Fasting and Post–Methionine Load Homocyst(e)ine Values Are Correlated With Microalbuminuria and Could Contribute to Worsening Vascular Damage in Non–Insulin-Dependent Diabetes Mellitus Patients

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The study aim was to assess the relationship between homocyst(e)inemia and microalbuminuria in non–insulin-dependent diabetes mellitus (NIDDM) patients. The study was performed on 33 NIDDM patients (16 males and 17 females), and 16 healthy control subjects (seven males and nine females). Plasma fasting and post–methionine load homocyst(e)ine (tHcy), together with other parameters that could modify tHcy levels, were assessed. There were no significant differences between NIDDM patients and controls for fasting tHcy (8.12 ± 3.17 *v* 7.19 ± 2.40 μmol/L) and post–methionine load tHcy (26.51 ± 11.50 *v* 25.06 ± 10.76 μmol/L). Moreover, there was a significant correlation between urinary albumin excretion (UAE) and fasting tHcy ($r = .340$, $P = .05$) and post–methionine load tHcy ($r = .502$, $P = .004$) in NIDDM patients. Fasting tHcy was correlated both with post–methionine load tHcy ($r = .429$, $P = .01$) and with vitamin B_{12} ($r = -.349$, $P = .04$) in NIDDM patients. Microalbuminuric NIDDM patients had higher fasting tHcy (9.05 ± 3.83 μmol/L) than normoalbuminurics (7.12 ± 1.95 μmol/L). In addition, NIDDM patients with complications presented higher fasting tHcy values than the group without complications (9.61 ± 3.34 *v* 6.53 ± 2.09 μmol/L, Kolmogorov-Smirnov two-sample test for nonparametric data [KS] = 1.794, $P = .003$), without any other significant differences in the parameters considered. tHcy could be an important risk factor worsening the prognosis in NIDDM patients, especially microalbuminuric patients. Microalbuminuric NIDDM patients could be particularly prone to hyperhomocyst(e)inemia, probably due to endothelial or renal dysfunction with a reduction in the scavenging of tHcy.

HOMOCYST(E)INE (tHcy) is an intermediate sulfur amino acid formed during the conversion of methionine to cysteine. tHcy can be metabolized via the transsulfuration pathway to form cystathionine or via a remethylation pathway to form methionine.[1] Only a small amount of tHcy circulates freely; most of it is protein-bound. Almost all of the population presents tHcy values from 5 to 15 μmol/L, but often, due to genetic or acquired factors, plasma tHcy may be increased.[1-4] This increase might be responsible for atherogenic and thrombotic tendencies as shown in homocystinuria, a rare autosomal recessive disease caused by a deficiency of cystathionine β-synthetase.[1] Premature atherosclerosis occurs in patients with increased levels of this sulfur amino acid, but many hypotheses have been advanced to explain this tendency. Among the possible causes are an induction of cyclin A gene expression in vascular smooth muscle cells[5]; endothelial dysfunction[6]; a reduced level of protein C, a natural anticoagulant involved in the blockage of factor V and VII of the clotting system[7]; inhibition of von Willebrand factor processing and secretion[8]; enhancement of lipid peroxidation[9]; direct endothelium damage caused by tHcy, due to a toxic sulfur-containing amino acid accumulation in endothelial cells[10]; interaction between nitric oxide (NO) and tHcy[11-12]; and finally, a reduction in serum antithrombin activity with a reduction of thrombomodulin, which links thrombin.[13] Microalbuminuria, defined as increased urinary albumin excretion (UAE) greater than 30 mg/24 h and less than 300 mg/24 h, is related to systemic disorders of the transcapillary escape rate and could be considered not only a cardiovascular risk factor but also a marker of endothelial impairment.[14] Our purpose was to evaluate the relationship between fasting tHcy and post–methionine load tHcy with increased urinary albumin excretion as a marker of endothelial or renal dysfunction in non–insulin-dependent diabetes mellitus (NIDDM) patients.

SUBJECTS AND METHODS

The study was performed on 33 NIDDM patients (16 males and 17 females; mean age, 60.9 ± 5.93 years; duration of disease, 7.75 ± 5.35 years) diagnosed as suggested in previous studies[15] and 16 healthy control subjects (seven males and nine females) matched for age, sex, and Quatelet index. All patients and controls provided informed consent to be enrolled in the study.

