

Fasting and Post-Methionine Homocysteine Levels in NIDDM

Determinants and correlations with retinopathy, albuminuria, and cardiovascular disease

YVO M. SMULDERS, MD
MELINA RAKIC, MD
ED H. SLAATS, PHD
MARCO TRESKES, PHD

ERIC J.G. SJBRANDS, MD
DIEGO A.M. ODEKERKEN, MD
COEN D.A. STEHOUWER, MD
JOSEPH SILBERBUSCH, MD

OBJECTIVE — The increased cardiovascular risk in subjects with NIDDM is partly explained by an association with established risk factors like hypertension, dyslipidemia, and obesity. Mild hyperhomocysteinemia has emerged as a new risk factor for cardiovascular disease. The purpose of this study was to assess its role in NIDDM.

RESEARCH DESIGN AND METHODS — We studied predictors of homocysteine levels and correlations between homocysteine and (micro-)albuminuria, retinopathy, and history of cardiovascular disease in normotensive NIDDM subjects under stable metabolic control. This was done in 85 NIDDM subjects by measuring fasting and post-methionine-loading homocysteine levels together with blood pressure, BMI, serum cholesterol, triglyceride, HDL cholesterol, folate, vitamin B12, pyridoxal-5-phosphate, HbA_{1c}, and (micro-)albuminuria and creatinine clearance in triplicate 24-h urine samples. The relationship between micro- and macrovascular complications and fasting homocysteine only was studied in an additional 65 subjects, giving a total of 150 subjects.

RESULTS — In multiple regression analysis, significant ($P < 0.05$) predictors of fasting homocysteine were low-normal values of creatinine clearance (threshold effect at $<80 \text{ ml} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$), folate ($<20 \text{ nmol/l}$), and vitamin B12 ($<350 \text{ pmol/l}$), and postmenopausal status in women. Determinants of post-methionine homocysteine were pyridoxal-5-phosphate levels $<80 \text{ nmol/l}$, creatinine clearance, and sex (higher levels in women). Hyperhomocysteinemia did not cluster with other cardiovascular risk factors, like hypertension, obesity, or dyslipidemia. Regarding cardiovascular complications, fasting homocysteine, but not post-methionine homocysteine, was higher in subjects with a history of cardiovascular disease. There was a stepwise increase in the prevalence of subjects with cardiovascular disease with increasing fasting homocysteine. The prevalence of cardiovascular disease was 19.4% in the bottom quartile of fasting homocysteine, versus 55.0% in the top quartile (P for trend <0.01). Neither fasting homocysteine nor post-methionine homocysteine correlated with (micro-)albuminuria or with retinopathy.

CONCLUSIONS — The findings suggest that homocysteine levels in NIDDM rise even with modest deterioration of renal function and when vitamin status is in the low to low-normal range. Fasting homocysteine correlates with macrovascular disease, but we found no evidence of a correlation with retinopathy or (micro-)albuminuria. Post-methionine homocysteine levels do not show a correlation with micro- or macrovascular complications.

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From the Departments of Internal Medicine (Y.M.S., M.R., E.J.G.S., J.S.), Clinical Chemistry (E.H.S., M.T.), and Cardiology (D.A.M.O.), Onze Lieve Vrouwe Gasthuis; and the Institute for Cardiovascular Research and Department of Medicine (C.D.A.S.), Academisch Ziekenhuis Vrije Universiteit, Amsterdam, the Netherlands.

Address correspondence and reprint requests to Y.M. Smulders, MD, Onze Lieve Vrouwe Gasthuis, 1e Oosterparkstraat 279, PO-box 95500, Amsterdam 1090 HM, the Netherlands.

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Abbreviations: Hcy, homocysteine; δ -Hcy, post-methionine homocysteine level minus fasting homocysteine level; HPLC, high-performance liquid chromatography; MTHFR, methylene-tetrahydrofolate reductase.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

NIDDM is associated with a sharply increased risk of cardiovascular disease and mortality. It is estimated that ~65% of all patients die from cardiovascular complications (1,2). Part of this increased risk is explained by clustering of NIDDM with established risk factors for atherosclerosis, such as hypertension, obesity, and dyslipidemia, in the insulin-resistance syndrome. It appears, however, that these associated risk factors explain only part of the excess cardiovascular disease in NIDDM patients (3,4). Whether the diabetic hyperglycemic state itself completes the risk profile or whether other factors are involved is a matter of debate. NIDDM patients with microalbuminuria are particularly at risk of developing cardiovascular disease (5–8). The underlying mechanism of this association is unknown, but it is clear that NIDDM patients with microalbuminuria have an even higher incidence of hypertension, dyslipidemia, and thrombotic disorders than do normoalbuminuric NIDDM patients (9–13).

Classic homocystinuria due to cystathionine β -synthase deficiency associates with early atherosclerosis and cardiovascular mortality. Recently, mild elevations of plasma homocysteine (Hcy) have been identified as an independent risk factor for early atherosclerotic vascular disease (14–19) and thromboembolic disease (20). Mild hyperhomocysteinemia has a complex etiology, including insufficient intake of vitamins B6 and B12 and folate (21) and genetic factors, of which a thermolabile form of methylene-tetrahydrofolate reductase (MTHFR) is probably the most important one. The mechanism of premature cardiovascular disease in this context is not precisely known, but may relate to increased vulnerability to lipid toxicity, vascular smooth muscle cell growth factor properties of Hcy, endothelial damage, or vasomotor dysfunction or to disorders of platelet aggregation and coagulation (22).

The possible role of mild hyperhomocysteinemia in NIDDM is unclear. Few studies have addressed this issue, with con-

flicting results (23–27). So far, measurements of post-methionine-load Hcy have been reported in only 18 NIDDM patients (24). The determinants of Hcy levels in diabetic patients have not been studied in detail. The present study was undertaken to address the following questions: First, what are the predictors of hyperhomocysteinemia in NIDDM, and do high Hcy levels cluster with other cardiovascular risk factors, like hypertension, obesity, and dyslipidemia? Second, do Hcy levels correlate with microvascular complications, like (micro-)albuminuria and retinopathy? And finally, what is the impact of Hcy levels on the prevalence of cardiovascular complications in NIDDM patients?

