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Plasma homocysteine is related to albumin excretion rate in patients with diabetes mellitus: a new link between diabetic nephropathy and cardiovascular disease?

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Summary The high risk of cardiovascular disease in patients with diabetes mellitus, particularly in those with nephropathy, is not completely explained by classical risk factors. A high plasma homocysteine concentration is an independent risk factor for cardiovascular disease but information on its association with diabetes is limited. Fasting homocysteine concentrations were measured in the plasma of 165 diabetic patients (75 with insulin-dependent [IDDM]; 90 with non-insulin-dependent diabetes [NIDDM]) and 56 non-diabetic control subjects. Other measurements included the prevalence of diabetic complications, glycaemic control, lipid and lipoprotein levels, vitamin status and renal function tests. Patients with NIDDM had higher homocysteine levels than control subjects, whereas IDDM patients did not (9.2 ± 4.5 vs 7.7 ± 2 μmol/l, $p < 0.01$; and 7.0 ± 3 vs 7.4 ± 2 μmol/l, NS). Univariate correlations and multiple regression analysis showed albumin excretion rate to be the parameter with the strongest independent association with homocysteine. Patients with both types of diabetes and nephropathy had higher plasma homocysteine concentrations than those without nephropathy. Increases of homocysteine in plasma were related to increases in the severity of the nephropathy. Fasting hyperhomocysteinaemia was considered as the mean of the plasma homocysteine for all control subjects (7.5 ± 2.1 μmol/l) + 2 SD (cut-off =11.7 μmol/l). Nephropathy was present in 80 % of diabetic patients with fasting hyperhomocysteinaemia. In conclusion, increases in fasting homocysteine in diabetic patients are associated with increased albumin excretion rate, especially in those with NIDDM, thus providing a potential new link between microalbuminuria, diabetic nephropathy and cardiovascular disease. [Diabetologia (1998) 41: 684–693]

Keywords Homocysteine, hyperhomocysteinaemia, diabetes mellitus, diabetic nephropathy, microalbuminuria, cardiovascular diseases.

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Abbreviations: AER, Urinary albumin excretion rate; BMI, body mass index; CVD, cardiovascular diseases; FPG, fasting plasma glucose; Hcy, homocysteine; HDLc; high density lipoprotein cholesterol; IDDM, insulin-dependent diabetes mellitus; LDLc, low density lipoprotein cholesterol; Lp(a), lipoprotein (a); NIDDM, non-insulin-dependent diabetes mellitus; PLP, pyridoxal 5′-phosphate; UPC, urinary protein concentration; VLDLc, very low density lipoprotein cholesterol.

Insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) increase the risk of cardiovascular disease (CVD) [1–3]. Nephropathy substantially increases this risk both in diabetic and non-diabetic patients [4–8]. The increased susceptibility of diabetic patients with nephropathy to atherosclerosis can be explained, in part, by risk factors such as hypertension, hyperlipidaemia and haemorrheological changes [8–13]. Hence, the identification and treatment of new risk factors is important to reduce the high rate of cardiovascular events in patients with diabetes, especially in those with nephropathy [14].

Hyperhomocysteinaemia is increasingly recognized as a risk factor for vascular disease affecting heart, brain and extremities. A considerable amount of genetic, biochemical, pathophysiological, clinical and epidemiological data suggest a causal role for hyperhomocysteinaemia in the development of atherosclerosis and thrombosis. Patients with severe inherited forms of hyperhomocysteinaemia are at a very high risk of CVD [15, 16]. Moderate hyperhomocysteinaemia is frequent in patients with CVD [17]. Several, though not all, prospective analyses, and one meta-analysis which included most clinical and epidemiological studies performed to date, support a causal relationship between moderate hyperhomocysteinaemia and CVD [18–20].

Hyperhomocysteinaemia is very frequent in renal failure which suggests that the kidney's function is crucial to homocysteine (Hcy) catabolism [21] and this has been supported recently by direct, experimental evidence [22].

Detecting hyperhomocysteinaemia in patients at high risk of CVD is of importance because safe and effective treatment is currently available. Administration of folate, vitamin B12, vitamin B6 and betaine are very effective in reducing Hcy concentrations in plasma [23, 24] although the efficacy in terms of reducing cardiovascular events has been demonstrated, to-date, only in patients with inherited forms of severe hyperhomocysteinaemia [15, 17, 24].

Despite the current interest, there is limited, and sometimes inconsistent, information regarding the prevalence of hyperhomocysteinaemia in IDDM and NIDDM. Further, its aetiology and its relationship with CVD and nephropathy is equivocal [25–28]. For clarification of these aspects, we determined the concentration of Hcy in the plasma of a group of IDDM and NIDDM patients and control subjects and assessed whether hyperhomocysteinaemia was related to nephropathy and to CVD.

Subjects, materials and methods

Patients and control subjects. A total of 165 diabetic patients (75 with IDDM and 90 with NIDDM) were recruited consecutively from among those attending the Diabetes Clinic of the *Hospital de la Santa Creu i Sant Pau* (Barcelona). Non-diabetic control subjects ($n = 56$) were recruited from among the clinical and laboratory staff and their families and were selected to match for age and gender distribution of the diabetic group as a whole. None of them had a history of CVD. Of the 56 control subjects, 28 were similar with respect to the characteristics of the IDDM group and 28 to the NIDDM group. Diabetes was diagnosed and classified according to the National Diabetes Data Group [29]. In diabetic patients, retinopathy, nephropathy and macroangiopathy were diagnosed and classified as follows. Retinopathy was diagnosed following a detailed ophthalmological examination by a specialist or when the patient had a documented history of the disease (retinal photocoagulation or vitrectomy, abnormal retinal fluorescein angiography). Nephropathy was recorded and classified as "incipient", "overt" or "renal failure" following at least two biochemical measurements in 24 h urine specimens (in the absence of urinary infection and haematuria) and in serum. Incipient nephropathy (microalbuminuria) was defined as persistent urinary albumin excretion rate (AER) in the range of 20–200 μg/min, while overt nephropathy was diagnosed when AER was over 200 μg/min or an abnormal urinary protein concentration (UPC) (proteinuria > 300 mg/day) was recorded. Renal failure was diagnosed when the serum concentration of creatinine was over 120 μmol/l. Since urine analysis had been performed as a part of a routine work-up that did not include determination of urinary creatinine concentration or freezing of the urine samples, creatinine clearances, when needed, were calculated with the Cockroft-Gault formula [30]. The patient was considered to be hypertensive when blood pressure was 140/90 mm Hg or more or antihypertensive treatment was prescribed. The following were considered proof of macroangiopathy: 1) coronary heart disease, defined by a history of angina with a positive exercise test or an abnormal coronary angiogram, or by a history of myocardial infarction (confirmed by biochemical and electrocardiographical criteria); 2) ischaemic stroke, confirmed by cerebral computerized axial tomography or nuclear magnetic resonance imaging or by a similarly documented history of the disease; 3) peripheral vascular disease, established by a history of intermittent claudication with abnormal Doppler pressure or a history of reconstructive vascular surgery or amputation. Four IDDM patients had a history of CVD, of whom three had coronary heart disease and two peripheral vascular disease. Thirty NIDDM patients had a history of CVD and, of them, there were 17 cases of coronary artery disease, 15 of peripheral vascular disease and 4 of ischaemic stroke. None of the patients studied had had a cardiovascular event for at least 3 months prior to the inclusion into the study. As part of the clinical work-up, the patients and the control subjects were fully informed of the purpose and protocol and the study was approved by the ethics committee of the hospital.