Subjects were defined as having hypertension if the systolic blood pressure was at least 160 mm Hg or diastolic blood pressure at least 95 mm Hg or if they were receiving drug treatment for hypertension. If they had hypertension or overt renal dysfunction, they were excluded from the study. Plasma tHcy levels were assessed at baseline in both patients and controls, as were folate and vitamin B_{12}; furthermore, microalbuminuria, hemoglobin A_{1c} (HbA_{1c}), creatinine, total cholesterol, high-density lipoprotein (HDL), triglycerides, leucocyte count, smoking, the presence of complications, and therapy were analyzed in NIDDM patients.

All patients and controls were subjected to the methionine load test because in some patients with impaired tHcy metabolism, fasting concentrations may be normal.[16] Participants were phlebotomized following an overnight fast (10 to 14 hours), and blood samples were taken at baseline and once again 5 hours after an oral load (0.1 g/kg body weight) of L-methionine.[16] All whole blood specimens for tHcy analysis were drawn into vacutainers containing EDTA and immediately refrigerated at 4°C. Within 2 hours of collection, blood samples were centrifuged, and then the EDTA plasma was separated and stored at −20°C. Total plasma tHcy was determined by high-performance liquid chromatography (HPLC) with fluorescence detection as previously described by Ubbink et al.[17] Prior to reversed-phase HPLC analysis, thiols were derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, a thiol-specific fluorogenic probe that is commercially available. The method is simple, sensitive, reproducible (between-run coefficient of variation, 6.6%), and very suitable for routine determination of plasma tHcy levels.

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Submitted May 6, 1997; accepted December 1, 1997.

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0026-0495/98/4708-0005$03.00/0

Urine samples were collected over a 24-hour period, and UAE was measured by nephelometry (Kone Microalbuminuria Diagnostics Kit, Eespoo, Finland). Microalbuminuria was defined as UAE greater than 30 mg/24 h and less than 300 mg/24 h. Vitamin B_{12} and folate levels were determined simultaneously in a single tube by the ICN Pharmaceuticals SimulTRAC-SNB Radioassay Kit (Costa Mesa, CA). All of the other parameters were analyzed as described in previous studies.[4,16,18]

A positive smoking history was recorded when the individual was still a smoker or had quit less than 1 year before being interviewed. All of the complications correlated with a prothrombotic state were recorded. Recorded documentation was used to establish the presence of atherothrombotic and cardiovascular complications, as well as any other kind of disease or complication related to endothelial disorders (eg, retinopathy or obliterative arteriopathy). Particular importance was assigned to the following events: acute myocardial infarction (if clearly documented admission to an intensive care unit was required or if Q waves were present on the electrocardiogram [ECG]); coronary artery disease (if a history of angina pectoris or repolarization defects were present); ischemic stroke (without considering extracranial or intracranial causes of stroke such as chronic atrial fibrillation, cardiac valve dysfunction or replacement, or presence of antiphospholipid antibodies, atherosclerotic plaque in the carotid artery, or intracranial aneurysm); arterial thromboembolism; peripheral vascular disease; and retinopathy. Through the use of ECG, computed tomographic (CT) scan, arteriography, B-mode/Doppler ultrasound, and measurement of the principal enzymatic levels, most of the complications were confirmed. All patients underwent an ultrasound test of the carotid artery with echocolor-Doppler to exclude hemodynamically significant stenosis. Patients who presented signs of complications or vascular involvement, particularly of cerebral origin, were subjected to a CT scan. The diagnosis of stroke was made in the presence of neurologic signs, with CT scan confirmation of cerebral damage. Moreover, arteriography was not performed as a matter of course in all of the patients or controls, but only in patients with clear signs of cerebral involvement, ie, in three patients with ischemic stroke and in one patient with obliterative arteriopathy, also in view of a possible surgical approach.

Descriptive values are expressed as the mean ± SD. Differences between groups were tested with Student's *t* test for unpaired data, and when the data were not normally distributed, the Kolmogorov-Smirnov two-samples test (KS) was used. Spearman's rank correlation coefficient was used to assess correlations between variables not normally distributed. Values for UAE and tHcy were natural logarithm–transformed as required to improve normality. Multiple stepwise regression models were executed to include variables significantly correlated with ln tHcy and with ln UAE. Multifactorial ANOVA was used to analyze the three groups (controls, microalbuminurics, and normoalbuminurics) after the logarithmic transformation, with multiple range analysis. Where appropriate, Fisher's test was used. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL). A value of P less than or equal to .05 was considered significant.