RESEARCH DESIGN AND

METHODS — Consecutive patients with a history of NIDDM for at least 1 year were recruited from the diabetes outpatient clinic of our hospital. NIDDM was defined according to World Health Organization criteria (28) and was treated by diet in combination with oral medication or insulin. Patients aged 30–75 years were considered for inclusion. Metabolic control had to be stable for a period of at least 1 year before the study. Patients were not included if one of the following conditions was present: serum creatinine $>130 \mu\text{mol/l}$, unregulated hypertension (systolic $>160 \text{ mmHg}$ and/or diastolic $>95 \text{ mmHg}$), congestive heart failure, major invalidating illness (end-stage pulmonary disease, cancer, etc.), or pregnancy.

During the first study phase, subjects came to the clinic after an overnight fast of 12 h. The following data were collected for each patient on a single day: height, weight, and blood pressure in the sitting position after 10 min of rest with a standard clinical sphygmomanometer. Blood was drawn and analyzed for HbA_{1c} , serum levels of creatinine, total cholesterol, triglycerides, HDL cholesterol, vitamin B12, folate, pyridoxal-5-phosphate, and total Hcy. Next, subjects were given 0.1 g/kg L-methionine powder dissolved in apple juice. Patients then took their usual dose of oral antidiabetic medication or insulin and were given a low protein breakfast with an individualized carbohydrate content. Blood was drawn again 6 h after methionine ingestion and was analyzed for serum total Hcy. During the 3 days before the study, subjects collected triplicate timed 24-h urine samples, which were analyzed for albumin and creatinine excretion. Microalbuminuria was diagnosed when the median albumin

excretion of these triplicate samples was 30–300 mg/24 h. Otherwise, subjects were classified as normo- ($<30 \text{ mg/24 h}$) or macroalbuminuric ($>300 \text{ mg/24 h}$).

In addition, information was collected regarding retinopathy and history of cardiovascular disease. Retinopathy was diagnosed or excluded only if subjects had been seen by an expert ophthalmologist within 1 year before the day of study. Cardiovascular disease was defined as documented myocardial infarction, angina pectoris confirmed by exercise tests, myocardial perfusion scintigraphy, or a $\geq 70\%$ coronary artery stenosis on cineangiography, a history of coronary bypass surgery, and/or identification of ischemic heart disease on the electrocardiogram (Minnesota codes I1–3, IV1–3, V1–3, VII1) (29). All electrocardiograms were seen by the same cardiologist, who was unaware of the subjects' history or laboratory results. A positive family history for cardiovascular disease was defined as ischemic heart disease in first-degree relatives.

After the first study phase was completed in 85 subjects, a less-intensive protocol was followed with an emphasis on studying further the relationships between microvascular and cardiovascular disease and fasting Hcy only. In this second study phase, subjects ($n = 65$) met the same inclusion and exclusion criteria and were evaluated according to the same protocol, with the exception of the methionine-loading test and the measurement of B vitamins and creatinine clearance. Thus, in the analysis and presentation of the results, predictors of Hcy levels and correlations of post-methionine and δ -Hcy (post-methionine Hcy level minus fasting Hcy level) with complications were analyzed using data from the 85 phase 1 study subjects. The analysis of complications with fasting Hcy is based on the combined data from study phases 1 and 2 (150 subjects).

Analytical methods

Plasma Hcy was measured with high-performance liquid chromatography (HPLC), using a previously described method (30). For better separation, chromatographic conditions were changed into gradient elution from 0 to 20% acetonitril in 0.1 mol/l KH_2PO_4 (pH 1.75). Serum and urinary creatinine was assayed with a modified Jaffé method. HbA_{1c} was measured using an automated HPLC analyzer (reference range 5.2–6.7% [Diamat; BioRad Laboratories, Hercules, CA]). Total cholesterol was meas-

ured using a fully enzymatic (cholesterol oxidase peroxidase [CHOD-PAP]) kinetic method. HDL cholesterol was measured with the same method after precipitation of VLDL and LDL with phosphotungstic acid and magnesium ions. Triglycerides were assayed using an enzymatic method. Serum vitamin B12 and folate were measured with a competitive protein-binding assay (Dual-count Solid Phase Boil assay; Diagnostic Products, Los Angeles, CA). Reference ranges are 150–665 pmol/l for vitamin B12 and 6–39 nmol/l for folate. Pyridoxal-5-phosphate was measured with an HPLC method and fluorimetric detection (reference range 20–100 nmol/l). Urinary albumin was measured using a laser-nephelometric method on a Behring Nephelometer (Behringwerke, Marburg, Germany).

Statistical methods

Hcy levels were expressed as fasting, post-methionine, and increase after methionine (δ -Hcy). Creatinine clearance was calculated as the mean of three 24-h creatinine clearances. Possible predictors of Hcy levels were first tested individually using Spearman's rank correlation test for continuous variables and the Mann-Whitney U test for nominal variables. Multiple linear regression analysis was used to identify independent predictors of Hcy levels. Because the number of potential predictors of Hcy (16 in total) was relatively large compared with the number of subjects in the study, the criterion for entering a variable into the multivariate model was set at a P value for bivariate correlation with Hcy levels ≤ 0.10 . To compare Hcy levels between subjects with normo-, micro- and macroalbuminuria, nonparametric ANOVA (Kruskal-Wallis) was used. Trends in the fraction of patients having (micro-)albuminuria, retinopathy, or cardiovascular disease among several quantiles of Hcy levels were tested using the χ^2 test for trend. The independent correlation of several risk factors with the presence of cardiovascular disease was tested by multiple logistic regression. The P value for initial entry in this logistic regression model was set at 0.20. In a second level of correction, known determinants of cardiovascular disease with P values in the bivariate tests >0.20 were also included in the model. All P values reported are two-sided. A P value of ≤ 0.05 was considered statistically significant.

RESULTS — Table 1 lists demographic and clinical characteristics of subjects included in both study phases. Less than 5%

Table 1—Demographic and clinical characteristics of study population of both study phases of subjects approached for either the phase 1 or phase 2 protocol refused to participate.

