We chose two different approaches to investigate the nature of the relation between tHcy and polyneuropathy, because it is not known whether this relation, if any, is linear or has a certain threshold. We calculated odds ratios and 95% confidence intervals (CI), both for serum tHcy as a categorical (to allow for a non-linear dose-response relation and to reduce the influence of outliers) and as a continuous variable, the latter expressed per 5 μmol/L (about 1 SD) increment of serum tHcy. For the analyses with serum tHcy as a categorical variable, we calculated odds ratios for tHcy divided in two (>14 μmol/L versus <=14 μmol/L) and in three categories (<=9.0 μmol/L, 9.1 to 14.0 μmol/L and >14.0 μmol/L). To test for trend, the three categories were entered in the model as an ordinal variable. (In the present study, we did not find a clear threshold above which risk of polyneuropathy increased. Therefore, we defined hyperhomocysteinemia as serum tHcy levels higher than 14 μmol/L.[21]) We used multiple logistic regression analysis to control for potential confounders, i.e.: age, sex, height, diabetes, HbA_{1c}, alcohol intake, and current smoking. We also tested models which in addition included blood pressure, serum total cholesterol, HDL cholesterol, triglycerides, fasting insulin, BMI, or waist-hip ratio. Moreover, the association between known diabetes duration and polyneuropathy was investigated in a subanalysis among subjects known to have diabetes mellitus. Finally, because a low vitamin B_6 is associated with neuropathy,[22] we investigated the relation between vitamin B_6 intake and polyneuropathy.

Table 1. Characteristics of the subjects

	No polyneuropathy		Any polyneuropathy		*P*-value*
N	500		95		
Men *%*	45.4		57.9		0.03
Age *years*	63.5	(7.2)	68.5	(5.6)	<0.001
Body height *cm*	167.4	(9.0)	171.7	(9.0)	<0.001
Body mass index kg/m^2	27.2	(4.0)	27.5	(3.7)	0.6
Waist-hip ratio	0.91	(0.09)	0.95	(0.08)	<0.001
Systolic blood pressure *mmHg*	139	(20)	142	(19)	0.2
Diastolic blood pressure *mmHg*	83	(10)	83	(10)	1.0
Current smoker *%*	28.6		30.5		0.7
Vitamin B_6 intake *mg/day*	1.4	(0.4)	1.5	(0.4)	0.03
Alcohol intake, >=moderate† *%*	7.9		8.7		0.8
Type 2 diabetes mellitus	24.2		43.2		<0.001
Duration of diabetes‡ *years*	6.7	(2.4-15.1)	6.3	(2.8-10.8)	0.4
HbA_{1c} *% of hemoglobin*	5.8	(1.1)	6.5	(1.9)	<0.001
Fasting insulin *pmol/L*	82	(62-117)	92	(68-135)	0.1
Total cholesterol *mmol/L*	6.7	(1.2)	6.4	(1.3)	0.02
HDL cholesterol *mmol/L*	1.3	(0.3)	1.2	(0.3)	0.05
Triglycerides *mmol/L*	1.5	(1.2-2.2)	1.6	(1.1-2.2)	0.7
Total homocysteine *µmol/L*	11.3	(9.2-14.0)	12.0	(10.1-14.4)	0.2

Data are presented as mean (SD), percentage of the total or median (interquartile range).

* Tested with Student's *t*-test or Wilcoxon's rank sum test for continuous variables and Pearson's chi-square test for frequencies.
† >30 g/day
‡ Diabetes duration of those subjects known with diabetes mellitus.

Possible interaction between tHcy and diabetes with regard to risk of polyneuropathy was assessed in a stratified analysis and with an interaction term. All analyses were performed with SPSS for Windows 7.5.2. A 95% CI not including 1.0 was considered to indicate statistical significance.

Results

Any polyneuropathy

The baseline characteristics of the study population are presented in Table 1. The prevalence of any polyneuropathy was 12.4% (33 of 266) in subjects with NGT, 12.6% (21 of 167) in those with IGT, and 25.3% (41 of 162) in diabetic subjects; it was 15.6% (69 of 442) in subjects with

serum tHcy levels <=14 μmol/L and 17.0% (26 of 153) in those with serum tHcy levels >14 μmol/L.

The median serum tHcy level was 12.2 μmol/L (IQR: 9.9-15.3) in men and 10.8 μmol/L (IQR: 9.0-13.3) in women. Serum tHcy levels correlated with age (r=0.17; P<0.001). After adjustment for age and sex, there was a weak inverse correlation between serum tHcy and vitamin B_6 intake (r=-0.08; P=0.05), but there was no correlation between serum tHcy and the following variables: body height (r=0.03; P=0.4), HbA_{1c} (r=-0.04; P=0.3), or duration of diabetes in subjects known with diabetes (r=-0.02; P=0.9).

High serum tHcy levels were not related to risk of any polyneuropathy, whereas known risk factors such as diabetes, HbA_{1c}, and body height were positively related. In addition, a high vitamin B_6 intake was also related to risk of any polyneuropathy (Table 2). Additional adjustment for other potential confounders did not affect the results (data not shown). After stratification for diabetes and adjustment for age and sex, the odds ratio (95% CI) per 5 μmol/L increment of serum tHcy for any polyneuropathy was 0.91 (0.60 to 1.39) in non-diabetic and 1.29 (0.83 to 2.00) in diabetic subjects (P=0.4 for interaction). After additional adjustment for HbA_{1c}, diabetes duration, and height, the odds ratio among diabetic subjects was 1.15 (0.67 to 1.99).

Definite polyneuropathy

Data on one diabetic subject were missing. The prevalence of definite polyneuropathy was 2.6% (7 of 266) in subjects with NGT, 2.4% (4 of 167) in those with IGT, and 8.7% (14 of 161) in diabetic subjects; it was 4.8% (21 of 442) in subjects with serum tHcy levels <=14 μmol/L and 2.6% (4 of 152) in those with serum tHcy levels >14 μmol/L. After adjustment for age and sex, the odds ratio (95% CI) for definite polyneuropathy was 0.62 (0.21 to 1.89) for serum tHcy >14 μmol/L, 0.94 (0.49 to 1.78) per category increment of serum tHcy level, 1.23 (0.75 to 2.02) per 5 μmol/L increment of serum tHcy level, 3.28 (1.44 to 7.47) for diabetes, 1.38 (1.13 to 1.68) per % increment of HbA_{1c}, 2.18 (0.68 to 6.49) per mg increment of daily vitamin B_6 intake, 1.05 (0.99 to 1.12) per cm increment of body height, 0.79 (0.29 to 2.20) for current smoking, and 0.76 (0.10 to 6.00) for >= moderate alcohol intake. Finally, we also performed multiple linear regression analysis with logtransformed VPT as the (continuous) dependent variable. After adjustment for age, sex, height and diabetes, there was no association between serum tHcy and VPT (P=1.0).

Table 2. Odds ratios (95% confidence intervals) for any polyneuropathy

Risk factors	Adjusted for age and sex	* Adjusted for age, sex, and other risk factors
Hyperhomocysteinemia (>14 vs <=14 µmol/L)	0.78 (0.46 - 1.32)	0.85 (0.49 - 1.50)
Total homocysteine (per category increment)†	0.90 (0.63 - 1.29)	0.93 (0.63 - 1.37)
Total homocysteine (per 5 µmol/L increment)‡	1.04 (0.77 - 1.40)	1.00 (0.72 - 1.39)
Diabetes (yes/no)	2.25 (1.39 - 3.63)	2.00 (1.19 - 3.34)
HbA_{1c} (per % increment)	1.37 (1.17 - 1.61)	1.34 (1.13 - 1.59)§
Body height (per cm increment)	1.10 (1.06 - 1.14)	1.10 (1.06 - 1.14)
Vitamin B_6 intake (per mg/day increment)	2.56 (1.34 - 4.89)	1.88 (0.96 - 3.70)
Current smoking (yes/no)	1.25 (0.75 - 2.09)	1.40 (0.81 - 2.40)
>=Moderate alcohol intake (yes/no)	1.12 (0.48 - 2.63)	1.19 (0.49 - 2.87)

* Adjusted for age, sex, body height, diabetes and vitamin B_6 intake. When these analyses were adjusted for homocysteine, homocysteine was entered as a 3-category variable in the models.
† Serum total homocysteine was divided in three categories: <=9.0 µmol/L, 9.1 to 14.0 µmol/L, and >14.0 µmol/L (see Methods).
‡ After exclusion of 5 outliers (5 subjects with normal glucose tolerance who had serum total homocysteine >35 µmol/L).
§ Not adjusted for diabetes.