RESULTS

Characteristics of the NIDDM group are summarized in Table 1. The diabetes appeared fairly well compensated, and 17 NIDDM subjects presented complications (five retinopathy, four coronary artery disease, three ischemic stroke, two previous myocardial infarction, one obliterative arteriopathy, one coronary artery disease plus retinopathy, and one arterial thromboembolism). All of them were treated with diet or hypoglycemic agents alone. Nine of the patients were smokers. Fasting tHcy values (8.12 ± 3.17 *v* 7.19 ± 2.40 μmol/L, KS = .319, P = .99) and post–methionine load tHcy values (26.51 ± 11.50 *v* 25.06 ± 10.76 μmol/L, KS = 0.495, P = .96) were similar in NIDDM subjects versus controls. Four NIDDM patients (12.1%) with fasting tHcy values less than 9 μmol/L showed impaired methionine tolerance (IMT) post–methionine load (greater than the 75th percentile for post–methionine load tHcy value).

Table 1. Characteristics of the NIDDM Patients (distribution analysis)

Characteristic	Mean ± SD	Median	25th Percentile	75th Percentile
Age (yr)	60.9 ± 5.93	61	58	65
Diabetes duration (yr)	7.75 ± 5.35	7	3	12
Glucose (mg/dL)	177.9 ± 46.1	176	153	209
Fasting tHcy (μmol/L)	8.12 ± 3.17	7.86	6.16	9.41
Postload tHcy (μmol/L)	26.5 ± 11.5	24.6	17.9	29.9
SBP (mm Hg)	138 ± 13.2	135	130	150
DBP (mm Hg)	80 ± 9.01	80	75	85
UAE (mg/24 h)	33.5 ± 25.7	30	17	38
HbA_{1c} (%)	8.04 ± 1.84	7.8	6.9	8.4
Folate (ng/mL)	74.5 ± 36.5	68	51	85
Vitamin B_{12} (pg/mL)	582.6 ± 207.6	559	435	690
Creatinine (mg/dL)	0.90 ± 0.29	0.9	0.8	1.0
Total cholesterol (mg/dL)	224.4 ± 28.2	226	203	242

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure.

For UAE, 17 of 33 NIDDM patients (51.5%) had values above 30 mg/24 h and were considered microalbuminurics, 16 were normoalbuminurics, and none had overt proteinuria. The distributions of tHcy, UAE, and all other parameters were analyzed, and since the distribution was not normal, Spearman's rank coefficient was evaluated. The most important correlations are summarized in Table 2. There were positive correlations between fasting tHcy and UAE (r = .340, P = .05; Fig 1), post–methionine load tHcy and UAE (r = .502, P = .004), and fasting tHcy and post–methionine load tHcy (r = .429, P = .015) and a negative correlation between fasting tHcy and vitamin B_{12} (r = −.349, P = .04). There were also significant correlations between blood glucose and HbA_{1c} (r = .374, P = .03), vitamin B_{12} and folate (r = .499, P = .004), mean arterial pressure and folate (r = −.352, P = .04), and age and blood glucose (r = .495, P = .005). To improve normality, UAE and tHcy were natural logarithm (ln)–transformed and the presence of correlations was reassessed; ln tHcy appeared to be inversely correlated with ln folate (r = −.404, P = .01). By analyzing different parameters that could modify the UAE rate, a stepwise logistic regression was made between ln UAE and all other parameters. Only ln post–methionine load tHcy reached a level

Table 2. Table of Correlations (Spearman's rank coefficient)

Parameters	*P*	Correlation Coefficient (*r*)
Vitamin B_{12} *v* folate	.0047	.499
HbA_{1c} *v* Blood Glc	.03	.374
Fasting tHcy *v* Vitamin B_{12}	.04	−.348
Fasting tHcy *v* UAE	.05	.340
Postload tHcy *v* UAE	.004	.502
Ln tHcy *v* Ln folate	.01	−.404*

Abbreviations: Glc, glucose; ln, logarithm.
*Linear regression analysis.