	Study phase 1	Study phase 2
<i>n</i>	85	65
Sex (male/pre-/postmenopausal female)	48/15/22	37/10/18
Age (years)	57.2 ± 10.2	55.8 ± 10.1
Actual smoking (no/yes)	65/20	46/19
Time since diagnosis of NIDDM (years)	10.7 (6.6)	10.7 (7.5)
Insulin/oral medication	59/26	47/18
BMI (kg/m ²)	29.1 (4.9)	30.4 (4.2)
Systolic blood pressure (mmHg)	142.1 (16.4)	137.6 (20.0)
Diastolic blood pressure (mmHg)	85.3 (6.9)	80.3 (10.1)
HbA _{1c} (%)	9.2 (1.8)	8.9 (1.6)
Serum creatinine (μmol/l)	93.4 (18.6)	93.8 (15.4)
Serum cholesterol (mmol/l)	5.9 (1.0)	5.4 (1.1)
Serum triglyceride (mmol/l)	1.9 (0.6–18.3)	1.8 (0.6–10.6)
Serum HDL cholesterol (mmol/l)	1.2 (0.4)	1.2 (0.4)
Vitamin B12 (pmol/l)	295.8 (122.6)	NA
Folate (nmol/l)	19.8 (9.5)	NA
Pyridoxal-5-phosphate (nmol/l)	59.2 (28.5)	NA
Creatinine clearance (ml/min)	81.4 (29.8)	NA
Hcy (μmol/l)		
Fasting	11.0 (4.0–23.0)	10.0 (6.0–32.0)
Post-methionine	38.0 (17.0–76.0)	NA
Increase after methionine	26 (11.0–66.0)	NA
(Micro-)vascular complications		
Normo-/micro-/macroalbuminuria	41/33/11	47/14/4
Retinopathy (yes/no)	39/37*	29/32*
History of cardiovascular disease (yes/no)	23/62	21/44

Data are *n*, means ± SD, or medians (range). *Adequate retinopathy data (funduscopy ≤1 year before study) were not available for nine subjects in study phase 1 and for four subjects in phase 2. NA, not available.

Predictors of Hcy levels

Table 2 displays the bivariate correlations between fasting, post-methionine, and δ-Hcy and other variables. Hcy levels were not different in subjects using insulin versus those using oral antidiabetic medication, nor were there effects of ACE-inhibitor or non-ACE-inhibitor antihypertensive agents. There were 31 subjects who were taking metformin. Vitamin B12 and Hcy levels were not different between this group and the group that was not on metformin. About half of all subjects included were non-Caucasians (mainly Creole and Hindustani). No relationship between ethnicity and Hcy was observed (data not shown).

The results of the multivariate analysis are displayed in Table 3. Vitamin B12, folate, creatinine clearance, and menopausal status in women were independent predictors of fasting Hcy. The effect of age on fasting Hcy in the entire group in bivariate analysis was fully accounted for by menopausal status in the female subgroup. Post-methionine Hcy was predicted by pyridoxal-5-phosphate levels and creatinine clearance. Also, men had lower post-methionine Hcy than women, whereas their fasting Hcy was sim-

Table 2—Bivariate correlations of fasting, post-methionine, and increase after methionine (Hcy levels with clinical and laboratory data in 85 NIDDM subjects)

	Fasting Hcy			Post-methionine Hcy			δ-Hcy		
	Mean difference	<i>r</i>	<i>P</i> value	Mean difference	<i>r</i>	<i>P</i> value	Mean difference	<i>r</i>	<i>P</i> value
Continuous variables									
Age (years)	—	0.40	0.003	—	0.20	0.07	—	0.08	0.49
Time since diagnosis of diabetes (years)	—	0.14	0.22	—	0.02	0.83	—	0.004	0.97
BMI (kg/m ²)	—	−0.09	0.41	—	0.12	0.29	—	0.17	0.11
Mean arterial blood pressure (mmHg)	—	0.15	0.18	—	−0.02	0.89	—	−0.05	0.65
HbA _{1c} (%)	—	−0.16	0.15	—	−0.14	0.18	—	−0.06	0.57
Serum cholesterol (mmol/l)	—	0.09	0.42	—	0.05	0.64	—	0.04	0.73
Serum triglycerides (mmol/l)	—	0.01	0.92	—	−0.02	0.86	—	−0.01	0.91
Serum HDL cholesterol (mmol/l)	—	0.19	0.09	—	0.06	0.60	—	0.02	0.82
Vitamin B12 (pmol/l)	—	−0.40	0.003	—	−0.20	0.06	—	−0.09	0.43
Folate (nmol/l)	—	−0.29	0.008	—	−0.14	0.19	—	−0.06	0.61
Pyridoxal-5-phosphate (nmol/l)	—	−0.22	0.06	—	−0.35	0.003	—	−0.33	0.005
Creatinine clearance (ml · min ^{−1} · 1.73 m ^{−2})	—	−0.48	<0.001	—	−0.38	<0.001	—	−0.26	0.02
Nominal variables									
Sex (M)	−0.05	—	0.93	−7.3	—	0.03	−7.2	—	0.004
Postmenopausal status in females	5.1	—	<0.001	9.0	—	0.06	3.9	—	0.27
Current smoking (yes)	1.6	—	0.18	3.7	—	0.05	2.1	—	0.11

P values for nominal variables were calculated with the Mann-Whitney *U* test. Hcy is given in micromoles per liter. *r*, Spearman's ρ correlation coefficient.