Discussion

This population-based study showed a relation between distal somatic polyneuropathy and known risk factors such as diabetes, hyperglycemia, and body height (a proxy for neuron length),[23] but not with hyperhomocysteinemia. The latter result of the present study is in agreement with Hofmann et al.,[24] but not with Araki et al.[25] Araki et al. found a positive association between high levels of serum tHcy and neuropathy among type 2 diabetic patients. However, the reported association was not adjusted for known risk factors such as age and hyperglycemia.

We considered several factors that may explain why we did not find a relation between serum tHcy levels and neuropathy. First, the intracellular metabolism of homocysteine is dependent on both enzymes and B vitamins. Tissue-specific patterns of homocysteine metabolism may result in tissue-specific pathologies. This may explain that hyperhomocysteinemia is related to risk of microalbuminuria, but not to risk of distal neuropathy, although both are thought to be an expression of microangiopathy.[26] On the other hand, the pathophysiological role of microangiopathy might be less important in the development of neuropathy compared to microalbuminuria. Second, the presence of neuropathy might not have been adequately assessed

in the present study. However, this is not supported by our finding that well-known risk factors such as diabetes and hyperglycemia were strongly related to risk of both any and definite polyneuropathy. We used clinical neurological examination of sensory functions and tendon reflexes, because this has proved to be a valid and sensitive method for the screening of neuropathy.[27] The absence of light touch sense and ankle reflexes are thought to be early indicators of neuropathy.[28] In this manner we intended to identify all subjects with any sign of polyneuropathy. Furthermore, we identified a group of subjects with definite neuropathy by assessing the VPT in addition to the clinical neurological examination.

The linear positive association between vitamin B_6 intake and neuropathy was an unexpected finding. As far as we know, there are no other studies that reported a similar result. We can offer three potential explanations for this finding. First, a very high (>100 mg/day) intake of vitamin B_6 is known to be neurotoxic,[29,30] and it is theoretically conceivable that much lower intakes have a similar effect. This possibility requires further investigation, because the desirability of an increase in the recommended daily intake of vitamin B_6 is currently debated. Second, it is possible that subjects with subjective complaints due to neuropathy changed their dietary habits, resulting in an increased vitamin B_6 intake. Finally, we cannot exclude that a high vitamin B_6 intake is a marker for some unmeasured variable that is in fact related to neuropathy. Therefore, our finding on vitamin B_6 intake and neuropathy should be interpreted with caution.

Although a weak relation (as judged from the confidence intervals) cannot be excluded, we conclude that hyperhomocysteinemia is probably not related to risk of distal somatic polyneuropathy.

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8

Does metformin increase the serum total homocysteine level in non-insulin-dependent diabetes mellitus?

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Abstract

Background An elevated serum total homocysteine level is a risk factor for atherosclerosis. Metformin decreases serum vitamin B_{12} and may thereby indirectly increase the serum total homocysteine level. The aim of this study was to estimate the effect of metformin on the serum total homocysteine level in non-insulin-dependent diabetes mellitus (NIDDM) patients.

Methods Fasting serum total homocysteine level was measured in 40 NIDDM patients who had received treatment with metformin (500 to 2550 mg/day) for at least 6 months and in 71 NIDDM patients not treated with metformin and matched for sex, age (± 5 years), serum creatinine (± 5 μmol/L) and current smoking habits. 'Exposed' patients were matched with 'nonexposed' patients. A two-way analysis of variance was performed.

Results The mean serum total homocysteine level was 11.5 μmol/L in the metformin-exposed patients and 10.6 μmol/L in the nonexposed patients. Thus, the metformin-exposed patients had slightly higher serum total homocysteine levels (difference 0.8 μmol/L, 95% confidence interval, -0.4 to 2.0 μmol/L). Results were similar in men and women. Finally, no dose-response relation between cumulative exposure to metformin (dose x duration of treatment) and the serum total homocysteine level could be demonstrated.

Conclusion We conclude that the effect of metformin on serum total homocysteine level in NIDDM patients, if any, is likely to be small.

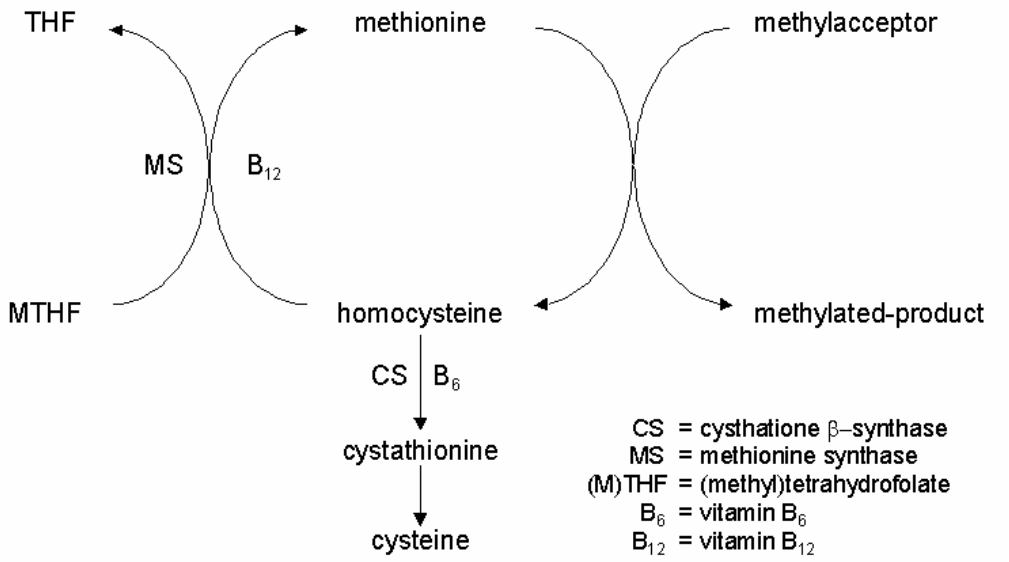

Figure 1. Methionine-homocysteine metabolism.

Introduction

Non-insulin-dependent diabetes mellitus (NIDDM) is accompanied by accelerated atherosclerosis which can be explained, in part, by concomitant hypertension and dyslipidemia. There is an approximately three-fold increased risk of cardiovascular disease in NIDDM patients.[1]

The most effective treatment for NIDDM is currently under debate. The University Group Diabetes Program reported that sulfonylureas[2] and a biguanide (phenformin)[3] appeared to increase cardiovascular mortality significantly as compared with diet alone. In addition, it is unknown whether intensive insulin treatment of NIDDM decreases the risk for cardiovascular complications by lowering glucose levels, or increases the risk by postulated direct atherogenic effects.[4] Finally, the United Kingdom Prospective Diabetes Study has not shown any specific therapy (sulphonylurea, insulin or metformin) to be advantageous or disadvantageous as compared with diet alone with respect to the incidence of major cardiovascular complications,[5] although metformin favourably modifies secondary clinical alterations due to insulin resistance, such as arterial hypertension, obesity and hyperlipidemia. A possible explanation for this apparent discrepancy could be a deleterious effect of metformin on serum total homocysteine levels.

An elevated serum total homocysteine level is a recently recognized risk factor for atherosclerosis that is independent of 'classical' risk factors.[6] It has not been extensively investigated whether an association exists between non-insulin-dependent diabetes mellitus and serum total homocysteine level.[7,8]

Treatment with metformin may increase serum total homocysteine levels in NIDDM. Metformin decreases serum vitamin B_{12} levels by up to 30% by inducing vitamin B_{12} malabsorption.[9,10] Low serum vitamin B_{12} may then increase the serum total homocysteine level[11] (Figure 1). Metformin may induce malabsorption of vitamin B_{12} by two different mechanisms. First, metformin can bind free calcium, which is required for the uptake of the vitamin B_{12}-intrinsic factor-complex in the ileum by its receptor.[12] The second mechanism is permanent and mediated by depression of intrinsic factor secretion.[13] In addition, subclinical vitamin B_{12} deficiency is common among the elderly[14-16] and it is therefore important to know whether the use of metformin aggravates the metabolic consequences of low vitamin B_{12} levels among elderly NIDDM patients. There are no data, however, on whether the use of metformin by NIDDM patients is associated with an increase in serum total homocysteine levels. Therefore, in order to estimate the effect of metformin on the serum total homocysteine level in NIDDM patients, we compared serum total homocysteine levels in a group of

metformin-treated NIDDM patients to those in a matched group of patients not treated with metformin.

Patients and methods

From 1992 to 1995 a cohort study investigating the influence of glycemic control on well-being in primary care NIDDM patients was carried out in Hoorn, the Netherlands.[17] Twenty seven of 31 general practitioners agreed to take part in this study, which was approved by the ethical review committee of the University Hospital Vrije Universiteit, Amsterdam. Informed consent was obtained from all participants. All participating general practitioners were encouraged to make treatment decisions according to a standardized step-up regimen based on the standard of the Netherlands College of General Practitioners. The main indication for treatment with metformin was NIDDM with a BMI >27 kg/m^2 if prior dietary measures (± sulphonylureas) did not normalize fasting blood glucose levels. Contraindications for metformin use were impairments in renal and/or liver function, or heart failure.