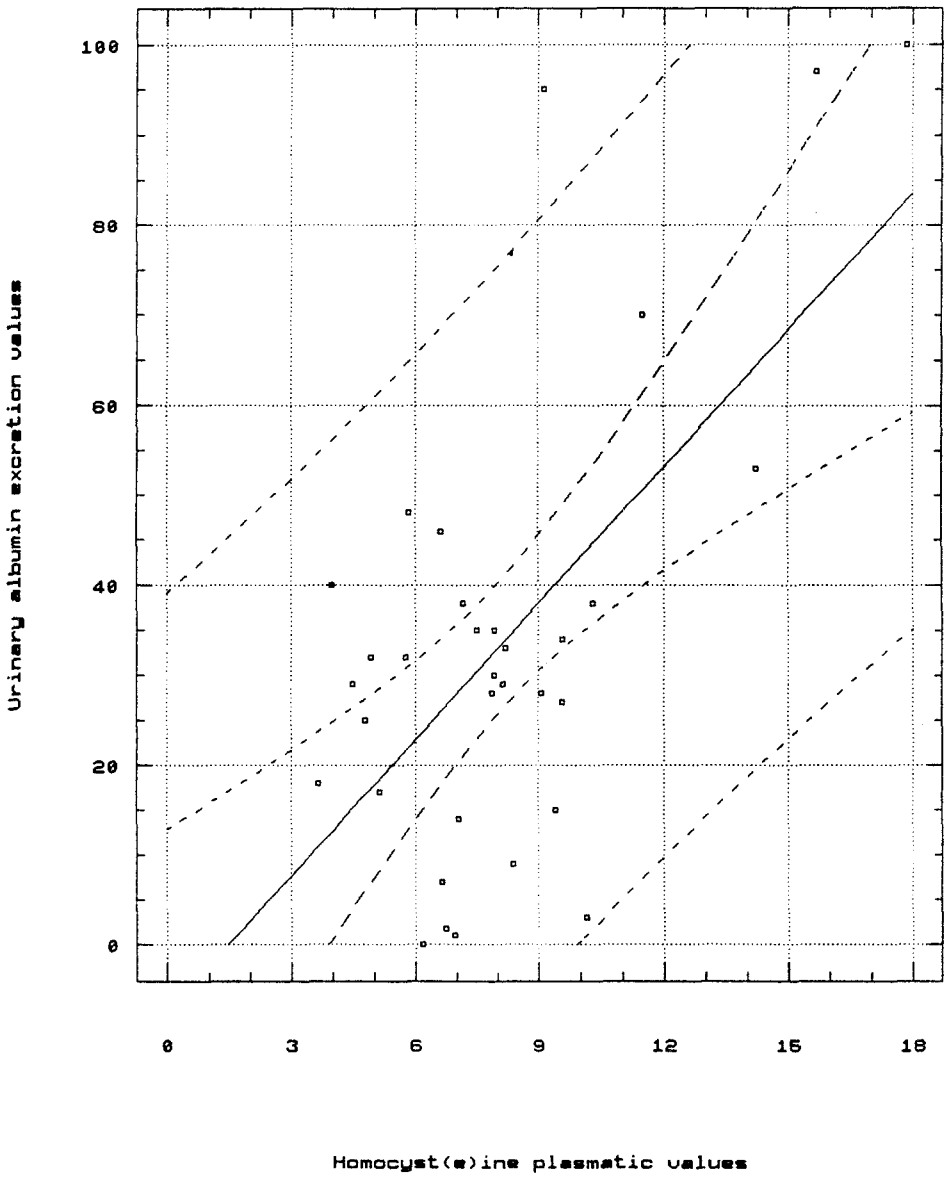

Fig 1. Scatterplots of fasting tHcy (μmol/L) and UAE (mg/24 h). Spearman's rank correlation coefficient = .340, $P = .05$.

of statistical significance (F = 6.06, $P = .01$), while all of the other parameters did not.