Laboratory analyses. Venous blood was obtained between 07.00 and 10.00 hours after an overnight fast. Measures to prevent the flow of Hcy from erythrocytes to plasma were strictly implemented [31]. Plasma and serum aliquots were quickly separated and frozen at –80 °C for batched analysis. Plasma Hcy concentration was determined by high-performance liquid chromatography (HPLC) (Millipore; Waters Chromatographic Division, Milfore, Mass., USA) and fluorescence detection (Kontron Instruments, Milan, Italy) and included all molecular forms that can be reduced to free Hcy [32]. Intra- and inter-assay coefficients of variation of the Hcy determination were 4.99 % and 7.28 %, respectively [32]. Serum vitamin B12, serum folate and erythrocyte folate concentrations were determined using commercial kits (ACS Ciba-Corning, catalogue # LKF01 and Immulite, DPC, catalogue # 672211, respectively) using automated chemiluminescent immunoassays adapted to the ACS:180 (Ciba-Corning Diagnostics Corp, Medfield, Mass., USA) and DPC Immulite (Diagnostic Products Corp, Los Angeles, Calif., USA). Plasma pyridoxal 5′-phosphate (PLP, active vitamin B6) concentrations were measured using a commercially available radioenzymatic assay (Bühlmann Laboratories AG, Allschwil, Switzerland). Glycated haemoglobin (HbA_{1c}) was measured by HPLC (Hi-Auto A1 c, Dic-Kyoto, Japan; reference range in our laboratory is 3.7–5.5 %) [33]. Serum creatinine, cholesterol, triglycerides and uric acid, as well as other basic biochemical blood tests, were measured by standard chemical and enzymatic commercial methods in a Hitachi 727 autoanalyzer (Boehringer Mann-

Table 1. Clinical characteristics in patients with diabetes mellitus and control individuals

	IDDM controls $n = 28$	IDDM patients $n = 75$	NIDDM controls $n = 28$	NIDDM patients $n = 90$
Age (years)	38 ± 4	33 ± 12	59.3 ± 12	60 ± 11
Males/females	16/12	44/31	15/13	51/39
Body mass index (kg/m^2)	25.6 ± 4	23.5 ± 3	26.7 ± 4	27.1 ± 4
Diabetes duration (years)	–	12.1 ± 11	–	10.5 ± 12
Hypertension (%)	0	6.7	14.3	26.7
Retinopathy (%)	ND	37.3	ND	47.8
Nephropathy (%)	ND	18.7	ND	44.4
Macroangiopathy (%)	ND	5.3	ND	33.3

Results are expressed as mean ± SD. There are no significant differences ($p < 0.05$) between the diabetic groups and their specific control groups

heim, Mannheim, Germany) [33]. The serum concentration of HDL cholesterol (HDLc) was measured after precipitation of the apoprotein B containing lipoproteins with phosphotungstic acid-$MgCl_2$ [34]. LDL cholesterol (LDLc) was calculated using the Friedewald formula [34]. When the triglyceride level was over 3.5 mmol/l, a combined ultracentrifugation-precipitation method was used following recommendations of the Lipid Research Clinics Laboratory [34]. Lipoprotein(a) (Lp(a)) concentrations in plasma were measured using a commercial enzyme-linked immunoabsorbent assay (Organon Teknica N. V., Turnhout, Belgium). UPC was determined using the Coomassie Brilliant Blue dye procedure adapted to an autoanalyser as has been described [35]. AER was determined by nephelometry using a commercial kit containing specific antibody (Behringwerke AG, Marburg, Germany).

Statistical analyses. Analyses were performed with the SPSS PC (+) statistical package ("SPSS Inc., Chicago, IL, USA"). All tests used were two-tailed and p less than 0.05 was considered as significant. The normality of distribution of any variable was assesed using the Kolmogorov-Smirnov test and where skewed (Hcy, Lp(a), triglycerides, VLDL cholesterol and vitamin B12) were logarithmically transformed to reduce kurtosis. Comparisons between groups were performed using the chi-square and the unpaired t-test, using log transformed means when the data were skewed. Pearson's correlation coefficient was used to assess the relationship between the log transformed Hcy of the following variables: age, body mass index (BMI), creatinine, AER, UPC, triglycerides, VLDL cholesterol (VLDLc), HDLc, LDLc, total cholesterol, Lp(a), HbA_{1c}, fructosamine, glucose, uric acid, serum folate and vitamin B12 and PLP. Also, all variables were included in a multiple regression stepwise analysis using the log transformed Hcy as the dependent variable. Hcy, triglycerides, VLDLc, Lp(a) and vitamin B12 were log transformed before inclusion in this analysis. The change in R^2 (explained variance) was also calculated.

Results

Table 1 shows some of the clinical characteristics of the individuals studied. There were no differences between the diabetic groups and their control subjects with respect to the distributions of age, gender and BMI. The prevalence of retinopathy was 1.3-fold more frequent in NIDDM patients than in IDDM whereas the prevalence of nephropathy and macroangiopathy was 2.4-fold and 6.3-fold more frequent in NIDDM than in IDDM patients. Based on a previous analysis of blood and urine performed in all diabetic patients it was concluded that 14 IDDM patients and 40 NIDDM patients had nephropathy (Table 1).

