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DETERMINING THE RELATIONSHIP BETWEEN HOMOCYSTEINEMIA AND BIOMARKERS OF INFLAMMATION, OXIDATIVE STRESS AND FUNCTIONAL KIDNEY STATUS IN PATIENTS WITH DIABETIC NEPHROPATHY

ISPITIVANJE ODNOSA HOMOCISTEINEMIJE I BIOMARKERA INFLAMACIJE, OKSIDATIVNOG STRESA I FUNKCIONALNOG STATUSA BUBREGA KOD BOLESNIKA SA DIJABETESNOM NEFROPATIJOM

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Summary

Background: One of the leading causes of terminal renal failure is diabetic nephropathy. The aim of this study was to determine the relationship between homocysteine levels and the biomarkers of renal function, inflammation and oxidative stress, as well as the incidence of macrovascular complications in patients with diabetic nephropathy.
Methods: Sixty-four patients with diabetic nephropathy were included in this study. They were divided according to their homocysteine levels into two groups: hyperhomocysteinemic (HHcy, n=47) and normohomocysteinemic patients (NHCy, n=17). The results were compared to a control group (n=20) with normal renal function and without diabetes. Besides homocysteine, cystatine C, creatinine, urea, albuminuria, creatinine clearance, lipid status parameters, apolipoprotein A-I and B, lipoprotein (a), CRP, fibrinogen, oxidative LDL were determined using appropriate methods. The incidence of macrovascular diabetic complications was also determined.
Results: The results indicate that the level of renal dysfunction is greater in HHcy than in NHcy patients ($p<0.05$). In HHcy patients levels of oxLDL were also higher compared to NHcy patients (119.3±140.4 vs. 71.4±50.8 ng/mL,

Kratak sadržaj

Uvod: Dijabetesna nefropatija jedan je od vodećih uzroka terminalne bubrežne insuficijencije. Cilj ove studije bio je ispitivanje odnosa homocisteinemije i biomarkera bubrežne funkcije, inflamacije i oksidativnog stresa, kao i učestalosti makrovaskularnih komplikacija kod bolesnika sa dijabetesnom nefropatijom.
Metode: U studiju je uključeno 64 ispitanika sa dijabetesnom nefropatijom koji su podeljeni u dve grupe u odnosu na homocisteinemiju: hiperhomocisteinemični (HHcy, n=47) i normohomocisteinemični (NHcy, n=17). Odgovarajućim metodama su pored nivoa homocisteina u krvi određivani i nivoi cistatina C, kreatinina, uree, albuminurije, klirensa kreatinina, parametara lipidskog statusa, apolipoproteina A i B, lipoproteina(a), CRP, fibrinogena, oksidisanog LDL, kao i učestalost makrovaskularnih komplikacija dijabetesne bolesti.
Rezultati: Dobijeni rezultati upoređivani su sa rezultatima kontrolne grupe ispitanika (n=20) sa urednom bubrežnom funkcijom, bez prisutne dijabetesne bolesti. Rezultati ukazuju da je stepen bubrežne disfunkcije veći kod HHcy nego kod NHcy pacijenata ($p<0,05$). Takođe, kod HHcy pacijenata nivo oxLDL je značajno viši u odnosu na NHcy pacijente

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List of abbreviations: AIP – atherogenic index of plasma, BMI – body mass index, BSA – body surface area, CAD – coronary arterial disease, C – cholesterol, CCr – creatinine clearance, DM – diabetes mellitus, DN – diabetic nephropathy, HHcy – hyperhomocysteinemia, IM – infarctus myocardii, LPI – lipid pentad index, LTI – lipid tetrad index, NHcy – normohomocysteinemia, oxLDL – oxidised low density cholesterol, PAD – peripheral artery disease, sdLDL – small dense low density cholesterol, TAS – systolic blood pressure, TAD – diastolic blood pressure, tHcy – total homocysteine, UAE – urinary albumin excretion.

p<0.05) as well as fibrinogen levels (4.3±1.3 vs. 3.7±0.8 g/L, p<0.05). The incidence of macrovascular complications is more frequent in HHcy than in NHcy patients (55.3. vs. 35.3 %, p>0.05), and in patients with macroalbuminuria compared to patients with microalbuminuria (65% vs. 39%, p<0.05).