For the present investigation we selected Caucasian patients between 40 and 75 years of age who had used metformin for at least 6 months. Forty NIDDM patients who had received treatment with metformin (500 to 2550 mg/day) for at least 6 months were matched with 71 NIDDM patients never treated with metformin. Matching was performed for the following determinants of the serum total homocysteine level: sex, age (± 5 years), serum creatinine (± 5 μmol/L) and current smoking habits. Each 'exposed' patient was matched with two 'nonexposed' patients, except in nine individuals who could be matched with only one nonexposed patient. We excluded patients using vitamin B_{12} or folic acid supplements, or medications known to interfere with folic acid metabolism; we also excluded patients with renal impairment (serum creatinine >120 μmol/L), previous gastric surgery, a past or current history of hypothyroidism, malignant disease, psychiatric disease, or treatment with antibiotics at the time of investigation. For the metformin users we collected data (see below) at the time of their longest metformin use, which could be precisely reconstructed from individual patients' records. For the matched patients, we collected data at the time that the variables for which matching was performed showed the smallest difference with the corresponding variables in the exposed group. We thus recorded age, sex, BMI, diabetes duration, and current smoking habits (yes or no). Blood pressure was defined as the average of two readings recorded on the right arm of seated patients after at least five minutes of rest

with a random zero mercury sphygmomanometer (Hawksley-Gelman, Lancing, Sussex, UK). Hypertension was defined as blood pressure >=160 mmHg systolic and/or >=95 mmHg diastolic and/or the current use of antihypertensive medication. We also assessed the previous cardiovascular history. A history of myocardial infarction, angina pectoris, stroke, transient ischemic attack or intermittent claudication was considered present if corroborated by written information from the patients' physician(s). Finally, a blood sample was taken in the fasted state.

We measured glycated hemoglobin (by HPLC; Bio-Rad, Veenendaal NL, the Netherlands; reference range, 4.3 to 6.1%), serum creatinine (modified Jaffé method), total cholesterol (enzymatic techniques; Boehringer-Mannheim, Germany) and serum total homocysteine. Fasting blood samples were centrifuged within one hour following collection. Fasting blood samples were stored a comparable time at -20°C; the median storage periods for the nonexposed patients and exposed patients were 1.9 and 1.5 years, respectively ($P=0.3$). There is good evidence that serum total homocysteine levels are stable in serum for 10 years or more.[14,18] To minimize the imprecision of the assay, all samples were analyzed in the same run in December 1995. Serum total (free plus protein bound) homocysteine level was measured by using tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by HPLC with fluorescence detection.[19] The intra- and interassay coefficients of variation are 2.1% and 5.1%, respectively. Reference values are 8 to 18 μmol/L for men, 6 to 15 μmol/L for premenopausal women and 6 to 19 μmol/L for postmenopausal women.

Statistical methods

The patients were categorized by exposure to metformin. Descriptive data are given as mean (SD), median (interquartile range) or number (percentage of the total). Differences in their baseline characteristics were then evaluated by means of a Mann-Whitney test for numerical variables, and a chi-square test for categorical variables.

A two-way analysis of variance was performed in order to estimate the effect of use of metformin on serum total homocysteine levels, with the matching structure of the study taken into account. The resulting 95% confidence interval for this effect is presented. This analysis was repeated for men and women separately; the resulting effect estimates were compared, so as to exclude the possibility of effect modification by sex.

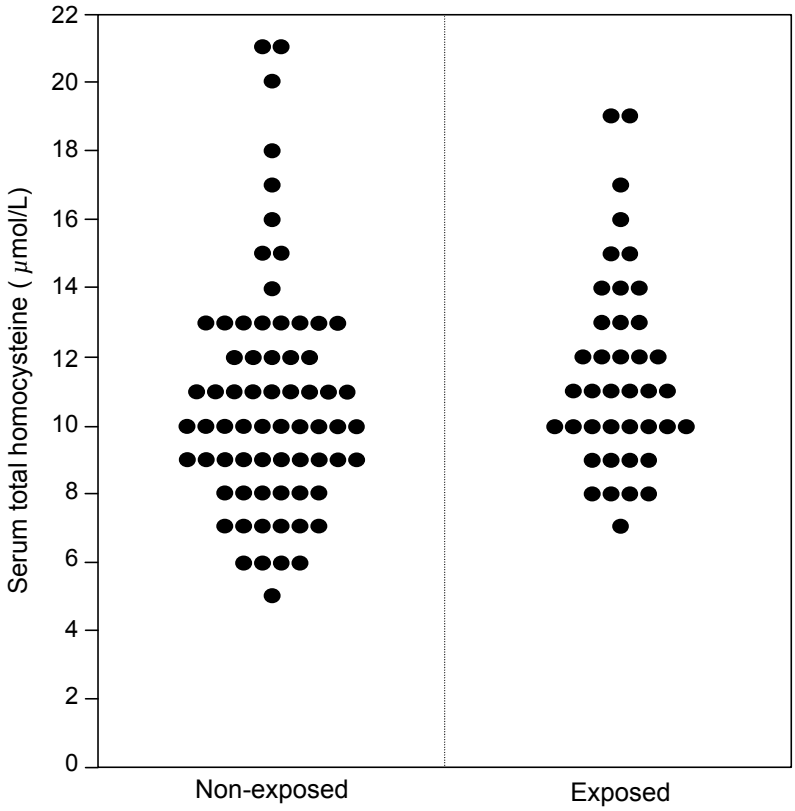

Figure 2. Serum total homocysteine levels in 71 NIDDM patients not exposed to metformin and in 40 patients exposed to metformin.
Values shown have been rounded.

In order to investigate the possible dose-response relation between exposure to metformin and serum total homocysteine level, we performed a linear regression analysis, with cumulative exposure to metformin, defined as prescribed dose x duration, as the independent variate. As the dependent variate we chose the difference between the serum total homocysteine level of an exposed patient and the mean of the serum total homocysteine levels of the corresponding matched nonexposed patients. We made this a weighted regression analysis, with lower weights allocated to matched pairs than to triplets. Statistical analyses were performed with SPSS for Windows 6.1. Two-sided *P*-values <0.05 were considered statistically significant.

Results

Table 1 shows the clinical and the laboratory data. The mean serum total homocysteine level in the exposed patients was 11.5 μmol/L (range, 7.0 to 19.2), and in the nonexposed patients was 10.6 μmol/L (range, 4.9 to 20.8). The serum total homocysteine levels of exposed and

Table 1. Patient characteristics by exposure to metformin

	Non-exposed		Exposed		*P*-value
N (*% men*)	71	(52)	40	(53)	
Age *years*	63	(9)	64	(9)	
NIDDM duration *years*	5	(3-9)	6	(4-10)	0.3
Current smokers	13	(18)	8	(20)	
BMI kg/m^2	28.4	(4.0)	29.6	(4.6)	0.2
Diabetes therapy					
Metformin *mg/day current*			1000 (500-2550)*		
Metformin *use years*			1	(0.5-2.0)*	
Sulfonylurea *%*	54	(76)	28	(70)	0.4
Insulin *%*	16	(23)	1	(3)	0.006
Diet only *%*	1	(1)			
History of cardiovascular events *%*	17	(24)	9	(23)	0.8
Angina pectoris *%*	8	(11)	2	(5)	
Myocardial infarction *%*	8	(11)	4	(10)	
Transient ischemic attack *%*	1	(3)			
Stroke *%*	1	(3)			
Intermittent claudication *%*	2	(3)	3	(8)	
Blood pressure *mmHg*	148/84	(24/14)	145/82	(21/14)	0.2/0.4
Hypertension *%*	45		55		0.2
HbA_{1c} *%*	7.4	(1.6)	7.4	(1.3)	0.8
Serum total cholesterol *mmol/L*	6.1	(1.2)	6.0	(1.0)	0.7
Serum creatinine *μmol/L*	81	(11)	81	(11)	
Serum total homocysteine *μmol/L*	10.6	(3.3)	11.5	(3.0)	0.1

Data are means (SD), *N* (%), or median (interquartile range) or (* range); *P*-values are reported for the non-matched variables only.

nonexposed patients are shown in Figure 2. The metformin-exposed patients had slightly higher serum total homocysteine levels (difference 0.8 μmol/L, 95% CI: -0.4 to 2.0). The difference was similar in men (0.4 μmol/L, 95% CI: -1.5 to 2.3) and women (1.2 μmol/L, 95% CI: -0.3 to 2.6). Furthermore, a dose-response relation between cumulative exposure to metformin and the serum total homocysteine level could not be demonstrated.

Discussion

We investigated the effect of metformin on the serum total homocysteine level in NIDDM patients and found that this effect, if any, appeared to be small.