Table 2 summarizes the results of Hcy as well as the vitamin, lipid and lipoproteins, renal function and glycaemic control parameters measured in the patients with diabetes and in the control groups. Patients with NIDDM had higher Hcy than their controls, whereas IDDM patients did not (9.2 ± 4.5 vs 7.7 ± 2 μmol/l, $p < 0.01$, and 7 ± 3 vs 7.4 ± 2 μmol/l, NS). Using the mean + 2 SD of the concentration of Hcy in plasma of the control group as a whole (7.5 ± 2.1), values of 11.7 μmol/l or higher were considered to constitute fasting hyperhomocysteinaemia. Using this statistical criterion, the incidence of fasting hyperhomocysteinaemia was 3.6 % in IDDM control subjects and 5.3 % in IDDM patients whereas it was 7.1 % in NIDDM control subjects and 17.8 % in NIDDM patients. Erythrocyte folate concentrations tended to be lower in patients than in control subjects. Serum folate and vitamin B12 tended to be higher in the diabetic groups than in control subjects. There were no differences between the PLP concentrations of the two diabetic groups and their specific control counterparts. The proteinuria of NIDDM patients was greater than that of patients with IDDM. UPC data from 11 patients (2 with IDDM and 9 with NIDDM) are not included in Table 2 because AER had not been measured but, if included, the difference in UPC between NIDDM and IDDM patients would have been increased (mean ± SD 410 ± 1111 mg/day, median 115, range 7.3–8700 in NIDDM vs 159 ± 172 mg/day, median 107, range 50–1119 in IDDM). Lastly, NIDDM and IDDM patients had significant increases of fasting plasma glucose with respect to their specific controls.

Univariate correlations and multiple regression analyses were performed to establish the principal

Table 2. Measured biochemical parameters in patients with diabetes and in control subjects

	IDDM controls $n = 28$	IDDM patients $n = 75$	NIDDM controls $n = 28$	NIDDM patients $n = 90$
Homocysteine (μmol/l)	7.4 ± 2	7.0 ± 3	7.7 ± 2	9.2 ± 4[a]
Erythrocyte folate (nmol/l)	604 ± 266	588 ± 170	736 ± 225	673 ± 290[a]
Serum folate (nmol/l)	15 ± 8	19 ± 8[a]	19 ± 7	24 ± 12[a]
PLP (nmol/l)	30 ± 8	31 ± 11	32 ± 10	29 ± 8
Vitamin B12 (pmol/l)	395 ± 118	550 ± 218[a]	466 ± 261	571 ± 290
Cholesterol (mmol/l)	5.2 ± 1	4.8 ± 1	5.6 ± 1	5.4 ± 1
Triglycerides (mmol/l)	1.3 ± 0.5	0.9 ± 0.6	1.0 ± 0.5	1.7 ± 1.3[a]
VLDL-c (mmol/l)	0.3 ± 0.5	0.5 ± 0.3	0.4 ± 0.5	0.7 ± 0.3[a]
LDL-c (mmol/l)	3.5 ± 1	2.8 ± 0.9[a]	3.7 ± 0.9	3.4 ± 1.0
HDL-c (mmol/l)	1.4 ± 0.5	1.4 ± 0.4	1.5 ± 0.5	1.2 ± 0.4
Lipoprotein(a) (mg/dl)	21 ± 35	22 ± 30	35 ± 43	27 ± 33
Uric acid (μmol/l)	294 ± 83	224 ± 74[a]	298 ± 71	301 ± 107
Creatinine (μmol/l)	88 ± 14	87 ± 14	91 ± 15	94 ± 37
UPC (mg/day)	ND	161 ± 173	ND	229 ± 442
AER (μg/min)	ND	50 ± 144	ND	40 ± 95
FPG (mmol/l)	4.9 ± 0.7	9.4 ± 4.9[a]	4.9 ± 0.7	9.7 ± 4.2[a]
HbA_{1c} (%)	ND	7.8 ± 2	ND	8.2 ± 2

ND, Not determined. Reference values: erythrocyte folate (212–1453), serum folate (6–39), PLP (20–160), vitamin B12 (150–1200), creatinine (< 114), UPC (< 300), AER (< 20), HbA_{1c} (< 5.5 %), FPG (4.1–6.4) Values are expressed as mean ± SD. [a] $p < 0.05$ compared to control subjects

Table 3. Determinants of Hcy in patients with diabetes mellitus: univariate correlations and multiple regression analysis

	Diabetic patients		IDDM		NIDDM	
Univariate correlations						
	r	*p*	*r*	*p*	*r*	*p*
Age	0.39	< 0.001	0.34	< 0.05	0.18	< 0.05
Body mass index	0.21	< 0.01				
Creatinine	0.40	< 0.001	0.35	< 0.01	0.42	< 0.001
UPC	0.36	< 0.001	0.27	< 0.05	0.37	< 0.001
AER	0.44	< 0.001	0.38	< 0.01	0.47	< 0.001
Triglycerides	0.30	< 0.001			0.22	< 0.05
VLDL-c	0.30	< 0.001			0.26	< 0.05
HDL-c	– 0.14	< 0.05				
Lp(a)	0.17	< 0.05			0.26	< 0.05
FPG					– 0.20	< 0.05
Uric acid	0.38	< 0.001			0.37	< 0.01
Multiple regression analysis						
	β[a]	*p*	β[a]	*p*	β[a]	*p*
AER	0.46	< 0.001	0.43	< 0.01	0.66	< 0.001
Age	0.37	< 0.001			0.54	< 0.001
Creatinine	0.24	< 0.05				

[a] β or slope given as a change in log transformed plasma Hcy expressed as μmol/l per change of unit of the independent variable

determinants of the concentrations of Hcy in plasma in the groups of patients with diabetes (Table 3). No significant correlation was found between Hcy and any of the vitamin parameters studied. All variables (independent of whether they were or were not significantly associated with Hcy levels in the univariate statistical analysis) were included in the multiple regression analysis which was performed using the stepwise method. Only AER ($R^2 = 0.19$, $p < 0.01$) in IDDM patients and AER and age ($R^2 = 0.62$, $p < 0.00001$) in NIDDM patients were independently related with plasma Hcy (Table 3). The same analysis was performed in the control groups. The parameters that reached significant levels of univariate correlation with plasma Hcy in the NIDDM control group were creatinine ($r = 0.44$) and vitamin B12 ($r = –0.39$) whereas those for the IDDM control group were age ($r = 0.92$), total cholesterol ($r = 0.70$), LDLc ($r = 0.47$) and creatinine ($r = 0.32$). The multiple regression analysis showed that only