Conclusions: It can be concluded that HHcy is significantly present in patients with diabetic nephropathy, especially if there is greater reduction of renal function. Besides that, significantly higher concentrations of inflammatory (fibrinogen) and oxidative stress (oxLDL) markers were present in HHcy patients with diabetic nephropathy compared to NHcy patients.Therefore in diabetic nephropathy patients it is useful to regularly monitor the levels of homocysteine, as well as inflammatory and markers of oxidative stress.

Keywords: homocysteine, biomarkers, diabetic nephropathy

(119,3±140,4 vs. 71,4±50,8 ng/mL, p<0,05), kao i nivo fibrinogena (4,3±1,3 vs. 3,7±0,8 g/L, p<0,05). Pojava makrovaskularnih komplikacija je učestalija kod HHcy nego NHcy pacijenata (55,3% vs. 35,3%, p>0,05), kao i kod pacijenata sa makroalbuminurijom u odnosu na pacijente sa mikroalbuminurijom (65% vs. 39%, p<0,05).

Zaključak: Na osnovu dobijenih rezultata može se zaključiti da je HHcy u značajnom stepenu prisutna kod bolesnika sa dijabetesnom nefropatijom, naročito ukoliko postoji izražena redukcija funkcionalne rezerve bubrega. Osim toga, u odnosu na NHcy bolesnike, kod HHcy bolesnika sa dijabetesnom nefropatijom značajno su više koncentracije inflamatornih (fibrinogena) i markera oksidativnog stresa u krvi (oxLDL). Stoga je kod bolesnika sa dijabetesnom nefropatijom poželjno redovno praćenje homocisteina u krvi, ali i inflamatornih markera, kao i markera oksidativnog stresa.

Ključne reči: homocistein, biomarkeri, dijabetesna nefropatija

Introduction

Diabetes mellitus (DM) type 2 is characterized by a significant rate of vascular complications (1), including diabetic nephropathy (DN), one of the leading causes of terminal renal failure. Besides that, in these patients there is a great incidence of cardiovascular diseases (2), especially if DN is present (3). In diabetic patients cardiovascular mortality is 2–4 times greater than in non-diabetic ones (4). Chronic vascular complications were present in 20% of type 2 DM patients at the moment of primary disease diagnosing (5). The most important factors leading to vascular complications are hyperglycemia, dyslipidemia, inflammation and oxidative stress (6). Homocysteine (Hcy) – the most intriguing of the so-called »vascular toxins« increases atherosclerotic potential in type 2 DM (7). Homocysteine is a sulfur-containing amino acid formed during the metabolism of methionine. It manifests direct toxic effects on endothelial cells and indirect ones on normal methylation in endothelial cells (8). This contributes to enhanced production of free radicals, stimulates proliferation of smooth muscle cells and changes of platelet activity (9) etc. Given that kidney has a leading role in the metabolism of Hcy, plasmatic levels of Hcy largely depend on renal function (10). In type 2 DM Hcy level can be elevated, decreased or unchanged compared to a general population of adequate age (11–13). This is due to glomerular hyperfiltration or hypofiltration which is characteristic of DM type 2. Hyperglycemia itself leads to activation of trans-sulfuric enzymes and affects plasmatic Hcy levels (14).

It is indicated in prospective studies that even moderately elevated plasmatic levels of Hcy in DM type 2 patients are a more significant risk factor for cardiovascular disease than in the non-diabetic population. Mechanisms of their synergistic action in premature atherosclerosis development are not yet known (15), as well as the mechanism by which hyperhomocysteinemia contributes to the development and progression of DM type 2 complications.

In order to assess the relationship between homocysteinemia and the biomarkers of inflammation, oxidative stress, functional renal status and the incidence of macrovascular complications of diabetes in DN patients, the subjects in this study were divided into two groups: with elevated (HHcy) and normal (NHcy) levels of homocysteine, and we evaluated creatinine clearance (CCr), cystatin C, albuminuria, lipid status parameters, apolipoprotein A-I and B, lipoprotein (a), levels of CRP, fibrinogen and oxidized LDL (oxLDL).

Materials and Methods

This cross-sectional study was carried out at the Clinical Center of Vojvodina, Novi Sad, Serbia. The study had previously been approved by an institutional ethics committee and all included subjects had approved their participation. A total of 84 subjects were included: 64 with diabetes mellitus type 2 (42 males/22 females) and with the presence of diabetic nephropathy (persistent urinary albumin excretion (UAE) >30 mg/24 h) (16, 17), divided into two groups according to their fasting plasma tHcy levels [the first group with HHcy (the normal range in our laboratory was 5–12 μmol/L), and the second with NHcy], and 20 healthy controls (12 males/8 females).