High concentrations of metformin accumulate in the wall of the gastrointestinal tract and may induce vitamin B_{12} malabsorption.[20] Evaluating the status of vitamin B_{12} by measuring serum concentrations or hemoglobin level and erythrocyte mean corpuscular volume can be misleading.[14,18,21,22] Therefore, even though there are, during metformin therapy, no changes in hemoglobin level or hematocrit,[9] this does not indicate that the intracellular vitamin B_{12} concentration is normal.[18] However, vitamin B_{12} is necessary for the metabolism of homocysteine. Thus, an elevated concentration of serum total homocysteine level has proven to be a highly sensitive indicator of tissue deficiency of vitamin B_{12}.[15,23] Therefore, and because an elevated serum total homocysteine level is a cardiovascular risk factor,[6,7] we chose to measure serum total homocysteine level in order to estimate intracellular vitamin B_{12} deficiency. Moreover, circulating vitamin B_{12} does not necessarily mirror intracellular vitamin B_{12}. It has been estimated that 5 to 10% of all patients with clinical vitamin B_{12} deficiency have normal to high serum levels of vitamin B_{12}.[23] This finding may be even more frequent in elderly patients.[16] In addition, it has been observed that 25 to 50% of patients with low serum vitamin B_{12} do not have evidence for tissue or clinical vitamin B_{12} deficiency. An explanation for this discrepancy may be the distribution of binding of vitamin B_{12} to transcobalamin (TC) I and II. A minor fraction of serum vitamin B_{12}, 10 to 20%, is bound to TCII, which plays an important role in cellular delivery of vitamin B_{12}. Therefore, a decrease in TCII-bound vitamin B_{12} may impair cellular vitamin B_{12} delivery without an important decrease in serum vitamin B_{12} levels.[24]

The prescription of metformin is related to the BMI, which, however, shows no association with the total homocysteine level.[25] Therefore, it is unlikely that the prescription practice confounded the results. We matched for important determinants of the serum total homocysteine level i.e., sex, age, serum creatinine and current smoking habits.[21,26-33] Finally, we decided to restrict this study to a Caucasian population, because there is evidence for differences in homocysteine metabolism among races. For example, blacks may metabolize homocysteine more effectively than whites.[34]

The difference of the serum total homocysteine level between exposed and nonexposed patients, 0.8 μmol/L, was small and not statistically significant. Possibly the mean duration (one year) and the mean dose

(1000 mg metformin/day) were too short and too small, respectively, to induce vitamin B_{12} deficiency. The total-body vitamin B_{12} pool has been estimated to be about 2 to 3 mg. Daily losses of vitamin B_{12} are approximately 0.1% of the body pool, resulting in a long half-life of vitamin B_{12} (480 to 1360 days).[35] The vitamin B_{12} pool is partly supplied by food and partly by reabsorption of vitamin B_{12} excreted into the bile. Therefore, malabsorption can induce vitamin B_{12} deficiency in a relatively short time (1 to 3 years).[24] It is reasonable to hypothesize that one year of metformin-induced malabsorption of vitamin B_{12} might increase serum total homocysteine levels. In addition, DeFronzo et al. have shown that even after 6 months of treatment with 2550 mg metformin per day a decrease of serum vitamin B_{12} levels by up to 30% can be induced.[9] Moreover, malabsorption of vitamin B_{12} can already be induced after 10 days of treatment with 3 grams of metformin per day.[10]

Another explanation for the small rise of the serum total homocysteine level could be the fact that homocysteine levels do not differ substantially between individuals with moderate and high serum vitamin B_{12} levels. If plasma vitamin B_{12} levels drop below 300 pg/mL, however, homocysteine levels rise dramatically.[36] Thus, if the included patients had moderate to high serum vitamin B_{12} levels prior to their metformin treatment, a decline of 30% of the serum vitamin B_{12} would not have raised the serum total homocysteine level by more than 1 to 2 μmol/L.[37] Serum storage conditions did not allow assessment of vitamin B_{12} levels; therefore, we could not analyze this issue in our study. Finally, a randomized placebo-controlled trial would have allowed more definite conclusions.

We conclude that treatment with 1000 mg of metformin per day during one year in Caucasian NIDDM patients is likely to have, if any, a small effect on serum total homocysteine level. The significance of small rises of serum total homocysteine level in diabetic patients is unknown, however, and requires further study. To put this into perspective, it has been estimated that a 1 μmol/L increment in the serum total homocysteine level elevates the risk of coronary artery disease by a factor of approximately 1.1.[6] Finally, we cannot exclude that more prolonged use of metformin does lead to an important increase of the serum total homocysteine level.

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9

General discussion

Table 1. Odds ratios (95% confidence intervals) for cardiovascular disease, mortality and microangiopathy per 5 µmol/L increment of serum total homocysteine

Endpoint	Adjusted for age and sex	Multivariate adjusted	Stratified analyses		*P for Interaction
			non-diabetic	diabetic	
Cardiovascular disease†	1.34 (1.12 - 1.60)	1.39‡ (1.15 - 1.68)	1.38§ (1.03 - 1.85)	2.33§ (1.11 - 4.90)	0.07
5-year overall mortality	1.31 (1.06 - 1.60)	1.26|| (1.02 - 1.55)	1.34|| (0.87 - 2.06)	2.51||¶ (1.07 - 5.91)	0.08
5-year cardiovascular mortality	1.55 (1.08 - 2.23)	1.45# (1.01 - 2.08)			not studied
Microalbuminuria†	1.30 (1.08 - 1.56)	1.33** (1.08 - 1.63)			0.9
Retinopathy†			0.97†† (0.42 - 2.82)	3.44†† (1.13 - 10.42)	0.08
Neuropathy†	1.04 (0.77 - 1.40)	1.00‡‡ (0.72 - 1.39)			0.4

* Interaction between hyperhomocysteinemia and diabetes with regard to investigated endpoint.
† Cross-sectional study.
‡ Adjusted for age, sex, diabetes, hypertension, hypercholesterolemia, and ever smoking.
§ Adjusted for age, sex, diabetes, hypertension, hypercholesterolemia, ever smoking and serum creatinine.
|| Adjusted for age, sex, diabetes, hypertension, hypercholesterolemia, current smoking, serum HbA_{1c} and albumin.
¶ Calculated for serum tHcy >14 µmol/L versus <=14 µmol/L.
# Adjusted for age, sex, diabetes, hypertension, hypercholesterolemia and current smoking.
** Adjusted for age, sex, glucose tolerance, hypertension, dyslipidemia, and current smoking.
†† Adjusted for age, sex, HbA_{1c} and hypertension. Odds ratio is calculated for serum tHcy >16 µmol/L versus <=16 µmol/L.
‡‡ Adjusted for age, sex, body height, diabetes, and daily vitamin B_6 intake.

Introduction

The preceding chapters of this thesis assess two important issues: whether hyperhomocysteinemia is a risk factor for cardiovascular morbidity, mortality, and microangiopathy independent of other cardiovascular risk factors, in particular type 2 diabetes; and whether hyperhomocysteinemia and type 2 diabetes interact (that is, amplify or attenuate each others' effect with regard to the endpoint under consideration). Finally, the effect of metformin (an oral glucose-lowering agent) on serum tHcy levels was investigated. In this final chapter a synthesis and general view will be presented. The main results of this thesis are summarized in Table 1.

Cardiovascular disease and mortality

In a 50- to 75-year-old general Caucasian population, hyperhomocysteinemia appeared to be a risk factor for cardiovascular disease independent of major cardiovascular risk factors such as type 2 diabetes, hypertension, hypercholesterolemia and smoking. The magnitude of the relation between hyperhomocysteinemia and cardiovascular disease was similar for peripheral arterial, coronary artery, and cerebrovascular disease (Chapter 2). In the same population we compared the strength of the relation between hyperhomocysteinemia and level of peripheral arterial obstruction (i.e., aortoiliacal vs femoropopliteal vs crural). After adjustment for major cardiovascular risk factors, hyperhomocysteinemia was related to aortoiliac but not to isolated crural arterial occlusive disease. This finding may partially explain the particular poor overall prognosis of subjects with proximal compared to distal peripheral arterial disease (Chapter 3). In a prospective case-control study, nested within the Hoorn Study (N=2484), we demonstrated that hyperhomocysteinemia, independently of type 2 diabetes and other major cardiovascular risk factors, was a risk factor for 5-year overall and cardiovascular mortality (Chapter 4). Taken together, these results support the hypothesis that hyperhomocysteinemia is a cardiovascular risk factor.

Microangiopathy

In the same population, we explored the relation between hyperhomocysteinemia and microangiopathy (i.e., microalbuminuria, retinopathy and neuropathy, where the latter is thought to be less exclusively due to microangiopathy than the first two). After adjustment for major risk factors,

hyperhomocysteinemia appeared to be a risk factor for microalbuminuria. This finding may partly explain the link between microalbuminuria and increased risk of cardiovascular disease. Moreover, this result may imply that hyperhomocysteinemia might not only be the consequence of impaired renal function due to a reduction of total body clearance of tHcy, but also a causal factor with regard to early nephropathy (Chapter 5).