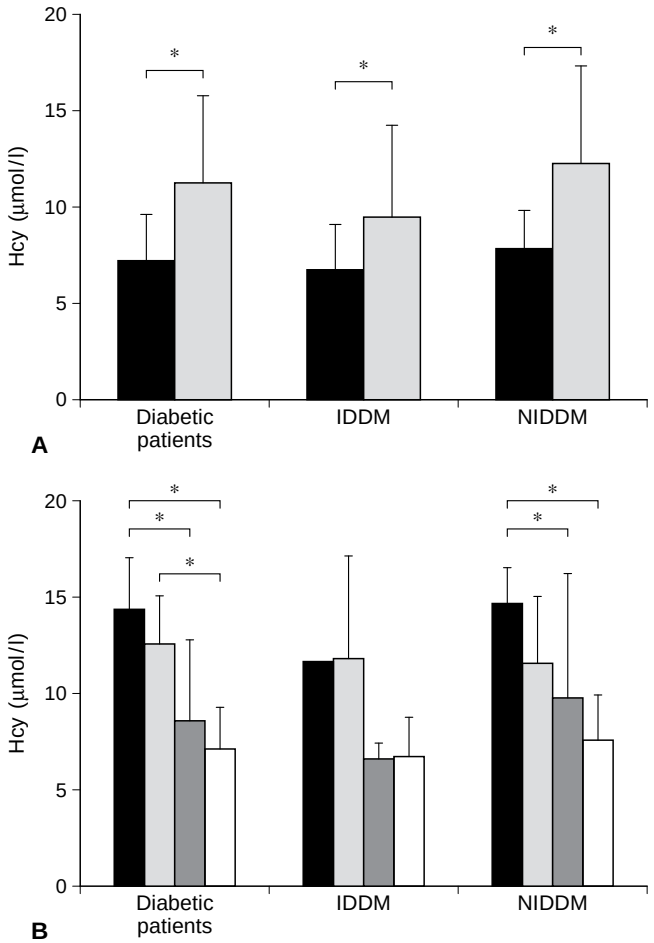

Fig. 1. A Hcy concentrations in diabetic patients without ■ and with □ nephropathy *$p < 0.01$. **B** Hcy concentrations in diabetic patients with different degrees of diabetic nephropathy. Group 1: Creatinine > 120 μmol/l (renal failure) ■; Group 2: UPC > 300 mg/day or AER > 200 μg/min (overt nephropathy) but creatinine < 120 μmol/l □; Group 3: AER 20–200 μg/min (incipient nephropathy) with creatinine < 120 μmol/l ▩; Group 4: AER < 20 μg/min and creatinine < 120 μmol/l (no nephropathy) □. In the whole diabetic group, $n = 9$ in Group 1, $n = 12$ in Group 2, $n = 21$ in Group 3, and $n = 88$ in Group 4. In IDDM patients, $n = 1$ in Group 1, $n = 2$ in Group 2, $n = 8$ in Group 3 and $n = 47$ in Group 4. In NIDDM patients, $n = 8$ in Group 1, $n = 10$ in Group 2, $n = 13$ in Group 3 and $n = 41$ in Group 4. *$p < 0.05$ between groups 1 and 4, 1 and 3, 2 and 4. $p < 0.05$ between groups 1 and 4, 1 and 3. UPC: urinary protein concentration. AER: albumin excretion rate

creatinine ($\beta = 0.44$) was independently associated with plasma Hcy in NIDDM control subjects whereas LDLc ($\beta = 0.71$) and creatinine ($\beta = 0.68$) were independently associated with plasma Hcy in the IDDM control group.

It is noteworthy that Figure 1 includes only those patients for whom AER, UPC and serum creatinine were measured simultaneously in what was, at least, the second biochemical evaluation of their renal function confirming the existence and classification of the nephropathy and, because of this data on fewer patients than shown in Table 1 are given. IDDM and NIDDM patients with nephropathy presented higher Hcy concentrations than those without nephropathy (Fig. 1A). Diabetic patients with and without nephropathy did not present any differences with respect to the vitamin variables measured (data not shown). The patients were then classified according to the existence and degree of nephropathy, but including the criterion of serum creatinine less than 120 μmol/l in the patients with incipient nephropathy and overt nephropathy to exclude the effect of renal failure on Hcy levels. Groups generated were: group 1, renal failure; group 2, incipient nephropathy with creatinine < 120 μmol/l; group 3, overt nephropathy with creatinine less than 120 μmol/l; group 4, no nephropathy. In the diabetic group as a whole and in NIDDM patients there was a significant increase in Hcy in relation to the presence and severity of the nephropathy. A similar trend, albeit non-significant, was found in the IDDM patients (Fig. 1B). The IDDM patients without nephropathy, incipient nephropathy, overt nephropathy and renal failure included in Figure 1 did not differ significantly with respect to the Cockroft-Gault derived creatinine clearances (94 ± 20 ml/min, 86 ± 3, 86 ± 49 and 63 [$n = 1$], respectively). However, the low number of patients in some of the subgroups limit the interpretation of these results. The NIDDM patients without nephropathy and NIDDM patients with renal failure included in Figure 1 had statistically different Cockroft-Gault derived creatinine clearances (77 ± 22 ml/min vs 43 ± 14, $p < 0.001$). However, these differences were not statistically different when NIDDM patients without nephropathy, NIDDM patients with incipient nephropathy and those with overt nephropathy (all included in Figure 1) were compared (77 ± 22 ml/min, 76 ± 26 and 69 ± 24, respectively).

IDDM and NIDDM patients with hyperhomocysteinaemia presented, in general, with higher values of creatinine, UPC, AER and Cockroft-Gault derived creatinine clearances than normohomocysteinaemic patients (Table 4). The only exception was the creatinine and the Cockroft-Gault derived creatinine clearance of IDDM patients, where the tendency of hyperhomocysteinaemic patients was towards higher values than those with normohomocysteinaemia; however, this difference did not reach statistical significance. The same analysis did not show any differences with respect to folates, vitamin B12 and PLP variables measured. The mean concentrations of serum vitamin B12 and folate were 795 pmol/l and 25 nmol/l in the patients with NIDDM and normohomocysteinaemia, whereas the corresponding values were 538 pmol/l and 23 nmol/l in patients with NIDDM and hyperhomocysteinaemia. The serum concentrations of vitamin B12 and folate in patients with IDDM and normohomocysteinaemia were 529 pmol/l and 20 nmol/l whereas the

Table 4. Renal function in diabetic patients classified as having normohomocysteinaemia (Hcy < 11.7 μmol/l) or hyperhomocysteinaemia (> 11.7 μmol/l)

Hcy (μmol/l)	Diabetic patients		IDDM		NIDDM	
	< 11.7	> 11.7	< 11.7	> 11.7	< 11.7	> 11.7
Creatinine (μmol/l)	85 ± 13 (84, 61–121)	130 ± 67[a] (113, 82–357)	86 ± 14 (85, 61–121)	100 ± 13 (102, 86–112)	85 ± 12 (84, 64–125)	137 ± 72[a] (119, 82–357)
UPC (mg/24 h)	174 ± 303 (108, 7–2990)	1127 ± 2101[a] (374, 40–8700)	130 ± 74 (108, 50–1119)	467 ± 471[a] (300, 102–999)	220 ± 424 (109, 7–2990)	1269 ± 2298[b] (448, 40–8700)
AER (μg/min)	21 ± 33 (11, 2–9)	229 ± 327[a] (55, 6–8982)	19 ± 24 (11, 6–772)	287 ± 421[a] (83, 6–772)	24 ± 40 (11, 2–211)	212 ± 319[a] (50, 7–892)
Creatinine clearance (ml/min)	86 ± 27 (83, 36–149)	56 ± 22[a] (50, 29–112)	93 ± 22 (88, 36–149)	66 ± 16 (78, 48–76)	78 ± 22 (79, 40–138)	55 ± 23[a] (48, 29–112)

Creatinine clearance: Cockroft-Gault derived creatinine clearances. Results are expressed as mean ± SD. Data between parentheses correspond to median and range. [a] $p < 0.001$, [b] $p < 0.01$ compared to patients with Hcy < 11.7 μmol/l

corresponding values were 642 pmol/l and 12 nmol/l in patients with IDDM and hyperhomocysteinaemia.