All patients with diabetic nephropathy were examined in the relevant clinics of the Clinical Center of Vojvodina in order to determine the presence of other chronic complications (University Eye Clinic and Clinic for Endocrinology, Diabetes and Metabolic Diseases). Retinopathy was diagnosed after ophthalmological examination (standard fundus eye examination for the presence of microaneurisms, neovascularisation, venous dilatation, cotton-wool spots or hemorrhage) or according to a positive history of dis-

ease. Clinical neuropathy was assessed by neurologic examination and interrogation about the symptoms of neuropathy (parestesia, pain in the legs and feet, etc). Coronary artery disease (CAD), stroke or peripheral artery disease (PAD) were considered as macroangiopatic complications. CAD was clinically assessed (positive history of cardiovascular events, and/or presence of angina pectoris, abnormal coronary angiogram) and evaluated by a cardiologist. Cerebrovascular disease was confirmed by cranial computerized tomography or magnetic resonance. A neurologist made the final diagnosis. PAD was confirmed by an endocrinologist and if there were present intermittent claudication, absence or reduced peripheral pulse (or both) or foot lesions due to vascular disease.

Patients with acute infection, thyroid dysfunction, liver disease and malignancies were excluded from the study.

In all subjects serum levels of creatinine, urea, uric acid, glucose, total cholesterol, HDL-cholesterol, triglycerides and creatinuria were measured using the standard biochemical methods. High-sensitive CRP, apolipoprotein A-I and B (Beckman-Coulter, Ireland), Lp (a) (Sentinel, Italy) and cystatin C (Dyazime, USA) were measured by immunoturbidimetry.

Creatinine clearance (mL/min/1.73 m^2 of body surface area (BSA)) was calculated using the same 24-h urine used for albuminuria determination by the formula (18):

$CCr=(U_{Cr} \times 24h$ urine volume (mL))/($S_{Cr} \times 1440$ (min/day))

U_{Cr} – urine creatinine (μmol/L), S_{Cr} – serum creatinine (μmol/L).

BSA was calculated by the formula (19): BSA (m^2) = $0.0235 \times$ height $(cm)^{0.42246} \times$ weight $(kg)^{0.51456}$.

Plasma tHcy level was determined by the FPIA method on an Axsym analyzer (Abbott, USA). Fibrinogen level was determined by the Claus metod in citrate plasma (20) on an ACL system (Instrumentation Laboratory, Italy). Glycated hemoglobin was measured as HbA_{1c} by the immuno-inhibitory test (Beckman-Coulter, Ireland). Albuminuria was determined using 24 h urine by the sandwich-immunometric method (NycoCard tests, Norway). OxLDL was determined by the ELISA method (Biomedica GmbH, Vienna, Austria).

Analyses were performed immediately after blood sampling, except for apoA-I, apoB, Lp(a) and oxLDL determination (samples were kept frozen at –20 °C no longer than one month before the determination of these parameters).

LDL-C, non-HDL-C, the traditional indexes LDL-/HDL-C, non-HDL-/HDL-C, and the bioindexes lipid tetrad index (LTI) = TG x total C x Lp(a)/HDL-C; lipid pentad index (LPI) = TG x total C x Lp(a) × apo B/A-I; atherogenic index of plasma (AIP) = log (TG/HDL-C) were calculated. All parameters were converted into SI units.

In addition, body mass index (BMI) was calculated for all subjects (21).

Statistics

Descriptive statistics, including median, arithmetic mean and standard deviation (SD) were used to describe the studied parameters. Differences in distributions of individual parameters between the study groups were analyzed using the parametric Student's t-test, or the nonparametric Mann-Whitney test in case a distribution showed a significant deviation. Linear regression analysis and Pearson coefficient of linear correlation were used to study correlation between variables. Statistical analysis was performed using the STATISTICA 8.0 software. $p<0.05$ was considered statistically significant.