NIDDM patients were then divided into two groups depending on whether they were microalbuminurics, normoalbuminurics, or controls, and reanalyzed. Microalbuminurics showed higher fasting tHcy values than normoalbuminurics (9.05 ± 3.83 *v* 7.12 ± 1.95 μmol/L), albeit only approaching significance ($P = .08$; Fig 2). Besides, as regards the ln post–methionine load tHcy, there was a clearly significant difference between microalbuminurics and normoalbuminurics (controls, 3.13 ± 0.44; normoalbuminurics, 3.04 ± 0.35; microalbuminurics, 3.34 ± 0.37; F = 2.629, t = −2.36, $P = .02$).

Moreover, in microalbuminurics, a tHcy value higher than 11.4 μmol/L, considered a significant cutoff for an increased risk to develop arterial carotid stenosis,[18] was reached in four patients (23.25%), while no normoalbuminurics had plasma tHcy values above 11.4 μmol/L ($P = .05$). In microalbumin-

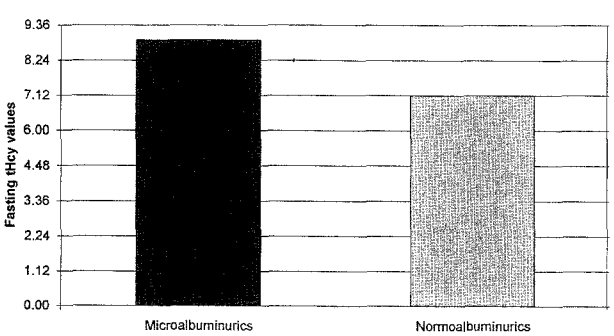

Fig 2. Fasting tHcy values in microalbuminurics (M) and normoalbuminurics (N) (M = 9.05 ± 3.83 μmol/L, N = 7.12 ± 1.95 μmol/L, $P = .08$). Kolmogorov-Smirnov 2-sample test for nonparametric data.

urics, the correlation between tHcy and UAE appeared stronger than in all of the other diabetic patients ($r = .655$, $P = .004$).

Taking into account the possible interference of nonnormal distribution, we analyzed whether patients with ln UAE values above 3.637, corresponding to the 75th percentile of ln UAE distribution, presented differences in ln tHcy and ln post–methionine load tHcy. Significant differences between the two groups of patients were observed both for ln tHcy (ln UAE $< 3.637 = 1.94 \pm 0.27$ *v* ln UAE $> 3.637 = 2.24 \pm 0.53$, $t = -2.08$, $P = .04$; Fig 3) and for ln post–methionine load tHcy (ln UAE $> 3.637 = 3.55 \pm 0.35$ *v* ln UAE $< 3.637 = 3.07 \pm 0.34$, $t = 3.282$, $P = .002$). The group with the highest UAE presented no particular differences in HbA_{1c} (UAE > 38 mg/24 h $= 7.36\% \pm 0.97\%$ *v* UAE < 38 mg/24 h $= 8.30\% \pm 2.08\%$, NS) or vitamin B_{12} and folate. Furthermore, patients with atherothrombotic complications had higher values for tHcy (9.61 ± 3.34 μmol/L) versus patients without complications (6.53 ± 2.09 μmol/L, KS = 0.506, $P = .003$; Fig 4) and did not present any other differences in the other parameters (Table 3).

DISCUSSION

The plasma tHcy concentration is regulated by several factors, both genetic and acquired.[1-4] The most important of these are the following: (1) genetic lower activity or clear deficiency of cystathionine β-synthetase (an enzyme involved in tHcy metabolism), which could lead to an increase of tHcy levels and to the appearance of homocystinuria; (2) genetic disorders of methylenetetrahydrofolate reductase, which could increase tHcy levels[1-4]; (3) senescence, which could lead to an increase in tHcy levels, perhaps as a result of an age-dependent reduction of cystathionine β-synthetase, although studies in rats demonstrate the opposite; (4) gender, since men have higher tHcy levels than women, probably due to greater muscle mass or different hormone patterns—in fact, levels in postmenopausal women approximate those in men; (5) deficiency of some nutritional factors (vitamin B_{12}, folate, and vitamin B_6), which appear to have a close negative correlation with tHcy levels; and (6) renal failure and serum creatinine levels, which are positively linked with tHcy. Elevated plasma tHcy concentrations in fasting conditions or post–methionine load conditions were correlated with several atherothrombotic and cardiovascular disorders: cerebrovascular disease, coronary artery disease, peripheral arterial occlusive disease, and acute myocardial infarction.[18-24] Previous studies have suggested that post–methionine load tHcy in the absence of fasting hyperhomocyst(e)inemia could account for over 40% of the total number of hyperhomocyst(e)inemic persons.[16] This was only partially confirmed in our NIDDM patients, as suggested by the presence of four NIDDM patients (12.1%) who presented with IMT and an increase of tHcy after the load test. If we take into account how methionine intake could be higher than the metabolic capacity, IMT could be considered a problem in diabetic