Hypertension could influence nephropathy and/or hyperhomocysteinaemia [36]. Of the total number of patients, 5 IDDM patients and 24 NIDDM patients were hypertensive. Of these, 27 were under antihypertensive therapy (20 were treated with angiotensin-converting enzyme inhibitors, 9 with calcium antagonists and 2 with diuretics) and 2 were controlled by diet. All IDDM patients with hypertension had nephropathy. NIDDM patients with hypertension ($n = 24$) had higher Hcy than NIDDM patients without hypertension ($n = 66$) (10.5 ± 5.6 μmol/l vs 6.9 ± 4.2, $p < 0.05$), both groups being similar with respect to age (61 ± 9 vs 60 ± 12, NS). NIDDM patients with hypertension had, in general, more severe biochemical signs of nephropathy than NIDDM patients without hypertension (AER 79 ± 184 μg/min vs 24 ± 89, NS; UPC 879 ± 1880 mg/day vs 152 ± 209, $p < 0.05$; serum creatinine 110 ± 61 μmol/l vs 74 ± 36, $p < 0.05$), but Cockroft-Gault derived creatinine clearances were not statistically different (75 ± 24 ml/min vs 75 ± 23, NS). Of the patients included in Figure 1, those with NIDDM, nephropathy and hypertension ($n = 15$) showed a tendency to have higher Hcy than NIDDM patients with nephropathy without hypertension ($n = 16$) (11.9 ± 6 μmol/l vs 6.6 ± 6.5, NS). On the other hand, patients with NIDDM without nephropathy but with hypertension ($n = 10$) had Hcy levels not statistically different from those NIDDM patients without nephropathy and without hypertension ($n = 31$) (8.4 ± 4 μmol/l vs 6.9 ± 3.4, NS).

Only 4 patients (1 IDDM and 3 NIDDM) of a total of 20 with hyperhomocysteinaemia (4 IDDM and 16 NIDDM) did not have nephropathy. However, only 3 of 11 IDDM patients with nephropathy and 13 of 31 NIDDM patients with nephropathy (Fig. 1) had hyperhomocysteinaemia. A comparison of diabetic patients with nephropathy who had hyperhomocysteinaemia with those who had not indicated that the former were older and had a more advanced nephropathy as judged by the severity of the renal function alterations (Table 5). The distribution of cases of hypertension was similar in the groups of patients with nephropathy and with or without hyperhomocysteinaemia.

Table 5. Differences observed between diabetic patients with nephropathy and hyperhomocysteinaemia (Hcy > 11.7 μmol/l) and those with nephropathy and normohomocysteinaemia (Hcy < 11.7 μmol/l)

	Nephropathy and Hcy > 11.7 μmol/l $n = 16$	Nephropathy and Hcy < 11.7 μmol/l $n = 26$	p value
Age (years)	60.4 ± 11	50.6 ± 15	< 0.05
AER (μg/min)	326 ± 355	80 ± 55	< 0.05
UPC (mg/day)	1452 ± 2326	492 ± 670	< 0.05
Creatinine (μmol/l)	141 ± 73	90 ± 13	< 0.05
Creatinine clearance (ml/min)	54 ± 24	88 ± 27	< 0.05

Creatinine clearance: Cockroft-Gault derived creatinine clearances. Results are expressed as mean ± SD

The prevalence of macroangiopathy was 25 % in IDDM patients with hyperhomocysteinaemia. In contrast, only 4.2 % of IDDM patients with Hcy less than 11.7 μmol/l had macroangiopathy ($p < 0.001$). IDDM patients with macroangiopathy ($n = 4$) had Hcy levels of 11.4 ± 4.4 μmol/l, while Hcy in IDDM patients without macroangiopathy ($n = 71$) was 6.8 ± 2.9 μmol/l ($p < 0.05$). There was no significant difference between Hcy values in NIDDM patients with macroangiopathy (9.9 ± 3.8 μmol/l, $n = 30$) and those without macroangiopathy (8.7 ± 4.7 μmol/l, $n = 60$).

IDDM and NIDDM patients with retinopathy had Hcy levels of 8.0 ± 3.6 μmol/l and 9.6 ± 4.0 μmol/l, respectively. However, when patients with nephropathy and macroangiopathy were excluded from the statistical analysis, patients with diabetes and retinopathy had Hcy concentrations of 6.5 ± 1.8 μmol/l.

Discussion

Fasting concentrations of plasma Hcy were higher in the NIDDM patients than in their control subjects (Table 2). Using the mean + 2 SD of the plasma concentrations of the total control group as a cut-off point, 5.3% and 17.8% of IDDM and NIDDM patients, respectively, were diagnosed as having hyperhomocysteinaemia while, by this statistical definition, 5.4% of the all control subjects presented Hcy values over 11.7 μmol/l. The use of two different cut-off points, calculated from each of the control groups, did not change the percentages of individuals with hyperhomocysteinaemia.

Hcy concentrations in populations have been demonstrated to be influenced by the concentrations of folate, PLP and vitamin B12, age and gender [15–17, 23, 37, 38] while, in other studies, Hcy concentrations in the population have correlated positively with blood pressure, creatinine, uric acid, VLDLc and/or triglyceride [39, 40]. The kidney plays a major role in the metabolism of Hcy [22] and, as such, would explain not only why renal failure is an important cause of hyperhomocysteinaemia but also why creatinine is one of the biochemical parameters that correlates best with Hcy levels [21, 39]. However, in this study and in others [41], the association of serum creatinine and Hcy was also found in control subjects with normal renal function. This is probably at least partly due to the requirement, in the synthesis of the precursor of creatinine (creatine), of the donation of methyl groups formed in the transformation of methionine to Hcy [41].