Results

General and laboratory characteristics of the study subjects are shown in *Table I* and *Table II*. In the group of HHcy diabetic patients, 91.5% used insulin and 70.2% used oral hypoglycemic agents, whereas among NHcy diabetic patients 82.4% used insulin and 76.5% oral hypoglycemic agents. Hypertension (BP>140/90 mm Hg) was registered in 91.5% of HHcy and 82.4% of NHcy diabetic patients, and 72.4% of HHcy and 52.9% of NHcy diabetic patients used ACE inhibitors ($p>0.05$).

Both HHcy and NHcy diabetic patients had a significantly higher mean BMI than the healthy controls (29.5±4.6 vs. 28.8±4.3 vs. 25.9±3.1 kg/m^2, $p<0.05$). Also, there was a similar significant difference for mean TAS (150.4±19.5 vs. 145.3±23.2 vs. 126.2±9.0 mmHg, $p<0.05$) and TAD (86.4±9.1 vs. 85±7.5 vs. 80.7±5.9 mmHg, $p<0.05$), while there were no significant differences in the measured parameters between the two groups of diabetic patients.

Macroalbuminuria was more prevalent among HHcy than NHcy diabetic patients (40.5% vs. 17.6%, $p>0.05$), and microalbuminuria was more prevalent in NHcy diabetic patients (59.5% vs. 82.4%, $p>0.05$).

Serum concentrations of cystatin C, creatinine, urea, uric acid were significantly higher, and creatinine clearance was significantly lower in HHcy diabetic patients than in the other groups. NHcy diabetic patients had significantly higher cystatin C levels compared to controls, and there were no significant differences in other parameters of renal function between these two groups of subjects.

Table I Characteristics of diabetic patients with hyperhomocysteinemia (HHcy), normohomocysteinemia (NHcy), and the control group.

	HHcy ($\bar{x}$±SD)	NHcy ($\bar{x}$±SD)	Control ($\bar{x}$±SD)
Age (years)	64.1±7.1^{b2}	61.1±6.5	60.6±3.2
Number (m/f)	47 (30/17)	17 (12/5)	20 (12/8)
tHcy (μmol/L)	17.3±4.6^{a3,b3}	10.2±1.2	10.4±1.4
tHcy range (μmol/L)	12.1–27.8	7.1–11.5	8.0–11.9
BMI (kg/m^2)	29.5±4.6^{b3}	28.8±4.3^{b1}	25.9±3.1
TAS (mmHg)	150.4±19.5^{b3}	145.3±23.2^{b2}	126.2±9.0
TAD (mmHg)	86.4±9.1^{b2}	85±7.5^{b1}	80.7±5.9
Duration of DM (years)	16.9±7.8	14.6±17.9	–
Neuropathy (%)	68.2	70.5	–
Retinopathy (%)	83.4	88.3	–
Fasting glucose (mmol/L)	9.7±4.7^{b3}	9.6±3.7^{b3}	5.7±0.5
HbA1c (%)	8.0±1.7^{b3}	8.4±1.5^{b3}	5.6±0.3

tHcy – total homocysteine, BMI – body mass index, TAS – systolic blood pressure, TAD – diastolic blood pressure, DM – diabetes mellitus, a1 – $p<0.05$, a2 – $p<0.01$, a3 – $p<0.001$ compared to NHcy; b1 – $p<0.05$, b2 – $p<0.01$, b3 – $p<0.001$ compared to control group.

Table II Laboratory characteristics of diabetic patients with hyperhomocysteinemia (HHcy), normohomocysteinemia (NHcy), and the control group.

	HHcy (±SD)	NHcy (±SD)	Control (±SD)
Albuminuria (mg/dU)	273.9±228.5^{a1,b3}	172.6±94.9^{b3}	14.3±8.3
Microalbuminuria (%)	59.5	82.4	–
Macroalbuminuria (%)	40.5	17.6	–
Cystatin C (mg/L)	1.95±0.92^{a3,b3}	1.15±0.18^{b2}	0.96±0.1
Creatinine (μmol/L)	147.8±90.4^{a3,b3}	91.1±14.2	84.3±12.2
CCr (mL/min/1.73 m^2)	66.9±33.3^{a3,b3}	95.2±31.4	95.8±21.1
Urea (mmol/L)	9.8±5.9^{a3,b3}	5.8±1.1	5.5±1.5
Uric acid (μmol/L)	371.1±107.8^{a3,b2}	278.5±72	290.0±90.9
CRP (mg/L)	4.9±5.9 (med 3.1)^{b3}	3.2±3.3 (med 1.8)	1.5±1.4 (med 1.15)
Fibrinogen (g/L)	4.3±1.3^{a1,b3}	3.7±0.8^{b2}	3.0±0.3

Hcy – homocysteine, CCr – creatinine clearance, a1 – $p<0.05$, a2 – $p<0.01$, a3 – $p<0.001$ compared to NHcy; b1 – $p<0.05$, b2 – $p<0.01$, b3 – $p<0.001$ compared to control group.