After adjustment for major risk factors, hyperhomocysteinemia was related to retinopathy in type 2 diabetic, but not in non-diabetic subjects (Chapter 6). Furthermore, we could not establish a relation between hyperhomocysteinemia and distal neuropathy, although a weak relation between hyperhomocysteinemia and neuropathy could not be excluded (as judged from the confidence intervals). (Chapter 7). The finding that hyperhomocysteinemia appeared to be related to microalbuminuria and retinopathy, but not to neuropathy, may reflect differences of Hcy metabolism between cells of various tissues. Another likely explanation could be that microangiopathy may have a more important role in the development of microalbuminuria and retinopathy than in that of neuropathy.

Hyperhomocysteinemia and type 2 diabetes

We did not find a relation between hyperhomocysteinemia and the diabetic state *per se* or insulin-resistance-related factors (Chapters 2 and 6), but this issue needs further investigation.[1]

Little is known about the effect of glucose-lowering agents on serum tHcy levels. Therefore, in a study design with matching for age, sex, serum creatinine and current smoking status, we estimated the effect of metformin (an oral glucose-lowering agent that may induce vitamin B_{12} malabsorption) on serum tHcy level. Serum tHcy was compared between 40 type 2 diabetic patients who had received treatment with metformin for at least 6 months and 71 type 2 diabetic patients not treated with metformin. The metformin-exposed patients had slightly, although not significantly, higher serum tHcy levels. Therefore, we concluded that the effect on serum tHcy level of treatment with 1000 mg of metformin during one year, if any, is likely to be small (Chapter 8).

Unfortunately, serum storage conditions did not allow assessment of vitamin B_6, vitamin B_{12} and folate levels, so that we were unable to explore whether the relation between B vitamins and serum tHcy levels differed between diabetic and non-diabetic subjects.

Hyperhomocysteinemia appeared to be a stronger risk factor among type 2 diabetic than among non-diabetic subjects with regard to risk of

cardiovascular disease, overall mortality and retinopathy. These findings may, in part, explain the increased risk of cardiovascular morbidity and mortality among type 2 diabetic patients, which cannot be explained by the high prevalence of major cardiovascular risk factors.

Interaction

A distinction is made between biological and statistical interaction.[2] The concept of 'biological interaction' specifically addresses the biological mechanism that underlies the relation under study. In the present thesis we used 'interaction' to indicate 'statistical interaction', which is alternatively called 'effect modification', 'effect-measure modification', or 'heterogeneity of effect'. Statistical interaction implies that the combined effect of two risk factors is different from what we would expect, knowing the effect of each risk factor separately. In this thesis interaction was assessed by means of logistic regression, which is a multiplicative model (with the odds ratio as effect measure). We showed that the relative risk of mortality associated with hyperhomocysteinemia is higher among diabetic than among non-diabetic subjects: the presence of diabetes modified the strength of the relation between homocysteine and mortality risk. Although statistical interaction does not elucidate the underlying biological mechanisms, it can be relevant from a public health perspective as it points to the existence of a high risk group for whom modification of the risk factor at issue (hyperhomocysteinemia) may be especially beneficial. The higher relative mortality risk for diabetic compared to the non-diabetic subjects, given the already increased baseline risk for diabetic patients, illustrates this issue.

Methodological considerations

Study design

All studies presented in this thesis were cross-sectional, except the study about the relation between hyperhomocysteinemia and 5-year mortality (Chapter 4). An important limitation of a cross-sectional study design is that one cannot rule out the possibility that elevated tHcy levels are the consequence rather than the cause of cardiovascular disease.

Potential Confounders

Inadequate adjustment for confounding is a threat to the validity of the results of an epidemiologic study. For cardiovascular disease, important risk

factors aside from age and male sex are hypercholesterolemia, hypertension, smoking and diabetes. In the present study serum cholesterol levels were adequately assessed. Blood pressure was also adequately assessed, but little was known about the history of hypertension and the duration of use of anti-hypertensive medication. With regard to smoking we could only assess whether subjects were current smoker (yes/no) or ever smoker (yes/no) of cigarettes, but there was no information available about the history of smoking habits. In contrast, glucose tolerance status was accurately assessed by means of two oral glucose tolerance tests. The latter provided the opportunity to adjust, in all analyses, as thoroughly as possible for glucose tolerance.

Exposure measurement

Homocysteine determinations were performed in serum obtained in the fasting state and stored at -20°C for 4 to 7 years. There is good evidence that serum tHcy levels are stable in frozen samples for 10 years or more.[3] Yet we cannot exclude the possibility that differences in storage duration of the serum samples may have affected serum tHcy concentrations in a dissimilar fashion. If so, the latter could have resulted in misclassification of exposure, which was most likely nondifferential with regard to disease status. Therefore, any bias introduced by such nondifferential exposure misclassification would likely bias towards the null value (of no relation).[4] Fasting serum tHcy levels are probably more reliable than non-fasting levels, since intake of food may influence serum tHcy levels[5,6] and the latter may thus increase measurement variability.

Methionine loading tests were not performed in the Hoorn Study. A methionine loading test would probably have resulted in more subjects being classified as having hyperhomocysteinemia, as compared to classification based on fasting serum tHcy alone.[7] Both fasting and post-methionine serum tHcy levels are related to risk of cardiovascular disease.[6] Therefore, post-load serum tHcy levels could have offered, in addition to fasting serum tHcy levels, insight in the relation between hyperhomocysteinemia and cardiovascular disease.

Outcome measurement

The history of cardiovascular disease (myocardial infarction, coronary artery bypass grafting, TIA/stroke, peripheral arterial reconstruction or limb amputation) was obtained by means of a self-administered questionnaire,

and, when positive, accepted only if confirmed by written information of the participant's general practitioner.

Self-reported myocardial infarction was confirmed in 58% (22 of 38) of the cases. The remaining cases appeared to be angina pectoris. Self-reported TIA/stroke was confirmed in 38% (12 of 32) of the cases. A limitation of the present study is that we could not distinguish between TIA and stroke, nor between ischemic and hemorrhagic stroke. Moreover, under-reporting of cardiovascular disease may have occurred. Misclassification which is not related to exposure, or nondifferential error, in general results in effect attenuation.[4] ECGs were coded according to the Minnesota code 1982 (MC).[8] A reproducibility study in a random sample ($N=50$) revealed a kappa coefficient (95% CI) for MC 1-1 or 1-2 (definite myocardial infarction) of 0.85 (0.56 to 1.00), indicating good agreement.

Overall mortality is an unequivocal endpoint. However, bias can occur due to loss of subjects during follow-up, which may be related to both the exposure and the outcome. In the present study there was no loss of follow-up. In contrast, death from a specific cause, such as cardiovascular mortality, might be affected by nondifferential misclassification and by competing mortality risks.

Graded versus threshold effect

It is not known whether the relation between serum tHcy levels and cardiovascular disease is graded or not.[9] Moreover, the nature of the relation between serum tHcy and cardiovascular disease may differ depending on the endpoint under consideration. For instance, a threshold of 18 μmol/L tHcy has been demonstrated for the relation with deep-vein thrombosis:[10] the risk of thrombosis did not increase among subjects with tHcy levels up to 18 μmol/L. In addition, the risk was increased greatly above 22 μmol/L. This finding indicates a threshold rather than a continuous dose-reponse relation.

In the present thesis we chose two different approaches to investigate the nature of the relation between tHcy and the endpoint under issue. We calculated odds ratios with tHcy both as a continuous and as a categorical variable. We acknowledge that the choice of the categories of serum tHcy in the separate studies is, to some extent, arbitrary. Although it is preferable to base the analysis on a large number of categories of serum tHcy, the limited number of cases often did not allow this. Therefore we regard the analyses in which tHcy was entered in the models as a categorical variable as additional analyses that may provide more insight in the nature of the relation between serum tHcy and the endpoint under issue. Obviously the present studies

were too small to solve the question whether the association between tHcy and the different endpoints is graded or has a certain threshold.