Table 3—Significant predictors of one or more types of Hcy levels in multivariate analysis

	Fasting Hcy		Post-methionine Hcy		δ-Hcy	
	<i>b</i>	<i>P</i> value	<i>b</i>	<i>P</i> value	<i>b</i>	<i>P</i> value
Postmenopausal status in females	4.45	0.002	—	—	—	—
Sex (M)	—	—	-5.4	0.02	-6.2	0.002
Vitamin B12 (per 10 pmol/l)	-0.07	0.04	—	—	—	—
Folate (per 1 nmol/l)	-0.1	0.01	—	—	—	—
Pyridoxal-5-phosphate (per 1 nmol/l)	—	—	-0.11	<0.001	-0.10	<0.001
Creatinine clearance (per 10 ml · min ⁻¹ · 1.73 m ⁻²)	-0.8	<0.001	-0.9	0.04	—	—

b, multivariate regression coefficient.

ilar. The correlation between smoking and post-methionine Hcy in Table 2 was reduced to a trend (*b* = 4.6, *P* = 0.08) after correction for other factors. Finally, only sex and pyridoxal-5-phosphate levels were predictors of δ-Hcy. Apart from the effect of current smoking on post-methionine Hcy, no other regression trends (0.05 < *P* ≤ 0.10) for any potential determinant of either fasting, post-methionine, or δ-Hcy were found in the multivariate analyses.

Regression plots of the continuous variables in Table 3 are shown in Fig. 1. As is evident from these plots, none of these correlations were linear. Threshold levels based on visual assessment are shown as vertical lines. The most powerful threshold levels in terms of the degree of statistical significance and size of the effect on Hcy levels were subsequently located in multivariate analysis and were found to be identical to those in Fig. 1 (data not shown).

From Fig. 1, one can observe that levels of vitamin B12 <350 pmol/l, folate <20 nmol/l, pyridoxal-5-phosphate <80 nmol/l, and creatinine clearance <80 ml · min⁻¹ · 1.73 m⁻² predispose to sharp increases in Hcy levels.

Correlations with micro- and macrovascular complications

In Table 4, fasting, post-methionine, and δ-Hcy are compared between subjects with various degrees of albuminuria and between those with and without retinopathy. These data provide no evidence of a correlation between Hcy levels and albuminuria or retinopathy. In successive quartiles of fasting Hcy levels, no trend toward an increase in the prevalence of either albuminuria (micro- and macroalbuminuria taken as a group) or retinopathy was observed (Fig. 2A). A similar result was seen for post-methionine Hcy and δ-Hcy (not graphically displayed).

As for cardiovascular disease, during the first study phase, a higher fasting Hcy level was observed in subjects with versus those without a history of cardiovascular disease

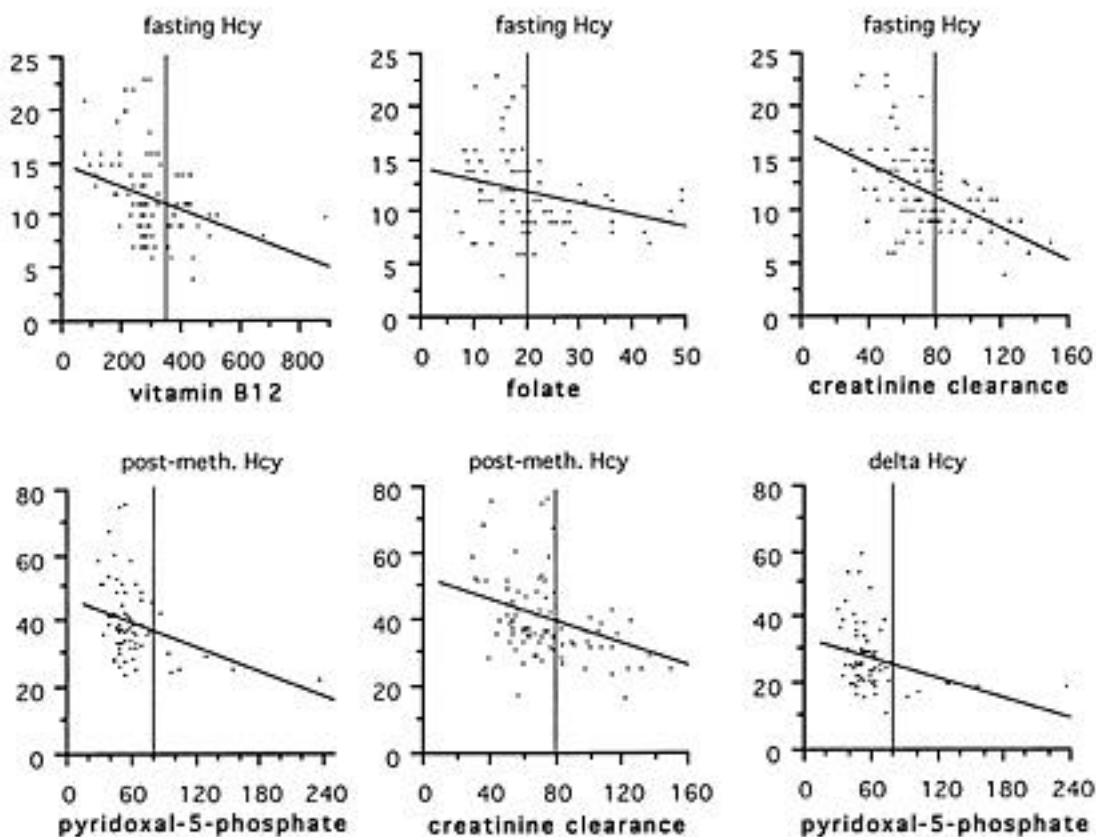


Figure 1—Regression plots of continuous variables significantly associated with fasting, post-methionine (post-meth.), and increase after methionine (delta) Hcy in multivariate analysis. Threshold levels are indicated by vertical lines. Units are as follows: Hcy levels, μmol/l; vitamin B12, pmol/l; folate, nmol/l; pyridoxal-5-phosphate, nmol/l; and creatinine clearance, ml · min⁻¹ · 1.73 m⁻².