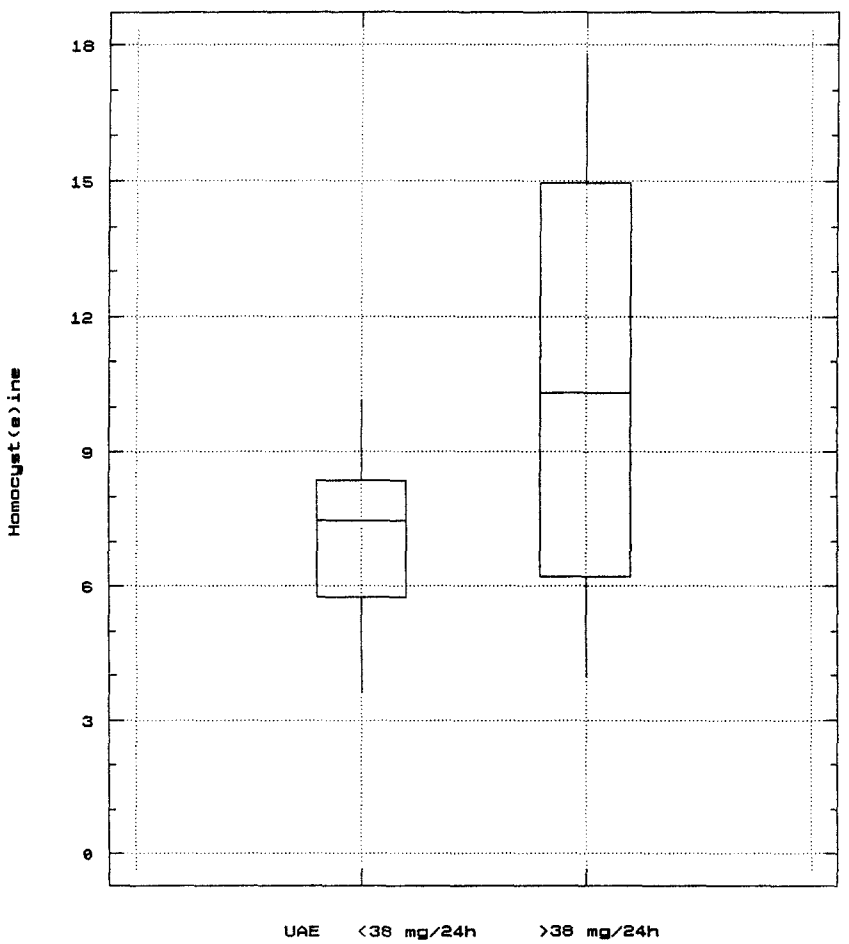

Fig 3. Multiple box plot of ln fasting tHcy obtained by dividing the patients into 2 groups with ln UAE higher or lower than 3.637 (75th percentile of ln UAE distribution; 1.94 ± 0.27 *v* 2.24 ± 0.53, $t = -2.08$, $P = .04$, Kolmogorov-Smirnov 2-sample test for nonparametric data).