To explore the probable cause(s) of fasting hyperhomocysteinaemia in our diabetic patients, we determined the vitamins that act as cofactors of key enzymes of Hcy metabolism. No significant correlations were found between any of these vitamins and Hcy concentrations and there were no differences between vitamin levels of diabetic patients (IDDM, NIDDM or both subgroups combined) with and without associated hyperhomocysteinaemia. Hence, variations in folate, vitamin B12 or PLP concentrations are not pertinent to the hyperhomocysteinaemia observed in the group of diabetic patients studied. This lack of correlation contrasts with other studies in which the levels of serum folate and vitamin B12 show a strong, non-linear inverse correlation with the concentration of plasma Hcy. This discrepancy could be explained in serveral ways. Most of the studies had been conducted in populations from central/northern Europe and north America and, as such, with considerable ethnic as well as dietary differences in respect to those of the present study. These differences may explain why our patients and control subjects had higher concentrations of serum folate and vitamin B12 than the individuals analysed in most of the reported studies [15–17, 23, 37, 38]. The consensus is that the detrimental effects of low serum folate and vitamin B12 on Hcy plasma levels become apparent only below certain levels, or cut-off points. These cut-offs points are situated at concentrations around 10 nmol/l of folate and 375 pmol/l of vitamin B12 [37, 42]. In our study, the mean of the concentrations of serum folate and vitamin B12 of the patients with diabetes are, in general, higher than these cut-off points (Table 2). Noticeably, the non-Gaussian distribution of these vitamin concentrations in our NIDDM and IDDM patients was due to the higher frequency of patients with high levels of these vitamins. Moreover, the serum concentrations of these vitamins were generally higher in the patient groups than in their respective controls (Table 2). The reason for the increased serum concentration of folate and vitamin B12 in the diabetic patients could be related to their treatment, especially the diet.

Around 19% of IDDM patients and 44% of NIDDM patients had a previous history of nephropathy, as indicated by elevated creatinine concentrations, proteinuria or microalbuminuria (Table 1). Univariate correlations and multiple regression analysis showed that AER was the independent parameter that correlated best with plasma Hcy values, both in IDDM and NIDDM patients (Table 3). We are not aware of any report indicating that hyperhomocysteinaemia induces nephropathy whereas, conversely, renal failure causing hyperhomocysteinaemia is well documented. Several of our findings support the concept that, mainly in the NIDDM patients, the existence of nephropathy was a major cause of fasting hyperhomocysteinaemia [21, 22]. Firstly, the Hcy concentrations in IDDM and NIDDM patients with nephropathy were higher than that in patients with IDDM and NIDDM without nephropathy (Fig. 1A). Secondly, the diabetic group as a whole and the NIDDM subgroup showed significant increases in Hcy values at nearly all levels where the severity of nephropathy increased (Fig. 1B). Thirdly, when the patients with IDDM and NIDDM were classified as having normohomocysteinaemia or hyperhomocysteinaemia, the latter had significant increases in most parameters indicative of nephropathy (Table 4). Fourthly, 80% of diabetic patients with fasting hyperhomocysteinaemia presented with nephropathy. However, 38% of patients with well-defined nephropathy did not have fasting hyperhomocysteinaemia. Comparing patients with nephropathy with and without hyperhomocysteinaemia, the main differences were that the patients with hyperhomocysteinaemia were older and had more severe nephropathy (Table 5).

One of the most interesting findings of our study relates to the tendency towards Hcy elevation in the stages of incipient and overt nephropathy, especially in NIDDM patients. Because patients with serum creatinine over 120 μmol/l were excluded from these

analysis (see legend to Fig. 1) and Cockroft-Gault derived creatinine clearances were not different from those of patients without nephropathy, this association does not seems to be due to an impairment of the glomerular filtration rate.

Given that age is another major independent factor associated with Hcy concentrations (Table 3), interactions between nephropathy and age would appear to be as critical in determining the concentration of plasma Hcy in the patients studied. This could explain, at least in part, why the association between nephropathy and hyperhomocysteinaemia was stronger in our NIDDM patients, who were older, with respect to our IDDM patients. Also, hypertension could favour hyperhomocysteinaemia by causing or favouring nephropathy, although other mechanisms are also possible [36].

Our results extend those of Hultberg et al. [25] who studied 79 IDDM patients and in which fasting hyperhomocysteinaemia was confined to those with serum creatinine over 115 μmol/l and/or ratio of albumin:creatinine clearance over 20. Agardh et al. [43] studied 76 IDDM patients and found a significant correlation between AER and Hcy. However, Hcy concentrations of normoalbuminuric and microalbuminuric patients were not significantly different. Robillon et al. [27] also studied fasting Hcy in 41 IDDM patients and observed lower levels of Hcy in IDDM patients than in control subjects. They did not find any correlation between Hcy values and the concentrations of vitamins and lipids nor with the presence of nephropathy. The reason for the discrepancy between these studies remains unclear. However, our protocol is the only one which measured AER in 24-h urine specimens and included patients with both types of diabetes. This aspect may be of importance since in our study: i) the association between AER and Hcy was stronger in NIDDM than in IDDM patients and; ii) age was an important factor in the increased Hcy values observed.

The molecular and cellular bases of the kidney's metabolism of Hcy remain largely unknown. The metabolism of Hcy involves two pathways, the remethylation to methionine and the transulphuration to cystathionine and cysteine. Fasting Hcy measurements appear to reflect mainly the remethylation pathway [44]. There are two ways in which Hcy is remethylated to methionine. In one, methyltetrahydrofolate is the donor of the methyl group. This compound is formed by the action of the methylenetetrahydrofolate reductase [45]. Vitamin B12 transfers this methyl group to Hcy to form methionine in a reaction catalysed by the enzyme methionine synthase [46]. An additional remethylation reaction uses betaine as the methyl donor, in a step catalysed by the enzyme betaine:homocysteine methyltransferase [47]. Both remethylation steps seem equally important in terms of Hcy conversion to methionine. While the enzymes methionine synthase and methylenetetrahydrofolate reductase are widely distributed in different tissues, betaine:homocysteine methyltransferase is restricted, in humans, to the liver and the kidney [45–48]. Thus, one or the other or both remethylation reactions could be altered in patients with diabetes and nephropathy. One explanation is that the renal lesion affects the synthesis of these enzymes. The correlation of Hcy with AER would suggest that the cell type dysfunction causing hyperhomocysteinaemia is localized mainly in the glomeruli. There is, as yet, little information on the mRNA expression of methylenetetrahydrofolate reductase and betaine:homocysteine methyltransferase in different tissues and cell types since their cloning and sequencing has been recent [45, 47]. A second possibility is that nephropathy affects the availability of the methyl donor substrates (folate and betaine) or vitamin B12 (the cofactor of methionine synthase). Our measurements of serum folate and vitamin B12 and erythrocyte folate did not correlate with plasma Hcy. We did not measure betaine. Therefore, a decrease in the availability of betaine in the kidneys of patients with diabetes and nephropathy remains a possibility, particularly since plasma betaine has been shown to be negatively correlated with microalbuminuria in diabetic subjects [49].