Table 4—Hcy levels in relation to microvascular complications

	Fasting Hcy ($\mu\text{mol/l}$)	<i>n</i>	Post-methionine Hcy ($\mu\text{mol/l}$)	<i>n</i>	δ -Hcy ($\mu\text{mol/l}$)
<i>n</i>	150	—	85	—	85
Microvascular disease					
Albuminuria					
Normoalbuminuria	11.0 (4–32)	89	37.0 (17–76)	41	26.0 (13–60)
Microalbuminuria	10.0 (6–23)	49	38.0 (18–76)	33	26.0 (11–66)
Macroalbuminuria	13.5 (9–22)	12	40.0 (29–75)	11	28.0 (19–54)
<i>P</i> value	0.65	—	0.58	—	0.76
Retinopathy					
No	11.0 (4–32)	71	37.0 (17–76)	39	26.0 (11–66)
Yes	11.0 (6–26)	66	37.0 (26–75)	37	26.0 (16–54)
<i>P</i> value	0.25	—	0.54	—	0.50

Data are median (range) or *n*. *P* values were obtained by the Kruskal-Wallis test for albuminuria categories and by the Mann-Whitney *U* test for retinopathy. Results are given for the combined data of the two study phases for fasting Hcy. *n* for post-methionine Hcy equals *n* for δ -Hcy.

(median fasting Hcy 14.0 $\mu\text{mol/l}$ [7–23] vs. 11.0 $\mu\text{mol/l}$ [4–22], *P* = 0.05.) The same was not true for post-methionine Hcy (39.0 $\mu\text{mol/l}$ [18–69] vs. 37.0 $\mu\text{mol/l}$ [17–76], *P* =

0.50) or for δ -Hcy (26.0 $\mu\text{mol/l}$ [11–46] vs. 26.0 $\mu\text{mol/l}$ [13–66], *P* = 0.80). In the combined analysis of study phases 1 and 2, fasting Hcy levels were again significantly higher

in subjects with those versus without a history of cardiovascular disease. Table 5 lists Hcy levels and other known risk factors for atherosclerotic disease in subjects with and without a history of cardiovascular disease. Age and family history of cardiovascular disease appear to be the main potential confounders of the relationship between fasting Hcy and cardiovascular disease in this data set. After adjustment for these factors by multiple logistic regression analysis, fasting Hcy remained independently correlated with cardiovascular disease (*P* = 0.02). Additional correction for cholesterol, blood pressure, duration of NIDDM, and smoking resulted in a *P* value for fasting Hcy of 0.06 (data not shown). To determine the nature of the correlation observed, the prevalence of cardiovascular disease in successive quartiles of fasting Hcy levels is shown in Fig. 2B. There was a stepwise increase in the prevalence of cardiovascular disease with increasing fasting Hcy (trend analysis, *P* < 0.01).

The diagnosis of cardiovascular disease was based on electrocardiographic abnormalities only in three subjects. Of the subjects with cardiovascular disease, six had an additional history of ischemic stroke, and five others had additional peripheral arterial occlusive disease. Of the subjects without cardiovascular disease, three had a history of ischemic stroke, and four others had peripheral arterial occlusive disease. Because the number of subjects suffering from other than cardiovascular atherosclerotic disease was so small, the analyses were restricted to cardiovascular disease only.

CONCLUSIONS — In this cross-sectional study, we systematically studied fasting and post-methionine Hcy and

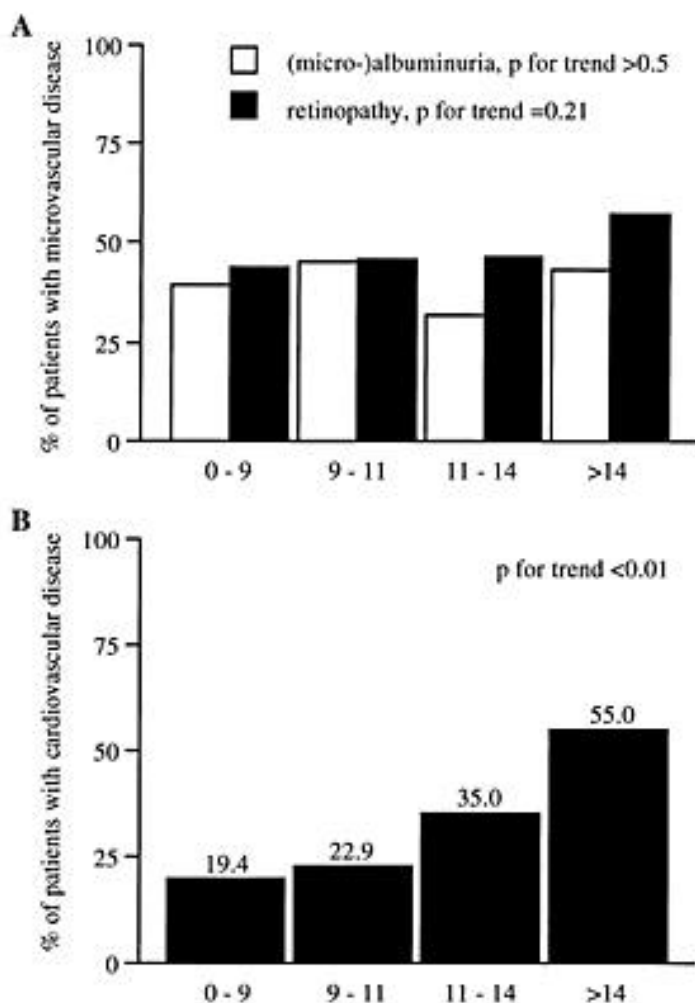


Figure 2—Percentage of subjects with (micro)albuminuria or retinopathy (A) and with a history of cardiovascular disease (B) in quartile groups of fasting Hcy levels. Values are by χ^2 test for trend.