Homocysteine-lowering therapy

Increased intake of folate, vitamin B_{12}, and vitamin B_6 can probably reduce the tHcy level in nearly all individuals by 15 to 40%, dependent on their pretreatment tHcy level. (Folate is the generic term for compounds that have vitamin activity similar to that of folic acid, the chemical that is added to supplements or fortified food. Folic acid is synthetic, heat stable, and approximately twice as bioavailable as the folate that occurs naturally in food.[11]) A recent meta-analysis[12] showed that treatment with 0.5 to 5.0 mg folic acid daily can lower serum tHcy by 15 to 40% within approximately six weeks. Vitamin B_{12} (mean 0.5 mg daily) produced an additional 7% reduction of the tHcy level. Vitamin B_6 (mean 16.5 mg daily) did not have a significant additional effect. The addition of oral vitamin B_{12} to folic acid would be expected to avoid the theoretical risk of neuropathy due to unopposed folic acid therapy in patients deficient in vitamin B_{12}, even if due to intrinsic factor deficiency or malabsorption.[13-15] The latter is relevant since vitamin B_{12} deficiency is common among the elderly, prevalence estimates in the general population (65 years and older) varying from 5 to 15%.[16-18]

Implications for patients with type 2 diabetes mellitus

The effects on cardiovascular morbidity and mortality risk of homocysteine-lowering treatment have yet to be assessed. It has been estimated that lowering tHcy by 5 μmol/L (about 1 SD) may reduce risk of cardiovascular death by about 10%.[9] This estimate may be even higher for diabetic subjects since the present thesis revealed an interaction between hyperhomocysteinemia and diabetes with regard to risk of cardiovascular morbidity and overall mortality. On the other hand, calculations with regard to the proportion of preventable cardiovascular mortality due to tHcy reduction may be an overestimation of the real achievable benefit. It is likely that subjects who presently have elevated tHcy levels that are amenable to an increased folic acid intake have had these elevations for varying lengths of their lifespan. It is unlikely that, after homocysteine-lowering therapy, previously hyperhomocysteinemic subjects will obtain the same low relative risk of cardiovascular disease as subjects who never had elevated tHcy levels. Vascular damage caused by earlier hyperhomocysteinemia will probably continue to influence atherogenesis even though the putative primary cause

(i.e., a high tHcy level) has been removed. Since atherosclerotic plaques evolve during an entire lifespan, late-in-life dietary changes can only be expected to yield limited benefits. Therefore, the degree of benefit will relate not only to the effectiveness of the intervention but also to the time in life when the intervention is introduced. However, understanding of factors that lead to atherosclerotic plaque instability causing thrombosis is increasing rapidly. It has become clear that the risk of developing an acute ischemic event depends on the number of unstable plaques, rather than the number of plaques overall.[19] Disbalance between production of connective tissue matrix proteins by smooth muscle cells and degradation by metalloproteases may result in a vulnerable plaque. Metalloproteases can probably be activated by oxidants. Since hyperhomocysteinemia is thought to increase oxidative stress,[20] it may also be involved in plaque stability. Taken together, this may imply that homocysteine-lowering therapy, even when started late in life, may exert beneficial effects.

Type 2 diabetes frequently develops after the age of 40 years, when development of atherosclerosis has probably already taken place for decades. Since type 2 diabetic patients are prone to accelerated development of atherosclerosis, it might be argued that vitamin B therapy, regardless of the initial homocysteine level, should be an integral part of diabetes treatment. Slowing down of the progression of atherosclerotic disease among type 2 diabetic patients, rather than prevention, might be a sensible goal.

Conclusions and implications for future research

We showed that hyperhomocysteinemia is related to risk of cardiovascular morbidity and mortality, especially among type 2 diabetic patients. In addition, hyperhomocysteinemia appeared to be related to risk of microalbuminuria in both non-diabetic and diabetic subjects, and to risk of retinopathy among type 2 diabetic subjects, but not to risk of neuropathy. Future, prospective, analyses of data of the Hoorn Study may provide further evidence as to whether hyperhomocysteinemia is indeed related to incidence of cardiovascular disease, microalbuminuria and retinopathy. Although there is strong evidence linking homocysteine to cardiovascular disease, the beneficial effect of homocysteine-lowering therapy in subjects with moderate hyperhomocysteinemia still has to be proven. Intervention trials with homocysteine-lowering therapy are currently ongoing.

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Abbreviations

ABPI	ankle brachial pressure index
ACR	albumin-to-creatinine ratio
AER	albumin excretion rate
B_6	vitamin B_6
B_{12}	vitamin B_{12}
BHMT	betaine-homocysteine methyltransferase
BMI	body mass index
CBS	cystathionine ß-synthase
CI	confidence interval
ECG	electrocardiogram
GFR	glomerular filtration rate
HbA_{1c}	glycosylated hemoglobin A_{1c}
Hcy	homocysteine
HDL	high density lipoprotein
HPLC	high-performance liquid chromatography
ICD	international classification of diseases
IGT	impaired glucose tolerance
IQR	interquartile range
LDL	low density lipoprotein
MA	microalbuminuria
MC	Minnesota code
MS	methionine synthase
MTHFR	methylenetetrahydrofolate reductase
N	number
NGT	normal glucose tolerance
NIDDM	non-insulin-dependent diabetes mellitus (type 2 diabetes)
OGTT	oral glucose tolerance test
OR	odds ratio
P	probability, tail probability (*P*-value)
PAD	peripheral arterial disease
PAR	population attributable risk
SAM	s-adenosylmethionine
SD	standard deviation
tHcy	total homocysteine
THF	tetrahydrofolate
TIA	transient ischemic attack
VPT	vibration perception threshold
WHO	World Health Organization
WHR	waist-hip ratio

Summary

Cardiovascular disease is the most common long-term complication of Type 2 (non-insulin-dependent) diabetes mellitus and accounts for 75 to 80% of the mortality among diabetic subjects. Cardiovascular mortality and morbidity rates are two to four times higher in diabetic patients than in non-diabetic subjects. Generally, the etiology of cardiovascular disease is thought to be multifactorial. The underlying mechanisms for the accelerated atherosclerosis in diabetes are poorly understood and cannot be fully explained by major cardiovascular risk factors such as the high prevalence of hypertension, hypercholesterolemia, smoking and/or hyperglycemia.

Hyperhomocysteinemia is a recently recognized modifiable risk factor for cardiovascular disease independent of major cardiovascular risk factors. In general, daily treatment with folic acid can lower homocysteine levels. Little is known about the impact of hyperhomocysteinemia on cardiovascular disease among type 2 diabetic patients. This thesis is focused on the relation between hyperhomocysteinemia on the one hand, and macro- and microangiopathy on the other hand in type 2 diabetic and non-diabetic subjects of a 50- to 75-year old general Caucasian population (The Hoorn Study). A general introduction is presented in *Chapter 1*.

In *Chapter 2* we investigated whether the strength of the association between hyperhomocysteinemia and cardiovascular disease is similar for peripheral arterial, coronary artery and cerebrovascular disease, in an age-, sex- and glucose tolerance-stratified random sample ($N=631$) from a 50- to 75-year-old general Caucasian population. Furthermore, we investigated the combined effect of hyperhomocysteinemia and diabetes mellitus with regard to cardiovascular disease.

The prevalence of fasting hyperhomocysteinemia (>14.0 μmol/L) was 25.8%. After adjustment for age, sex, hypertension, hypercholesterolemia, diabetes and smoking, the odds ratios [OR; 95% confidence intervals (CI)] per 5 μmol/L [about 1 standard deviation (SD)] serum total homocysteine increment were 1.44 (1.10 to 1.87) for peripheral arterial, 1.25 (1.03 to 1.51) for coronary artery, 1.24 (0.97 to 1.58) for cerebrovascular and 1.39 (1.15 to 1.68) for any cardiovascular disease. After stratification by glucose tolerance category and adjustment for the classical risk factors and serum creatinine, the ORs per 5 μmol/L serum total homocysteine increment for any cardiovascular disease were 1.38 (1.03 to 1.85) in normal glucose tolerance, 1.55 (1.01 to 2.38) in impaired glucose tolerance, and 2.33 (1.11 to 4.90) in non-insulin-dependent diabetes mellitus ($P=0.07$ for interaction). We conclude that the magnitude of the association between

hyperhomocysteinemia and cardiovascular disease is similar for peripheral arterial, coronary artery and cerebrovascular disease. In addition, high serum total homocysteine may be a stronger (1.6-fold) risk factor for cardiovascular disease in subjects with type 2 diabetes than in non-diabetic subjects.

In *Chapter 3* we studied whether the strength of the association between hyperhomocysteinemia and peripheral arterial disease differs according to the localization of the anatomical obstruction, in an age-, sex- and glucose-tolerance stratified random sample (N=631) of a 50- to 75-year-old general Caucasian population. After adjustment for age, sex, systolic blood pressure, current smoking, serum cholesterol and diabetes mellitus, the ORs (95% CI) per 5 μmol/L serum total homocysteine increment were 1.41 (1.05 to 1.89) for aortoiliac, 1.03 (0.70 to 1.52) for femoropopliteal and 0.82 (0.59 to 1.15) for crural obstructions. We conclude that hyperhomocysteinemia is associated with aortoiliac but not with isolated crural arterial occlusive disease.