In our study, the Hcy levels were higher in patients with IDDM and macroangiopathy than in patients with IDDM without macroangiopathy. This difference was not observed in NIDDM. These results should be viewed with caution. In the case of the IDDM group there were only four patients with macroangiopathy; while in the case of the NIDDM group a higher cardiovascular mortality rate for patients with hyperhomocysteinaemia cannot be ruled out [50]. To our knowledge, the only previously published data on the incidence of macroangiopathy in diabetic patients are the studies of Araki et al. [26] and Munshi et al. [28]. Araki et al. [26] studied 136 patients with NIDDM, 38.2 % of whom had macroangiopathy. Fasting Hcy concentrations in patients with diabetes and macroangiopathy (10.8 ± 3.8 μmol/l) were higher than Hcy values in patients with diabetes but without macroangiopathy (8.3 ± 3.1 μmol/l) or in control subjects (7.5 ± 2.1 μmol/l) [26]. Munshi et al. [28] studied fasting and post-methionine load Hcy in 11 patients with diabetes without macroangiopathy (5 IDDM and 6 NIDDM), 17 patients with diabetes and macroangiopathy (5 IDDM and 12 NIDDM). NIDDM patients with macroangiopathy presented with higher post-methionine Hcy values than patients with NIDDM without macroangiopathy or control individuals. No differences were observed in the fasting Hcy values between the different groups [28].

The results of the present study suggest that incipient and overt nephropathy, in the absence of renal failure, is associated with elevations of plasma Hcy in patients with NIDDM. This is of particular impor-

tance because these patients are at high risk of developing CVD and effective therapy for the hyperhomocysteinaemia is available. Prospective studies are needed to evaluate the relationship between nephropathy and hyperhomocysteinaemia and to know whether hyperhomocysteinaemia has a causal role in the development of macroangiopathy in patients with diabetes. It would be of interest, also, to assess whether the association between AER and plasma Hcy concentration holds true in non-diabetic subjects in whom microalbuminuria is also an important cardiovascular risk factor but the underlying mechanism of which is unknown. Hyperhomocysteinaemia has been shown to impair endothelial function and is, therefore, a potential candidate for explaining the link between microalbuminuria, endothelial disfunction and CVD as proposed by the Steno hypothesis [51–55].

Addendum. While this manuscript was under review, Hofmann et al. [56] reported a high prevalence of hyperhomocysteinaemia and endothelial dysfunction in patients with IDDM of long duration (mean of 22 years), also associated with nephropathy.

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References

1. Morrish NJ, Stevens LK, Fuller JH, Jarret RJ, Keen H (1991) Risk factors for macrovascular disease in diabetes mellitus: the London follow-up up to the WHO multinational study of vascular disease in diabetes. Diabetologia 34: 590–594
2. Donahue RP, Orchard TJ (1992) Diabetes mellitus and macrovascular complications: an epidemiological perspective. Diabetes Care 15: 1141–1155
3. Pyörälä K, Laakso M, Uusitapa M (1987) Diabetes and atherosclerosis: an epidemiological view. Diab Metab Rev 3: 463–524
4. Borch-Johnsen K, Kreiner S (1987) Proteinemia: value as a predictor of cardiovascular mortality in insulin-dependent diabetes mellitus. BMJ 294: 1651–1654
5. Jensen T, Borch-Johnsen K, Kofoed-Enevoldsen A, Deckert T (1987) Coronary heart disease in young type 1 (insulin-dependent) diabetic patients with and without diabetic nephropathy: incidence and risk factors. Diabetologia 30: 144–148
6. Krolewski AS, Kosinski EJ, Warram JH et al. (1987) Magnitude and determinants of coronary artery disease in juvenile-onset, insulin-dependent diabetes mellitus. Am J Cardiol 59: 750–755
7. Mattock M, Morrish N, Viberti GC, Keen H, Fitzgerald A, Jackson G (1992) Prospective study of microalbuminuria as predictor of mortality in NIDDM. Diabetes 41: 735–741
8. Miettinen H, Haffner SM, Lehto S, Rönnemaa T, Pyörälä K, Laakso M (1996) Proteinuria predicts stroke and other atherosclerotic vascular disease events in nondiabetic and non-insulin-dependent diabetic subjects. Stroke 27: 2033–2039
9. Pinkney JH, Foyle WJ, Denver AE, Mohamed-Ali AV, Mckinlay S, Yudkin JS (1995) The relationship of urinary albumin excretion rate to ambulatory blood pressure and erytrocyte sodium-lithium countertransport in NIDDM. Diabetologia 38: 356–362
10. Krolewski AS, Canessa M, Warram JH et al. (1988) Predisposition to hypertension and susceptibility to renal disease in insulin-dependent diabetes mellitus. N Engl J Med 318: 140–145
11. Lahdenpera S, Groop PH, Tilly-Kiesi M et al. (1994) LDL subclasses in IDDM patients: relation to diabetic nephropathy. Diabetologia 37: 681–688
12. Hirano T, Naito H, Kurokawa M et al. (1996) High prevalence of small LDL particles in non-insulin-dependent diabetic patients with nephropathy. Atherosclerosis 123: 57–72
13. Knöbl P, Schernthaner G, Schnack C et al. (1993) Thrombogenic factors are related to urinary albumin excretion in type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetic patients. Diabetologia 36: 1045–1050
14. Diabetes Care and Research in Europe (1992) The St Vincent declaration action program. Copenhagen: WHO Regional Office for Europe
15. Mudd SH, Levy HL, Skovby F (1995) Disorders of transulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular basis of inherited disease, 7th edn. McGraw-Hill, New York, pp 1279–1327
16. Rosenblatt DS (1995) Inherited disorders of folate transport and metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular basis of inherited disease, 7th edn. McGraw-Hill, New York, pp 3111–3128
17. Kang S-S, Wong PWK (1996) Genetic and nongenetic factors for moderate hyperhomocyst(e)inemia. Atherosclerosis 119: 135–138
18. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. JAMA 274: 1049–1057
19. Perry IJ, Refsum H, Morris RW, Ebrahim SB, Ueland PM, Shaper AG (1995) Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. Lancet 346: 1395–1398
20. Arnesen E, Refsum H, Bonaa KH, Ueland PM, Forde OH, Nordrehaug JE (1995) Serum total homocysteine and coronary heart disease. Int J Epidemiol 24: 704–709
21. Bostom AG, Shemin D, Lapane KL et al. (1995) Hyperhomocysteinaemia and traditional cardiovascular disease risk factors in end-stage renal disease patients on dialysis: a case-control study. Atherosclerosis 114: 93–103
22. Bostom A, Brosnan JT, Hall B, Nadeau MR, Selhub J (1995) Net uptake of plasma homocysteine by the rat kidney in vivo. Atherosclerosis 116: 59–62
23. Franken DG, Boers GHJ, Blom HJ, Cruysberg JRM, Trijbels FJM, Kloppenborg PWC (1994) Treatment of mild hyperhomocysteinaemia. Arterioscler Thromb 14: 465–470