Table 5—Fasting Hcy levels and other risk factors in relation to presence of cardiovascular disease in 150 NIDDM subjects

	Cardiovascular disease		P
	Absent	Present	
n	106	44	—
Age (years)	54.8 ± 10.3	59.1 ± 9.2	0.02
Sex (M/F)	60/46	25/19	0.98
BMI (kg/m ²)	30.0 ± 4.9	28.9 ± 3.8	0.94
Systolic blood pressure (mmHg)	140.3 ± 18.1	139.8 ± 18.6	0.94
Diastolic blood pressure (mmHg)	82.9 ± 8.9	80.7 ± 9.6	0.23
Time since diagnosis of NIDDM (years)	9.5 (1–31)	8.5 (1–30)	0.60
HbA _{1c} (%)	8.8 ± 1.8	8.9 ± 1.7	0.63
Total cholesterol (mmol/l)	5.7 ± 1.0	5.7 ± 1.2	0.74
Triglyceride (mmol/l)	1.8 (0.8–6.9)	2.2 (0.6–18.4)	0.26
HDL cholesterol (mmol/l)	1.3 ± 1.2	1.2 ± 0.4	0.58
Actual smoking (no/yes)	77/29	35/9	0.33
Family history of cardiovascular disease (yes/no)	31/75	21/23	0.02
Fasting Hcy (μmol/l)	10.0 (4–32)	13.0 (7–26)	0.02

Data are means ± SD, medians (range), or n. P values were obtained by the Mann-Whitney U test and the χ^2 test.

correlating factors in a defined group of NIDDM subjects. The results indicate that in NIDDM, the patient's vitamin status influences Hcy levels. It is important to note that Hcy levels increase with values of vitamin B12, pyridoxal-5-phosphate, and folate that are conventionally regarded to be safely within their normal ranges. A similar conclusion can be drawn for creatinine clearance, which is associated with sharply increasing Hcy levels as soon as it falls below a level of 80 ml · min⁻¹ · 1.73 m⁻². Furthermore, postmenopausal status in women associates with higher fasting Hcy, and women show larger increases in serum Hcy after a methionine load. The data provide no evidence for a tendency of hyperhomocysteinemia to cluster with other cardiovascular risk factors, particularly those present in the insulin resistance syndrome, including obesity, hypertension, and dyslipidemia.

Determinants of Hcy levels have been the subject of intensive research in nondiabetic subjects. Our findings are largely in line with the results of these studies. However, we found cut-off values for vitamin levels and creatinine clearance below which Hcy rises sharply that are higher than those generally reported in the literature (21,31–33). Although we did not study a nondiabetic control group, a comparison with data from the literature suggests that Hcy levels may rise in response to decreasing vitamin levels and glomerular filtration earlier in NIDDM patients than in nondia-

betic subjects. The observed effect of menopausal status on fasting Hcy in women is widely recognized. This cannot be said for the sharper rise in post-methionine Hcy in women (compared with men), which to our knowledge, has only been described once (34).

No correlation was found between hyperhomocysteinemia and degree of albuminuria. Although we acknowledge the intrinsic limitations of cross-sectional data in terms of testing for causal relationships, our data suggest that hyperhomocysteinemia cannot help explain why NIDDM patients with microalbuminuria have such a sharply increased risk of dying from cardiovascular complications. As is the case for albuminuria, Hcy levels did not correlate with the presence of diabetic retinopathy.

An important finding was the relationship between fasting Hcy and cardiovascular disease. The data show a gradual increase in the fraction of subjects with such a history with increasing fasting Hcy levels. Why only fasting Hcy correlates with cardiovascular disease in our study, and post-methionine or δ -Hcy does not, is unclear. It is, however, evident that the roles of post-methionine Hcy and δ -Hcy are less well defined than that of fasting Hcy, since prospective studies addressing the role of Hcy in atherosclerotic disease have not used post-methionine levels (14–16,18). Although measuring only fasting Hcy may fail to identify a fraction of subjects with high post-methionine levels

(35,36), the clinical relevance of isolated post-methionine hyperhomocysteinemia is unproven in prospective studies.

Previous studies of Hcy levels in IDDM or NIDDM have been relatively scarce. In 1991, Hultberg et al. (37) studied Hcy in IDDM patients and found a correlation with increased serum creatinine and increased albumin excretion rates. Another study showed lower Hcy levels in IDDM patients than in control subjects and failed to show a correlation of Hcy levels with renal function, albuminuria, or retinopathy (38). Agardh et al. (39) studied IDDM patients and found no correlation of Hcy with either retinopathy or the presence of microalbuminuria. In contrast to these studies, retinopathy was recently reported to correlate with mild hyperhomocysteinemia in a small (n = 25) number of patients (40). This finding was supported by a larger study, which also showed higher Hcy levels in IDDM patients versus nondiabetic control subjects. In addition, hyperhomocysteinemia in this study correlated with neuropathy, macroangiopathy, and albuminuria, although the latter correlation was not corrected for creatinine clearance (41). Finally, Hcy levels were recently reported to correlate with serum creatinine, albuminuria and folate, but not with retinopathy, in 50 IDDM patients (42). Unfortunately, the apparent correlation between albuminuria and Hcy was again not corrected for serum creatinine, let alone for creatinine clearance. In conclusion, for IDDM, the literature shows conflicting conclusions regarding a possible influence of the diabetic state per se on Hcy and regarding correlations of Hcy with various manifestations of diabetic organ damage.

As for studies in NIDDM, one report showed higher fasting Hcy levels in patients with macroangiopathy and did not reveal a correlation between Hcy and retinopathy or nephropathy, defined as proteinuria or increased serum creatinine (23). A recent study in 28 diabetic patients, only 18 of whom had NIDDM, suggested that post-methionine, and not fasting, hyperhomocysteinemia is correlated with the presence of vascular disease (24). The small number of subjects included somewhat limits the value of this last study. Retinopathy in NIDDM patients was found to correlate with the thermolabile MTHFR gene mutation, but the authors failed to report Hcy levels (43). Hoogeveen et al. (25) recently found an interaction effect of fasting hyperhomocysteinemia and NIDDM in terms of risk of macrovascular disease. In the same

study population, which included only Caucasian subjects, Hcy levels did show a correlation with albuminuria (26). The apparent discrepancy with the results that we obtained can possibly be explained by the different ethnic background of our population or by the fact that our study may have been underpowered to detect a threshold level of Hcy above which albuminuria increases. Finally, the recent report by Chico et al. (27) in 75 IDDM and 90 NIDDM subjects suggests that albuminuria correlates with fasting Hcy levels. However, in this study, the authors have chosen the rather questionable approach of analyzing albumin excretion rate as a covariate of serum creatinine, considering both as determinants of Hcy levels. The result was that serum creatinine did not qualify as a significant determinant of Hcy levels, whereas albumin excretion rate did. We believe that it is more appropriate to consider (micro)albuminuria as a possible complication of hyperhomocysteinemia, rather than the other way round.