CRP levels were significantly higher in HHcy diabetic patients compared to controls (4.9±5.9 vs. 1.5±1.4 mg/L, $p<0.05$) but not compared to NHcy diabetic patients (4.9±5.9 vs. 3.2±3.3 mg/L, $p>0.05$). Fibrinogen levels were significantly higher in HHcy diabetic patients compared to both NHcy diabetic patients and controls (4.3±1.3 vs. 3.7±0.8 vs. 3.0±0.3 g/L, $p<0.05$).

The two groups of diabetic patients had similar lipid status parameters, and there was a significant difference between them and controls in triglyceride, HDL-C and apoA-I levels (*Table III*).

Both groups of diabetic patients had significantly higher non-HDL-C/HDL-C ratio and AIP and LTI compared to the control group, and did not differ from each other in these parameters.

Table III Lipid parameters in diabetic patients with hyperhomocysteinemia (HHcy), normohomocysteinemia (NHcy), and the control group.

	HHcy ($\bar{x}$±SD)	NHcy ($\bar{x}$±SD)	Control ($\bar{x}$±SD)
C (mmol/L)	6.01±1.84	5.96±1.51	5.98±0.71
Triglycerides (mmol/L)	2.05±1.52^{b3}	2.05±1.19^{b3}	1.19±0.52
HDL-C (mmol/L)	1.13±0.26^{b1}	1.06±0.14^{b2}	1.32±0.29
LDL-C (mmol/L)	3.88±1.35	3.92±1.09	4.26±0.89
nonHDL-C (mmol/L)	4.82±1.73	4.94±1.44	4.8±0.93
apoA-I (g/L)	1.31±1.39^{b2}	1.31±1.41^{b1}	1.45±0.18
apoB (g/L)	1.04±0.34	1.04±0.27	1.04±0.34
Lp(a) (g/L)	0.36±0.56	0.34±0.35	0.18±0.19
oxLDL (ng/mL)	119.3±140.4^{a1,b1}	71.4±50.8	69.1±41.1
LDL/HDL-C	3.44±1.01	3.59±0.71	3.41±1.0
nonHDL/HDL-C	4.34±1.41^{b1}	4.62±1.32^{b1}	3.82±1.22
AIP	0.18±0.29^{b2}	0.23±0.23^{b2}	–0.03±0.25
LTI	3.47±7.5 (med 1.15)^{b1}	3.5±3.46 (med 2)^{b2}	0.88±0.84
LPI	4.17±11 (med 0.84)	3.39±3.9 (med 2.1)^{b1}	0.90±1.02
oxLDL/LDL	36.3±56.6^{a1,b1}	18.4±11.4	16.8±10.8

C – cholesterol, AIP – atherogenic index of plasma, LTI – lipid tetrad index, LPI – lipid pentad index, a1 – p<0.05, a2 – p<0.01, a3 – p<0.001 compared to NHcy; b1 – p<0.05, b2 – p<0.01, b3 – p<0.001 compared to control group.