In *Chapter 4* we investigated in a prospective case-control study, nested within the Hoorn Study (N=2484), whether hyperhomocysteinemia is a risk factor for 5-year overall and cardiovascular mortality among type 2 diabetic patients. Fasting serum total homocysteine concentration was measured in 171 subjects who died (cases; 76 of cardiovascular disease) and in 640 survivors (controls). After adjustment for major cardiovascular risk factors, HbA_{1c} and serum albumin, the OR (95% CI) for 5-year mortality was 1.56 (1.07 to 2.30) for hyperhomocysteinemia (>14 μmol/L) and 1.26 (1.02 to 1.55) per 5 μmol/L (about 1 SD) increment of serum total homocysteine. The OR (95% CI) for 5-year mortality for hyperhomocysteinemia was 1.34 (0.87 to 2.06) in non-diabetic and 2.51 (1.07 to 5.91) in diabetic subjects (P=0.08 for interaction). We conclude that hyperhomocysteinemia, independently of other major risk factors, is related to 5-year mortality and appears to be a stronger (1.9-fold) risk factor for mortality in type 2 diabetic patients than in non-diabetic subjects.

In *Chapter 5* we studied the association between hyperhomocysteinemia and microalbuminuria. The urinary albumin-to-creatinine ratio (ACR) was measured in an age-, sex- and glucose tolerance-stratified random sample of a 50- to 75-year-old general Caucasian population (N=653). Microalbuminuria was defined as ACR >3.0 mg/mmol. After adjustment for age, sex, glucose tolerance category, hypertension, dyslipidemia and smoking, the OR (95% CI) for microalbuminuria per 5 μmol/L serum total homocysteine increment was 1.33 (1.08 to 1.63). Additional adjustment for protein intake and serum creatinine did not

attenuate the association between microalbuminuria and serum total homocysteine. A 0.1 g/kg.day increment of protein intake was also associated with an increased risk for microalbuminuria after adjustment for age, sex, classical risk factors and serum total homocysteine [OR (95% CI); 1.20 (1.08 to 1.32)]. We conclude that both hyperhomocysteinemia and protein intake are related to microalbuminuria independent of type 2 diabetes and hypertension. Furthermore, the positive association between hyperhomocysteinemia and microalbuminuria may partly explain the link between microalbuminuria and the increased risk of cardiovascular disease. Finally, this result may imply that hyperhomocysteinemia might not only be the consequence of impaired renal function due to a reduction of total body clearance of serum total homocysteine, but also a causal factor with regard to early nephropathy.

In *Chapter 6* we studied the association between hyperhomocysteinemia and retinopathy. Retinal vascular changes ('retinopathy') were assessed by means of ophthalmoscopy and/or fundus photography in an age-, sex- and glucose tolerance-stratified random sample of a 50- to 75-year-old general Caucasian population ($N=625$). After stratification for diabetes and adjustment for age, sex, HbA_{1c} and hypertension, the OR (95% CI) for the association between retinopathy and hyperhomocysteinemia (>16 μmol/L) was 0.97 (0.42 to 2.82) in non-diabetic and 3.44 (1.13 to 10.42) in diabetic subjects ($P=0.08$ for interaction). We conclude that hyperhomocysteinemia may be a risk factor for retinopathy in type 2 diabetic, but probably not in non-diabetic subjects.

In *Chapter 7* we studied the association between hyperhomocysteinemia and polyneuropathy in an age-, sex- and glucose tolerance-stratified random sample of a 50- to 75-year-old general Caucasian population ($N=595$). Polyneuropathy was defined as the absence of at least two of the three following sensory modalities or reflexes of either foot: light touch sense, ankle reflex, and vibration sensation. After adjustment for age, sex, HbA_{1c} and hypertension, the OR (95% CI) for polyneuropathy per 5 μmol/L (about 1 SD) serum serum total homocysteine increment was 1.00 (0.72 to 1.39). We conclude that although a weak relation (as judged from the confidence intervals) cannot be excluded, hyperhomocysteinemia is probably not related to risk of distal somatic polyneuropathy.

Metformin, an oral glucose-lowering agent, may induce vitamin B_{12} malabsorption and therefore increase the serum serum total homocysteine level. Serum serum total homocysteine level was compared between 40 type 2 diabetic patients who had received treatment with metformin for at

least 6 months and 71 type 2 diabetic patients not treated with metformin. The metformin-exposed patients, as compared to the nonexposed patients, had slightly, although not significantly, higher serum serum total homocysteine levels. Therefore, we concluded that the effect on serum serum total homocysteine level of treatment with 1000 mg of metformin during one year, if any, is likely to be small (*Chapter 8*).

Finally, in *Chapter 9* a synthesis and general view is presented. The prevalence of hyperhomocysteinemia (>14 μmol/L) was more than 25% in a general 50- to 75-year-old Caucasian population. We showed that hyperhomocysteinemia is related to risk of cardiovascular morbidity and mortality, especially among type 2 diabetic patients. In addition, hyperhomocysteinemia appeared to be related to risk of microalbuminuria in both non-diabetic and diabetic subjects, and to risk of retinopathy among type 2 diabetic subjects, but not to risk of polyneuropathy. These findings add to accumulating evidence that hyperhomocysteinemia is a common and independent cardiovascular risk factor. They also suggest that hyperhomocysteinemia is an especially important risk factor in patients with type 2 diabetes, because it is linked to an increased risk of both micro- and macrovascular disease, i.e. the major causes of morbidity and mortality in type 2 diabetes.

Samenvatting

Hart- en vaatziekten vormen de belangrijkste complicatie van type-2-diabetes (niet-insuline-afhankelijke diabetes mellitus) en is bij 75 tot 80% van de patiënten de doodsoorzaak. Patiënten met type-2-diabetes hebben, ten opzichte van mensen zonder diabetes, een twee- tot viermaal verhoogd risico om te sterven aan hart- en vaatziekten. Over het algemeen wordt verondersteld dat de oorzaak van hart- en vaatziekten multifactoriëel is. Het precieze mechanisme van de versnelde atherosclerose die bij patiënten met diabetes optreedt is niet geheel opgehelderd. De verhoogde prevalentie van hypertensie, hypercholesterolemie, roken en de hyperglycemie spelen zeker een belangrijke rol bij de versnelde atherosclerose die bij type-2-diabetes optreedt, maar kunnen het toch niet geheel verklaren.

Hyperhomocysteïnemie, een verhoogd homocysteïnegehalte in het bloed, is een recent ontdekte risicofactor voor hart- en vaatziekten, onafhankelijk van de klassieke risicofactoren. Over het algemeen leidt een verhoging van de dagelijkse inname van foliumzuur tot een verlaging van het homocysteïnegehalte in het bloed. Er is weinig bekend over het effect van hyperhomocysteïnemie op hart- en vaatziekten bij patiënten met type-2-diabetes. Dit proefschrift beschrijft de samenhang tussen hyperhomocysteïnemie en macro- en microangiopathie bij mensen met en zonder type-2-diabetes in een 50- tot 75-jarige Caucasische bevolking (de Hoorn Studie). In *hoofdstuk* 1 wordt een algemene inleiding gegeven.

In *hoofdstuk* 2 bestudeerden wij of de sterkte van de associatie tussen hyperhomocysteïnemie en hart- en vaatziekten gelijksoortig is voor perifeer, coronair en cerebrovasculair arteriëel vaatlijden in een voor leeftijd, geslacht en glucosetolerantie gestratificeerde willekeurige steekproef ($N=631$) uit een 50- tot 75-jarige algemene Caucasische bevolking. Vervolgens onderzochten wij het gecombineerde effect van hyperhomocysteïnemie en type-2-diabetes op het risico van hart- en vaatziekten. De prevalentie van hyperhomocysteïnemie (>14 μmol/L) was 25,8% in deze 50- tot 75-jarige algemene Caucasische bevolking. Na correctie voor leeftijd, geslacht, hypertensie, hypercholesterolemie, diabetes en roken, was de oddsratio [OR; 95% betrouwbaarheidsinterval (BI)] per 5 μmol/L [ongeveer 1 standaarddeviatie (SD)] toename van het homocysteïnegehalte 1,44 (1,10 tot 1,87) voor perifeer, 1,25 (1,03 tot 1,51) voor coronair, 1,24 (0,97 tot 1,58) en voor cerebrovasculair vaatlijden en 1,39 (1,15 tot 1,68) voor hart- en vaatziekten in het algemeen. Na stratificatie voor glucosetolerantiecategorie, en correctie voor klassieke risicofactoren en serumcreatinine, was de OR per 5 μmol/L toename van het homocysteïnegehalte voor hart- en vaatziekten

1,38 (1,03 tot 1,85) bij personen met een normale glucosetolerantie, 1,55 (1,01 tot 2,38) bij personen met een gestoorde glucosetolerantie en 2,33 (1,11 tot 4,90) bij patiënten met type-2-diabetes (P=0,07 voor interactie). We concludeerden dat de sterkte van de relatie tussen hyperhomocysteïnemie en hart- en vaatziekten gelijksoortig was voor perifeer, coronair en cerebrovasculair arteriëel vaatlijden. Daarnaast bleek hyperhomocysteïnemie een sterkere (1,6 maal) risicofactor voor hart- en vaatziekten bij patiënten met dan bij mensen zonder type-2-diabetes.