Our study has several advantages compared with these previous studies. First, no other study has systematically measured both fasting and post-methionine Hcy in a substantial number of NIDDM subjects. Also, the relationship of Hcy with levels of pyridoxal-5-phosphate, vitamin B12, folate, and creatinine clearance was not previously studied in detail in a diabetic population. The sample size of $n = 150$ to study the relationship between fasting Hcy and micro- and macrovascular complications is unmatched by other studies in NIDDM. Finally, assessment of the level of albuminuria should preferably be based on multiple urine samples, as was done in our study, because of the high day-to-day variability of urinary albumin excretion (44).

The limitations of our study are largely inherent to cross-sectional studies in general. The causal relationship between fasting Hcy and the presence of cardiovascular disease cannot be proven using cross-sectional data. On the other hand, cross-sectional correlations between hyperhomocysteinemia and cardiovascular disease have usually been confirmed by prospective studies. The subjects in our study were recruited from an outpatient clinic for diabetes patients and are, consequently, not representative of the general population of NIDDM patients. The reasons for referral were mostly related to difficulties in achieving acceptable metabolic control with oral anti-diabetic agents, with initiation or follow-up of patients tak-

ing insulin or with comorbidity in the field of internal medicine. Consequently, none of our findings should be interpreted as describing the typical NIDDM patient. Rather, the value of our study is in the exploration of pathophysiological mechanisms. Finally, the absence of a nondiabetic control group limits interpretations regarding the extent to which determinants of hyperhomocysteinemia may behave in a manner that is typical for NIDDM.

The practical implications of our conclusions are that practitioners treating NIDDM patients should be aware of the risk of hyperhomocysteinemia, especially in the very early stages of nephropathy. Attention should be given to dietary measures ensuring that levels of vitamin B12, folate, and pyridoxal-5-phosphate are well within the upper range of their respective normal values. Whether all NIDDM patients should be screened for mild hyperhomocysteinemia is debatable. It seems justified, however, to measure fasting Hcy in NIDDM patients with minor reductions in creatinine clearance and in those who have already experienced a cardiovascular event. Treatment of hyperhomocysteinemia is relatively easy with folate supplementation, either alone or in combination with cyanocobalamin or pyridoxine (36), and is probably just as feasible in diabetic as in nondiabetic patients. Prospective studies are needed to evaluate whether therapeutic interventions aimed at lowering Hcy levels can reduce cardiovascular morbidity and mortality.

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References

- Panzram G: Mortality and survival in type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 30:123-131, 1987
- Zimmet PZ, Alberti KGMM: The changing face of macrovascular disease in non-insulin-dependent diabetes mellitus: an epidemic in progress. *Lancet* 350 (Suppl. 1):S11-S13, 1997
- Stamler J, Vaccaro O, Neaton JD, Wentworth D: Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care* 16:434-444, 1993
- Nathan DM, Meigs J, Singer DE: The epidemiology of cardiovascular disease in type 2 diabetes mellitus: how sweet it is...or is it? *Lancet* 350 (Suppl. 1):S14-S19, 1997
- Jarrett RJ, Viberti GC, Argyropoulos A, Hill RD, Mahmud U, Murrells TJ: Microalbuminuria predicts mortality in non-insulin dependent diabetes. *Diabet Med* 1:17-19, 1984
- Mogensen CE: Microalbuminuria predicts clinical proteinuria and early mortality in maturity onset diabetes. *N Engl J Med* 310:356-360, 1984
- Schmitz A, Vaeth M: Microalbuminuria: a major risk factor in non-insulin dependent diabetes: a 10-year follow-up study of 503 patients. *Diabet Med* 5:126-134, 1988
- Gall MA, Borch-Johnsen KB, Hougaard P, Nielsen FS, Parving HH: Albuminuria and poor glycaemic control predict mortality in NIDDM. *Diabetes* 44:1303-1309, 1995
- Mathiesen ER, Ronn B, Jensen T, Storm B, Deckert T: Relationship between blood pressure and urinary albumin excretion in development of microalbuminuria. *Diabetes* 39:245-249, 1990
- Niskanen L, Uusitupa M, Sarlund H, Siitonen O, Voutilainen E, Penttilä I, Pyörälä K: Microalbuminuria predicts the development of serum lipoprotein abnormalities favouring atherogenesis in newly diagnosed type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 33:237-243, 1990
- Knöbl P, Schernthaner G, Schnack C, Pietschmann P, Griesmacher A, Prager R, Müller M: Thrombogenic factors are related to urinary albumin excretion rate in type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 36:1045-1050, 1993
- Standl E, Stiegler H: Microalbuminuria in a random cohort of recently diagnosed type 2 (non-insulin-dependent) diabetic patients living in the greater Munich area. *Diabetologia* 36:1017-1020, 1993
- Alzaid AA: Microalbuminuria in patients with NIDDM: an overview. *Diabetes Care* 19:79-89, 1996
- Stampfer MJ, Malinow R, Willet WC, Newcomer LM, Upson B, Ullmann D, Tishler PV, Hennekens CH: A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *JAMA* 268:877-881, 1992
- Perry I, Refsum H, Morris R, Ebrahim S, Ueland P, Shaper A: Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. *Lancet* 346:1395-1398, 1995
- Arnesen E, Refsum H, Bønaa KH, Ueland PM, Førde OH, Nordrehaug JE: Serum total homocysteine and coronary heart disease. *Int J Epidemiol* 24:704-709, 1995
- Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG: A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *JAMA* 274:1049-1057, 1995
- Nygård O, Nordrehaug JE, Refsum H,