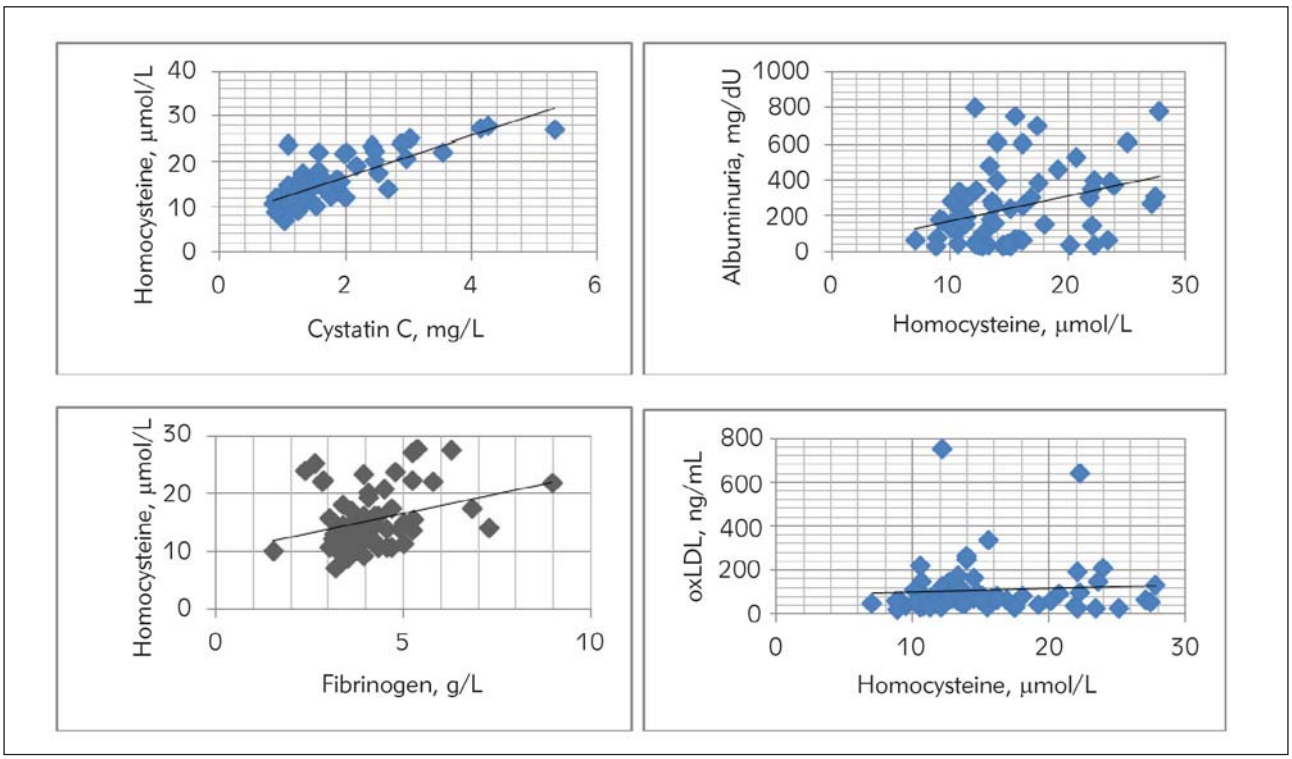

Figure 1 Correlation of tHcy with levels of cystatin C (r=0.78, p<0.0001), fibrinogen (r=0.31, p=0.013), oxLDL (r=0.06, p=0.61), and albuminuria (r=0.33, p=0.008).

Serum oxLDL levels was significantly higher in HHcy diabetic patients than in NHcy diabetic patients and controls, and not significantly different between NHcy patients and controls (119.3±140.4 vs. 71.4±50.8 vs. 69.1±41.1 ng/mL, $p<0.05$).

Macroangiopathic complications were more frequent in HHcy patients than in NHcy patients (55.3% vs. 35.3%, $p>0.05$), whereas the prevalence of neuropathy and retinopathy were similar in the two groups (68.2% vs. 70.5%, and 83.4% vs. 88.3%, $p>0.05$). Macroangiopathic complications were significantly more frequent in macroalbuminuric than in microalbuminuric diabetic patients (65.% vs. 39%, $p<0.05$), and tHcy levels were also significantly higher in macroalbuminuric subjects (18.1±5.35 vs. 13.9±4.26 μmol/L, $p<0.01$).

Cystatin C ($r=0.78$, $p<0.0001$), albuminuria ($r=0.33$, $p=0.008$) and fibrinogen ($r=0.31$, $p=0.013$) showed a direct correlation with tHcy (*Figure 1*). Although oxLDL levels were significantly higher in HHcy patients than in NHcy patients, statistical analysis did not show a linear correlation between the two parameters ($r=0.06$, $p=0.61$) (*Figure 1*).

There was a direct correlation between oxLDL levels and albuminuria ($r=0.37$, $p=0.002$).

Discussion

Cardiovascular complications present a leading cause of mortality in patients with diabetes mellitus type 2 (14). In addition to traditional risk factors, a significant role in the development of vascular complications in these patients may be ascribed to hyperhomocysteinemia (HHcy).