In *hoofdstuk* 3 bestudeerden we de sterkte van de associatie tussen hyperhomocysteïnemie en het niveau van de perifere arteriële obstructie (nl. aortoiliacaal versus femoropopliteaal versus cruraal) in een voor leeftijd, geslacht en glucosetolerantie gestratificeerde willekeurige steekproef (N=631) van een 50- tot 75-jarige algemene Caucausische bevolking. Na correctie voor leeftijd, geslacht, systolische bloeddruk, roken, serumcholesterol en diabetes, was de OR (95% BI) per 5 μmol/L toename van het homocysteïnegehalte 1,41 (1,05 tot 1,89) voor een aortoiliacale obstructie, 1,03 (0,70 tot 1,52) voor een femoropopliteale obstructie en 0,82 (0,59 tot 1,15) voor een crurale obstructie. We concludeerden dat hyperhomocysteïnemie geassocieerd is met aortoialacale, maar niet met geïsoleerde crurale obstructies.

In *hoofdstuk* 4 onderzochten we in een prospectief case-controlonderzoek, genest in de Hoorn Studie (N=2484), of hyperhomocysteïnemie een risicofactor is voor 5-jaarssterfte (zowel totale als cardiovasculaire sterfte) bij mensen met en zonder type-2-diabetes. Nuchter serumtotaalhomocysteïne werd bepaald bij 171 overleden mensen (cases; 76 ten gevolge van hart- en vaatziekten) en 640 overlevenden (controles). Na correctie voor belangrijke risicofactoren voor hart- en vaatziekten, HbA1c en serumalbumine, was de OR (95% BI) voor 5-jaarssterfte 1,56 (1,07 tot 2,30) voor hyperhomocysteïnemie (>14 μmol/L) en 1,26 (1,02 tot 1,55) per 5 μmol/L (ongeveer 1 SD) toename van het homocysteïnegehalte. De OR (95% BI) voor 5-jaarssterfte voor hyperhomocysteïnemie was 1,34 (0,87 tot 2,06) bij mensen zonder diabetes en 2,51 (1,07 tot 5,91) bij mensen met type-2-diabetes (P=0,08 voor interactie). We concludeerden dat hyperhomocysteïnemie, onafhankelijk van type-2-diabetes en andere belangrijke risicofactoren voor hart- en vaatziekten, een risicofactor is voor 5-jaarssterfte. Bovendien bleek hyperhomocysteïnemie een sterkere (1,9 maal) risicofactor voor sterfte te zijn bij patiënten met type-2-diabetes dan bij mensen zonder diabetes.

In *hoofdstuk* 5 bestudeerden we de associatie tussen hyperhomocysteïnemie en microalbuminurie. De albumine-creatinineratio (ACR) werd bepaald bij een voor leeftijd, geslacht en glucosetolerantie gestratificeerde

willekeurige steekproef (*N*=653) van een 50- tot 75-jarige algemene Caucausische bevolking. Microalbuminurie was gedefinieerd als een ACR >3,0 mg/mmol. Na correctie voor leeftijd, geslacht, glucosetolerantiecategorie, hypertensie, dyslipidemie en roken, was de OR (95% BI) voor microalbuminurie per 5 μmol/L (ongeveer 1 SD) toename van het homocysteïnegehalte 1,33 (1,08 tot 1,63). Aanvullende correctie voor eiwitinname en serumcreatinine beïnvloedde de associatie tussen hyperhomocysteïnemie en microalbuminurie niet. Een toename van de eiwitinname van 0,1 g/kg/dag was ook geassocieerd met het risico op microalbuminurie na correctie voor leeftijd, geslacht, klassieke risicofactoren en homocysteïne [OR (95% BI); 1,20 (1,08 tot 1,32)]. We concludeerden dat zowel hyperhomocysteïnemie als de dagelijkse eiwitinname zijn gerelateerd aan microalbuminurie, onafhankelijk van type-2-diabetes en hypertensie. De positive associatie tussen hyperhomocysteïnemie en microalbuminurie zou deels kunnen verklaren waardoor het hebben van microalbuminurie gepaard gaat met een verhoogd risico op hart- en vaatziekten. Bovendien kan dit resultaat impliceren dat hyperhomocysteïnemie niet alleen het gevolg zou kunnen zijn van een verslechterde nierfunctie (waarbij de totale lichaamsklaring van het homocysteïne afneemt), maar ook een oorzaak van microalbuminurie en dus van nierfunctieverlies.

In *hoofdstuk* 6 bestudeerden we de associatie tussen hyperhomocysteïnemie en retinopathie. Met behulp van ofthalmoscopie en/of fundusfotografie werd de aan- danwel afwezigheid van retinopathie vastgesteld bij een voor leeftijd, geslacht en glucosetolerantie gestratificeerde willekeurige steekproef (*N*=625) van een 50- tot 75-jarige algemene Caucausische bevolking. Na stratificatie voor diabetes en correctie voor leeftijd, geslacht, HbA1c en hypertensie, was de OR (95% BI) voor de associatie tussen retinopathie en hyperhomocysteinemie (>16 μmol/L): 0,97 (0,42 to 2,82) bij mensen zonder diabetes en 3,44 (1,13 to 10,42) bij patiënten met type-2-diabetes (*P*=0,08 voor interactie). We concludeerden dat hyperhomocysteïnemie mogelijk een risicofactor is voor retinopathie bij patiënten met type-2-diabetes, maar waarschijnlijk niet voor retinopathie bij mensen zonder diabetes.

In *hoofdstuk* 7 bestudeerden we de associatie tussen hyperhomocysteïnemie en polyneuropathie in een voor leeftijd, geslacht en glucosetolerantie gestratificeerde willekeurige steekproef (*N*=595) van een 50- tot 75-jarige algemene Caucausische bevolking. Polyneuropathie was gedefinieerd als de afwezigheid van tenminste twee van de volgende drie sensorische modaliteiten of reflexen van tenminste één van beide voeten:

aanrakingszin, vibratiezin en achillespeesreflex. Na correctie voor leeftijd, geslacht, HbA1c en hypertensie, was de OR (95% CI) voor polyneuropathie per 5 μmol/L (ongeveer 1 SD) toename van het homocysteïnegehalte 1,00 (0,72 tot 1.39). We concludeerden dat er waarschijnlijk geen associatie is tussen hyperhomocysteïnemie en polyneuropathie.

Metformine, een oraal toegediend glucoseverlagend medicijn, kan vitamine-B12-malabsorptie veroorzaken en daardoor een toename van de homocysteïnespiegel in het bloed. We vergeleken de serumhomocysteïnespiegels tussen twee groepen patiënten met type-2-diabetes die wel of geen metformine gebruikten. De patiënten die wel metformine gebruikten hadden een iets, maar niet significant, verhoogde homocysteïnespiegel in het bloed. Dit wijst erop dat metformine, bij een gemiddelde dagdosering (1000 mg) en gebruiksduur (1 jaar) zoals in dit onderzoek, geen wezenlijke invloed heeft op het serumhomocysteïnegehalte.

In *hoofdstuk 9* worden de resultaten van dit proefschrift samengevat en in perspectief geplaatst. De prevalentie van hyperhomocysteïnemie (>14 μmol/L) was ruim 25% in deze 50- tot 75-jarige algemene Caucasische bevolking. We toonden aan dat hyperhomocysteïnemie samenhangt met het risico op hart- en vaatziekten en de sterfte daaraan, met name onder patiënten met type-2-diabetes. Tevens bleek hyperhomocysteïnemie te zijn gerelateerd aan microalbuminurie bij zowel mensen met als zonder type-2-diabetes, en aan retinopathie bij type-2-diabetes patiënten, maar niet aan polyneuropathie. De bevindingen ondersteunen de hypothese dat hyperhomocysteïnemie een veel voorkomende en onafhankelijke risicofactor is voor hart- en vaatziekten. Hyperhomocysteïnemie lijkt met name voor patiënten met type-2-diabetes een belangrijke risicofactor, omdat hyperhomocysteïnemie zowel samenhangt met micro- als met macrovasculaire afwijkingen: de belangrijkste oorzaken van ziekte en sterfte onder patiënten met type-2-diabetes.

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About the author

Ellen Karen Hoogeveen was born on October 9, 1961, in Amsterdam, the Netherlands. In 1980 she graduated from secondary school (Montessori Lyceum Amsterdam). She started her medical study at the Erasmus University Medical School, Rotterdam, in 1985 and obtained her propaedeutic degree in 1986 (cum laude) and her medical degree in 1991 (cum laude).

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