- Ueland PM, Farstad M, Vollset SEE: Plasma homocysteine levels and mortality in patients with coronary heart disease. *N Engl J Med*337:230–236, 1997
19. Graham IM, Daly LE, Refsum HM, Robinson K, Brattström LE, Ueland PM, Palma-Reis RJ, Boers GHJ, Sheahan RG, Israelson B, Uitterwaal CS, Meleady R, McMaster D, Verhoef P, Witteman J, Rubba P, Bellet H, Wautrecht JC, Valk HWD, Sales-Luis AC, Parrot-Roulaud M, Tan KS, Higgins J, Gargcon D, Medrano MJ, Candito M, Evans AE, Andria G: Plasma homocysteine as a risk factor for vascular disease. *JAMA* 277:1775–1781, 1997
 20. D'Angelo A, Selhub J: Homocysteine and thrombotic disease. *Blood*90:1–11, 1997
 21. Selhub J, Jacques PF, Wilson PWF, Rush D, Rosenberg IH: Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA*270:2693–2698, 1993
 22. Bellamy MF, McDowell IFW: Putative mechanisms for vascular damage by homocysteine. *J Inher Metab Dis*20:307–315, 1997
 23. Araki A, Sako Y, Ito H: Plasma homocysteine concentrations in Japanese patients with non-insulin-dependent diabetes mellitus: effect of parenteral methylcobalamin treatment. *Atherosclerosis*103:149–157, 1993
 24. Munshi M, Stone A, Fink L, Fonseca V: Hyperhomocysteinemia following a methionine load in patients with non-insulin-dependent diabetes mellitus and macro-vascular disease. *Metabolism*45:133–135, 1996
 25. Hoogeveen EK, Kostense PJ, Beks PJ, Mackaay AJC, Jakobs C, Bouter LM, Heine RJ, Stehouwer CDA: Hyperhomocysteinemia is associated with an increased risk of cardiovascular disease, especially in non-insulin-dependent diabetes mellitus. *Arterioscler Thromb Vasc Biol*18:133–138, 1998
 26. Hoogeveen EK, Kostense PJ, Jager A, Heine RJ, Jakobs C, Bouter LM, Donker AJM, Stehouwer CDA: Serum homocysteine level and protein intake are related to the risk of microalbuminuria: the Hoorn study. *Kidney Int*54:203–209, 1998
 27. Chico A, Pérez A, Córdoba A, Arcélus R, Carreras G, Leiva AD, González-Sastre F, Blanco-Vaca F: Plasma homocysteine is related to albumin excretion rate in patients with diabetes mellitus: a new link between diabetic nephropathy and cardiovascular disease? *Diabetologia*1:684–693, 1998
 28. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*20:1183–1197, 1997
 29. Blackburn H, Keys A, Simonson E, Rautaharju P, Punsar S: The electrocardiogram in population studies. *Circulation*21:1160–1175, 1960
 30. Vester B, Rasmussen K: High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. *Eur J Clin Chem Clin Biochem*29:549–554, 1991
 31. Brattström L, Lindgren A, Israelsson B, Andersson A, Hultberg B: Homocysteine and cysteine: determinants of plasma levels in middle-aged and elderly subjects. *J Intern Med*236:633–641, 1994
 32. Pancharutini N, Lewis CA, Sauberlich HE, Perkins LL, Go RCP, Alvarez JO, Macaluso M, Acton RT, Copeland RB, Cousins AL, Gore JB, Cornwell PE, Roseman JM: Plasma homocyst(e)ine, folate, and vitamin B12 concentrations and risk for early-onset coronary artery disease. *Am J Clin Nutr* 59:940–948, 1994
 33. Arnadottir M, Hultberg B, Nilsson-Ehle P, Thysell H: The effect of reduced glomerular filtration rate on plasma total homocysteine concentration. *Scand J Lab Clin Invest* 56:41–46, 1996
 34. Andersson A, Brattström L, Israelsson B, Isaksson A, Hamfelt A, Hultberg B: Plasma homocysteine before and after methionine loading with regard to age, gender, and menopausal status. *Eur J Clin Invest* 22:79–87, 1992
 35. Bostom A, Jacques P, Nadeau M, Williams R, Ellison R, Selhub J: Post-methionine load hyperhomocysteinemia in persons with normal fasting total plasma homocysteine: initial results from the NHBLI Family Heart Study. *Atherosclerosis*116:147–151, 1995
 36. Berg MV, Boers GHJ: Homocystinuria: what about mild hyperhomocysteinemia? *Postgrad Med J*72:513–518, 1996
 37. Hultberg B, Agardh E, Andersson A, Brattström L, Isaksson A, Israelsson B, Agardh C: Increased levels of plasma homocysteine are associated with nephropathy, but not severe retinopathy in type 1 diabetes mellitus. *Scand J Clin Lab Invest*51:277–282, 1991
 38. Robillon JF: Type 1 diabetes mellitus and homocyst(e)ine. *Diabete Metab*20:494–496, 1994
 39. Agardh CD, Agardh E, Andersson A, Hultberg B: Lack of association between plasma homocysteine levels and microangiopathy in type 1 diabetes mellitus. *Scand J Clin Lab Invest*54:637–641, 1994
 40. Vaccaro O, Ingrosso D, Rivellesse A, Greco G, Riccardi G: Moderate hyperhomocysteinemia and retinopathy in insulin dependent diabetes. *Lancet*349:1102–1103, 1997
 41. Hofmann MA, Kohl B, Zumbach MS, Borcea V, Bierhaus A, Henkels M, Amiral J, Fiehn W, Ziegler R, Wahl P, Nawroth PP: Hyperhomocyst(e)inemia and endothelial dysfunction in IDDM. *Diabetes Care* 20:1880–1886, 1997
 42. Hultberg B, Agardh CD, Agardh E, Lövestram-Adrian M: Poor metabolic control, early age at onset, and marginal folate deficiency are associated with increasing levels of plasma homocysteine in insulin-dependent diabetes mellitus: a five-year follow-up study. *Scand J Clin Lab Invest*57:595–600, 1997
 43. Neugebauer S, Baba T, Kurokawa K, Watanabe T: Defective homocysteine metabolism as a risk factor for diabetic retinopathy. *Lancet*349:473–474, 1997
 44. Mogensen CE, Vestbo E, Poulsen PL, Christiansen C, Damsgaard EM, Eiskjaer H, Frøland A, Hansen KW, Nielsen S, Pedersen MM: Microalbuminuria and potential confounders. *Diabetes Care*18:572–581, 1995