In recent years HHcy has been recognized as an independent risk factor for early atherosclerotic changes both in the general population (22, 23) and in patients with type 2 DM (12, 15), in whom HHcy has been associated with renal dysfunction, i.e. development of nephropathy, whereas diabetes mellitus per se most likely is not the cause of increased plasma homocysteine levels (24). HHcy may occur also in microalbuminuric patients with mild forms of renal dysfunction (7, 17, 25).

In the present study 64 patients with type 2 DM with DN were examined. HHCy was found in 73.4% of all patients. Markers of renal function (CCr, creatinine, urea, cystatin C, uric acid) indicated a significantly higher degree of renal impairment in patients with HHCy compared to patients with NHcy (*Table I*). In addition, the degree of albuminuria was higher in HHcy patients (40.5% macroalbuminuric and 59.5% microalbuminuric) than in NHcy patients (17.6% macroalbuminuric and 82.4% microalbuminuric). Albuminuria significantly correlated with tHcy ($r=0.33$, $p=0.008$), and so did cystatin C ($r=0.78$, $p<0.0001$). Even stronger correlations between tHcy and albuminuria were found in some previous studies (7, 16). These findings corroborate the presumption that overt diabetic nephropathy is a major determinant of hyperhomocysteinemia in diabetic patients.

Experimental data, however, suggest that hyperhomocysteinemia may be involved in the development of glomerular and interstitial renal disease proportional to plasma homocysteine levels (26). A possible mechanism by which hyperhomocysteinemia causes progression of renal dysfunction is nitric oxide-dependent endothelial dysfunction via reduced activity of the enzyme involved in asymmetric dimethylarginine metabolism (26).

Furthermore, it has been shown that HHcy may contribute to the development and progression of vascular complications in type 2 DM (27). The major part of circulating homocysteine is bound to albumin, and the free fraction (the reduced form – rHcy) expresses potent vasculotoxic effects (28). The blood level of rHcy is elevated in diabetics because the process of nonenzymatic glycation decreases the amount of albumin available to homocysteine, whereby reducing the level of the bound fraction of homocysteine. Such an increase in rHcy levels could be one of the major mechanisms for the development and progression of vasculopathies in diabetes (29). In our study macroangiopathic changes were more frequent in HHcy patients than in NHcy patients (55.3% vs. 35.3%, $p>0.05$). In a study by Buysschert et al. (11) macroangiopathic complications were more frequent in HHcy patients with type 2 DM than in NHcy patients (70% vs. 42%, $p<0.01$). Okumura et al. (7) found significantly higher tHcy levels in patients with type 2 DM and macroangiopathy compared to patients without macroangiopathy (10.4±3.7 vs. 8.5±2.8 μmol/L). Moreover, it has been shown that the risk of coronary events increases by 28% for every 5 μmol/L rise of plasma tHcy (30), and that HHcy is a significant risk factor for the development of cardiovascular disease in patients with type 2 DM (2, 31, 32). However, in spite of the substantial findings indicating a causal relationship between HHcy and vascular impairment, especially in diabetic patients, it has been suggested that HHcy is a marker, rather than a cause, of early atherosclerotic changes, as some studies failed to demonstrate a relationship between homocysteinemia and carotid intima-media thickness in diabetic patients (33) or prove beneficial effects of tHcy lowering therapy on the cardiovascular risk (34).

In the present study macroangiopathic complications were more frequent in macroalbuminuric than in microalbuminuric diabetic patients (65.2% vs. 39%, $p<0.05$), regardless of the degree of homocysteinemia, and homocysteine levels were significantly higher in macroalbuminuric patients (18.1±5.35 vs. 13.88±4.26 μmol/L, $p<0.01$). Okumura et al. also found an increased prevalence of macroangiopathic complications as well as higher tHcy levels in diabet-

ic patients with macroalbuminuria compared to diabetic patients with micro- and normoalbuminuria (44% vs. 25% vs. 20%, $p>0.05$; and 11.5±3.36 vs. 8.93±2.64 vs. 7.93±2.66 μmol/L, $p<0.05$, respectively) (7). In addition, the HOPE study (35) data show that the presence of microalbuminuria is an independent predictor of cardiovascular disease. Pathological albuminuria accompanied with HHcy may therefore be a significant predictor of future vascular events in diabetic patients.