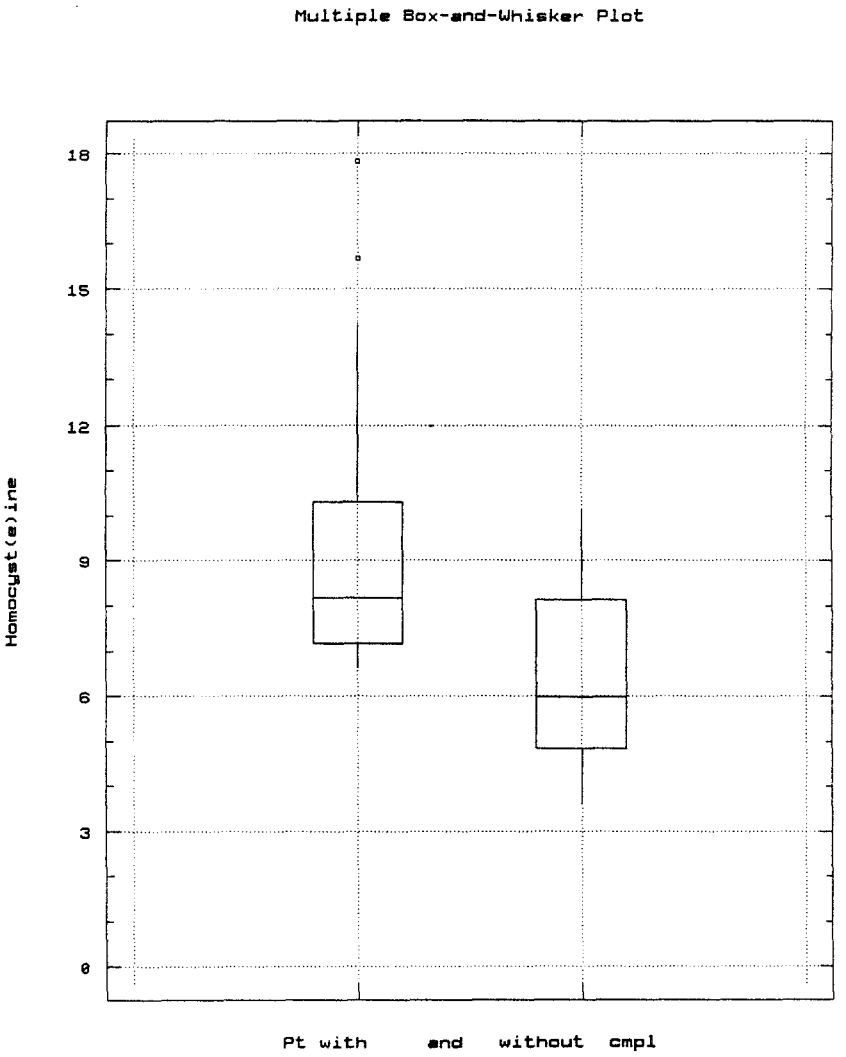

Fig 4. Multiple box plot of fasting tHcy values (μmol/L) obtained by dividing the patients into 2 groups with or without complications (9.61 ± 3.34 *v* 6.53 ± 2.09 mmol/L, KS = 0.506, *P* = .003; Kolmogorov-Smirnov 2-sample test for nonparametric data).

patients. Previous studies[25] have demonstrated that methionine-rich meals normally cause a slight increase in the plasma tHcy concentration. The American diet, particularly rich in eggs and packaged meals, may contain a methionine intake (up to 2 g/d) that is higher than the normal metabolic capacity (~1 g/d). This indicates a transient but constant increase in tHcy concentration.

Table 3. Characteristics of NIDDM Patients Divided According to the Presence of Complications (Kolmogorov-Smirnov two-sample test for nonparametric data)

Characteristic	With Complications	Without Complications	*P*
Fasting tHcy (μmol/L)	9.61 ± 3.34	6.53 ± 2.09	.003
Postload tHcy (μmol/L)	28.46 ± 14.09	24.45 ± 7.84	.95
UAE (mg/24 h)	41.2 ± 31.8	25.3 ± 14.1	.29
HbA_{1c} (%)	8.08 ± 1.91	8.00 ± 1.82	.26
Folate (ng/mL)	73 ± 36.9	76 ± 37.16	.59
Vitamin B_{12} (pg/mL)	579.5 ± 231.8	585.8 ± 186.0	.81
Creatinine (mg/dL)	0.92 ± 0.37	0.88 ± 0.17	.30
MAP (mm Hg)	99.3 ± 9.06	99.4 ± 9.78	.66
Glc (mg/dL)	171.7 ± 44.0	184.5 ± 48.7	.87
DD (yr)	8.17 ± 5.65	7.31 ± 5.16	.86
Age (yr)	62.4 ± 5.4	59.3 ± 6.1	.13

Abbreviations: MAP, mean arterial pressure; Glc, blood glucose; DD, duration of disease.