24. Stampfer MJ, Malinow MR (1995) Can lowering homocysteine levels reduce cardiovascular risk? N Engl J Med 332: 328–329
25. Hultberg B, Agardh E, Andersson A et al. (1991) 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
26. Araki A, Sako Y, Ito H (1993) Plasma homocysteine concentrations in Japanese patients with non-insulin-dependent diabetes mellitus: effect of parenteral methylcobalamin treatment. Atherosclerosis 103: 149–157
27. Robillon JF, Canivet B, Candito M et al. (1994) Type 1 diabetes mellitus and homocysteine. Diabete Metab 20: 494–496
28. Munshi MN, Stone A, Fink L, Fonseca V (1996) Hyperhomocysteinaemia following a methionine load in patients with non-insulin-dependent diabetes mellitus and macrovascular disease. Metabolism 45: 133–135
29. National Diabetes Data Group (1979) Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes 28: 1039–1057
30. González-Tallada A, González-Sastre F (1994) Evaluación de la función renal y del aparato urinario. In: González-Sastre F (ed) Bioquímica Clínica, 1st edn. Barcanova, Barcelona, pp 193–212
31. Andersson A, Lindgren A, Hultberg B (1995) Effect of thiol oxidation and thiol export from erythrocytes on determination of redox status of homocysteine and other thiols in plasma from healthy subjects and patients with cerebral infarction. Clin Chem 41: 361–366
32. Córdoba-Porras A, Sánchez-Quesada JL, González-Sastre F, Ordóñez-Llanos J, Blanco-Vaca F (1996) Susceptibility of plasma low- and high-density lipoproteins to oxidation in patients with severe hyperhomocysteinaemia. J Mol Med 74: 771–776
33. Sánchez-Quesada JL, Pérez A, Caixàs A et al. (1996) Electronegative low density lipoprotein subform is increased in patients with short-duration IDDM and is closely related to glycaemic control. Diabetologia 39: 1469–1476
34. Manual of laboratory operations. Lipid Research Clinics Program. Lipid and lipoprotein analysis (1974) DHEW Publications. NIH 75–628. Washington DC, US Government Printing Office
35. Cortés M, Blanco F, Merce J, Queraltó JM (1987) Adaptación del método Azul Brillante Coomassie al RA–1000 para la determinación de proteínas en orina. Clin Chem Newslett 1: 41–44
36. Sutton-Tyrrell K, Bostom A, Selhub J, Zeigler-Johnson C (1997) High homocysteine levels are independently related to isolated systolic hypertension in older adults. Circulation 96: 1745–1749
37. Selhub J, Jacques PF, Bostom AG et al. (1996) Relationship between plasma homocysteine, vitamin status and extracranial carotid-artery stenosis in the Framingham study. J Nutr 126: 1258S–1265S
38. Guttormensen AB, Ueland PM, Nesthus I et al. (1996) Determinants and vitamin responsiveness of intermediate hyperhomocysteinaemia (> 40 μmol/liter). J Clin Invest 98: 2174–2183
39. Malinow MR, Levenson J, Giral P et al. (1995) Role of blood pressure, uric acid, and hemorrheological parameters on plasma homocysteine concentration. Atherosclerosis 114: 175–183
40. Nygärd O, Vollset SE, Refsum H et al. (1995) Total homocysteine and cardiovascular risk profile. JAMA 274: 1526–1533
41. Lussier-Cacan S, Xhignesse M, Piolot A, Selhub J, Davignon J, Genest J Jr (1996) Plasma total homocysteine in healthy subjects: sex-specific relation with biological traits. Am J Clin Nutr 64: 587–593
42. Pancharutini N, Lewis CA, Sauberlich HE et al. (1994) Plasma homocyst(e)ine, folate, and vitamin B-12 concentrations and risk for early-onset coronary artery disease. Am J Clin Nutr 59: 940–948
43. Agardh CD, Agardh E, Andersson A, Hultberg B (1994) Lack of association between plasma homocysteine levels and microangiopathy in type 1 diabetes mellitus. Scand J Clin Lab Invest 54: 637–641
44. Miller JW, Nadeau MR, Smit D, Selhub J (1994) Vitamin B6 deficiency vs folate deficiency: comparison of responses to methionine loading in rats. Am J Clin Nutr 59: 1033–1039
45. Goyette P, Summer JS, Milos R et al. (1994) Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. Nature Genet 7: 195–200
46. Chen LH, Liu M-L, Hwang H-Y, Chen L-S, Korenberg J, Shane B (1997) Human methionine synthase. cDNA cloning, gene localization, and expression. J Biol Chem 272: 3268–3634
47. Garrow TA (1996) Purification, kinetics properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase. J Biol Chem 271: 22831–22838
48. McKeever MP, Weir DG, Molloy A, Scott JM (1991) Betaine homocysteine methyltransferase: organ distribution in man, pig, and rat and subcellular distribution in the rat. Clin Sci 81: 551–556
49. Lever M, Sizeland PCB, Bason LM, Haymer CM, Robson RA, Chambers ST (1994) Abnormal glycine betaine content of the blood and urine of diabetic and renal patients. Clin Chim Acta 230: 69–79
50. Nygärd O, Nordrehaug JE, Refsum H, Ueland PM, Farstad M, Vollset SE (1997) Plasma homocysteine levels and mortality in patients with coronary artery disease. New Engl J Med 337: 230–236
51. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Enevoldsen A (1989) Albuminuria reflects widespread vascular damage: the Steno hypothesis. Diabetologia 32: 219–226
52. Parving H-H, Nielsen FS, LE Bang et al. (1996) Macro-microangiopathy and endothelial dysfunction in NIDDM patients with and without diabetic nephropathy. Diabetologia 39: 1590–1597
53. Lentz SR, Sobey CG, Piegors DJ et al. (1996) Vascular dysfunction in monkeys with diet-induced hyperhomocysteinaemia. J Clin Invest 98: 24–29
54. Tawakol A, Omland T, Gerhard M, Wu JT, Creager MA (1997) Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. Circulation 95: 1119–1121
55. Upchurch GR Jr, Welch GN, Fabian AJ et al. (1997) Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 272: 17012–17017
56. Hofmann MA, Kohl B, Zumbach MS et al. (1997) Hyperhomocyst(e)inemia and endothelial dysfunction in IDDM. Diabetes Care 20: 1880–1886