Another subject of the present study was the relationship between homocysteinemia and the inflammation markers, CRP and fibrinogen, in patients with DN. CRP levels were significantly higher only in HHcy diabetic patients as compared to control subjects (median: 3.1 vs. 1.15 mg/L, $p<0.05$) and not significantly different between NHcy and HHcy diabetic patients (median: 3.1 vs. 1.8 mg/L, $p<0.05$). Previous studies have indicated a direct correlation between homocysteine and CRP in patients with type 2 DM (36, 37). Holven et al. (38) found that serum CRP and IL-6 levels were significantly higher in nondiabetics with HHcy compared to healthy controls. The findings of the present and previous studies show that although in patients with type 2 DM with DN the inflammation may be caused by the main disease, HHcy may play a significant contributory role in increasing the inflammatory response in these patients. It is worth noting that CRP is not a marker of inflammation only, but it may contribute in various ways to the development of endothelial dysfunction.

Although some previous studies failed to demonstrate an association between homocysteinemia and fibrinogenemia (39, 40), in the present work their correlation was significant ($r=0.31$, $p=0.013$), as it was in the studies by De Luis et al. (16) ($r=0.3$, $p<0.05$) and Kuch et al. (41) ($r=0.34$, $p<0.01$). In line with the results obtained by De Luis et al., (16) showing significantly higher plasma fibrinogen levels in HHcy than in NHcy patients (4.08±1.06 vs. 3.58±0.69 g/L, $p<0.05$), our findings were similar (4.3±1.3 vs. 3.7±0.8 g/L, $p<0.05$). Fibrinogen is an independent risk factor for cardiovascular disease (42), and the association between homocysteinemia and fibrinogenemia in our study concurs with previous findings indicating activity of the coagulation system in the setting of HHcy (43–45).

In accord with most previous findings (11, 16), we did not detect an association between HHcy and lipid status parameters in diabetic patients, whereas the values of AIP, an indirect marker of the level of small, dense LDL (sdLDL) particles (46), were significantly higher in both groups of diabetic patients compared to controls.

In diabetes mellitus the production of reactive oxygen radicals is increased, which has been confirmed by a large number of clinical trials demonstrating a rise of plasma concentrations of certain markers of oxidative stress in DM (47–51). One of the more prominent markers of oxidative stress in DM is oxidised LDL (oxLDL). Increased production of free oxygen radicals, higher oxLDL levels, along with stimulation of oxLDL uptake by macrophages and the ensuing promotion of early atherosclerotic changes might be contributed by rHcy.

In the present study, serum oxLDL levels were significantly higher in HHcy diabetic patients than in either NHcy diabetic patients or controls. Koubaa et al. (6) also found higher oxLDL levels in diabetic patients with higher levels of Hcy, which supports the fact that HHcy may contribute to an increased production of oxLDL. Hence, along with hyperglycemia, dyslipidemia and decreased activity of antioxidative enzymes, HHcy also may be one of the major factors contributing to lipid oxidation in diabetes.

In addition, we found a positive correlation ($r=0.37$, $p=0.002$) between oxLDL levels and albuminuria. In the study by Koubaa et al. (6) oxLDL levels were significantly increased in diabetic patients with a preserved renal functional reserve compared to healthy controls (142.3±49.8 vs. 95.3±37.8 ng/mL, $p<0.001$). Ujihara et al. (52) reported significantly higher serum oxLDL levels only in diabetics with macroalbuminuria, compared to normoalbuminuric and microalbuminuric patients and healthy controls (16.8±7.5 vs. 13.7±3.9 vs. 12.8±3.9 vs. 12.5±4.2 U/mL, respectively). These findings may suggest an association between oxLDL and progression of renal dysfunction in diabetic patients, which may also be corroborated by the fact that oxLDL stimulates collagen IV production in mesangial cells (53).

In summary, HHcy is common in patients with DN, especially in the setting of a more severe reduction of the renal functional reserve. Chronic macrovascular diabetic complications are more frequent in HHcy patients with DN, and particularly in those with macroalbuminuria. Compared to NHcy patients, HHcy patients with DN have significantly higher blood levels of biomarkers of inflammation (fibrinogen) and oxidative stress (oxLDL). It is therefore recommended that type 2 DM patients with DN should be regularly monitored for increases in blood levels of homocysteine as well as inflammation and oxidative stress biomarkers.

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Conflict of Interest Statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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