Previous studies have discussed the potential interaction between tHcy and glucose intolerance as a risk factor for atherosclerosis,[26-29] but none have been able to demonstrate a relationship between diabetes mellitus and hyperhomocyst(e)inemia, and this was confirmed in our study. Differences were not found between NIDDM patients and the control group for fasting or post–methionine load tHcy; this confirms the findings of previous studies.[26-29] Our NIDDM patients did not show particular signs of renal function impairment, ie, they were without increased serum creatinine levels at the time of study, so this was unlikely to be an element of confusion (Table 1).

NIDDM patients with complications showed an increase of fasting tHcy levels compared with NIDDM patients without complications (Fig 4). Moreover, no differences were observed for the post–methionine load tHcy, HbA_{1c}, UAE, folate, vitamin B_{12}, creatinine, total cholesterol, HDL cholesterol, triglycerides, smoking, and therapy (Table 3). All complications appeared to be endothelial disorders, so this could strengthen the hypothesis of a tHcy interference with the endothelial antiatherothrombotic capacity. tHcy could be an important risk factor worsening the

prognosis in NIDDM patients even if they are well treated and well compensated. In our study, tHcy is negatively correlated with vitamin B_{12} levels; moreover, ln tHcy is negatively correlated with ln folate (Table 2), so an adequate intake of both of these elements could be a first step to reduce hyperhomocyst(e)inemia.

An interesting finding of our study is the relationship between UAE and both fasting tHcy (Fig 1) and post–methionine load tHcy in NIDDM patients (Table 2). This correlation appeared to be stronger in the microalbuminurics among our diabetic patients. This means that microalbuminurics are the real target for a prevention policy in terms of hyperhomocyst(e)inemia. Microalbuminuric NIDDM patients had increased tHcy values (Fig 2) compared with normoalbuminurics, and they presented a significant difference in post–methionine load tHcy. If we considered the 75th percentile of UAE distribution, the difference between microalbuminurics and normoalbuminurics for fasting tHcy was strengthened (Fig 3). Two hypotheses can be made to explain the relationship between tHcy and UAE.

1. Increased tHcy as a Cause of Endothelial Dysfunction

The incipient renal dysfunction often seen in diabetic patients could lead to an increased UAE rate. The kidney is one of the principal sites of tHcy metabolism, and microalbuminuria as a marker of this renal damage might be expected to be accompanied by an increased tHcy concentration in fasting and postload conditions. In NIDDM patients, when renal function is only slightly affected (eg, with low values for microalbuminuria), the ability of the kidney to metabolize tHcy is partly maintained, but not under the additional stress of a methionine load. Besides, it must be remembered that in the course of life dietary methionine intake can be variable, so a partial increase of tHcy may be common. Moreover, increased UAE could reflect a systemic dysfunction of the vascular endothelium. Microalbuminuria is associated with the accumulation of extracellular matrix in glomeruli and large vessel walls[30] and with the proliferation of mesangial and myomedal cells.[31] It has been suggested that microalbuminuria could be a marker for vascular damage and is thus an early finding in atherosclerosis.[32] Microalbuminuria is a prognostic indicator not only of proteinuria and diabetic nephropathy[33] but also of early mortality, principally due to cardiovascular diseases,[34-35] although the real cause of this association is not clear. The increase of tHcy associated with the renal incipient dysfunction could explain the link between microalbuminuria and cardiovascular events.

2. Increased tHcy as a Consequence of Endothelial Dysfunction

NIDDM patients presented a decreased synthesis of endothelial NO and an increased inactivation of NO.[36] NO could be reduced by oxygen-derived free radicals or advanced glycosylation end products. Previous studies have shown that in vitro thiols (such as tHcy) react in the presence of NO to form *S*-nitrosothiols, compounds that also possess vasodilatory properties.[12] This *S*-nitrosothiol does not support H_2O_2 formation in the same manner as tHcy; in this way, the normal endothelium modulates the potential adverse effects of tHcy via the intervention of NO.[12] Microalbuminurics could therefore be considered as a subgroup of NIDDM patients with advanced endothelial dysfunction, in which NO no longer performs its normal role in scavenging tHcy.

It has to be verified whether microalbuminuria can be considered simply as a marker of endothelial dysfunction in all hyperhomocyst(e)inemic patients, or whether it might contribute to worsening the deleterious effects of tHcy on the cardiovascular system.

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