Turkish Journal of Endocrinology and Metabolism, (2003) 1 : 11-17 **ORGINAL ARTICLE**

Homocysteine Concentrations in Type 2 Diabetes Mellitus Patients Without Cardiovascular Disease: Relationship to Metabolic Parameters and Diabetic Complications

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The increased coronary heart disease (CAD) risk in subjects with type 2 diabetes was partly explained by an association with established risk factors like hypertension, hyperlipidemia and obesity. An increased plasma homocysteine level was accepted as an important risk factor for vascular disease, including coronary atherosclerosis. However, there was no data about the importance of homocysteine levels in type 2 diabetic patients without CAD. The aim of this study was, to determine the association between plasma homocysteine concentrations and metabolic parameters and diabetic complications in type 2 diabetic patients without CHD.

Thirty eight (23 women, 15 men) type 2 diabetic patients without CHD were included to the study. In patient group, routine biochemical and hematological tests, thyroid function tests, HgbA1c, microalbuminuria, vit B12, folic acid and homocysteine levels were assessed. Patients were evaluated for diabetic complications. Age and sex matched, 25 (15 women, 10 men) healthy control subjects were included to study, in order to compare the homocysteine levels with patient group.

There was no statistically significant difference in plasma homocysteine concentrations between patient and control group. However, there were more hyperhomocysteinemic patient in diabetic group than control subjects (10.5% vs 4%). There was no association between plasma homocysteine levels and age, duration of diabetes, fasting plasma glucose, post-prandial plasma glucose, urea, total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol and HgbA1c levels. There was an association between homocysteine and serum creatinine and also microalbuminuria levels ($p<0,05$). Homocysteine concentrations were not associated with diabetic retinopathy and neuropathy. A relation between homocystein levels and hypertension was observed. As a result, type 2 diabetes mellitus does not alter the homocysteine concentrations but with the occurance of complications like hypertension and microalbuminuria, concentrations tend to increase.

Key words: Type 2 diabetes mellitus, homocysteine, coronary heart disease

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Introduction

Cardiovascular disease, being 2-4 fold more prevalent in Type 2 Diabetes Mellitus, is still keeping its importance as the leading cause of death in this patient group. As obesity, hypertension and dyslipidemia, which are known to be

frequently associated with Type 2 Diabetes Mellitus, are insufficient in explaining this increase in risk; researchers are focusing on investigating new risk factors. In recent years homocysteine, which is an amino-acid containing thiol, has been described as an independent risk factor for cardiovascular disease. The fact that hyperhomocysteinemia can be prevented by various vitamin therapies has attracted researchers' interest.

Studies have been shown that plasma homocystein levels are elevated in diabetic as well as non-diabetic patients with cardiovascular disease. It has also been proven that homocysteine metabolism is affected by different factors directly related to diabetes such as hyperinsulinemia, renal damage, plasma vitamin levels and the use of some oral anti-diabetic agents. However, study results about the relationship of homocysteine levels with micro and macro vascular complications are contradictory.

The aim of this study was to investigate the relationship of diabetes, diabetic complications and factors known to be affecting homocysteine metabolism with homocysteine levels in type 2 diabetic patients without cardiovascular disease. For this purpose, homocysteine levels of type 2 diabetes mellitus patients without cardiovascular disease have been compared with healthy controls. Furthermore, the relationship of homocysteine levels with various factors like age, diabetes duration, body mass index (BMI), diabetic control, lipid profile, creatinine, HbA_1c, folic acid, vitamin B_{12} levels, microalbuminuria, neuropathy and retinopathy were evaluated.

Materials And Methods

After approval of the study protocol by the local ethical committee, the study was conducted at Kocaeli University Medical School Division of Endocrinology and Metabolism. Inclusion criterion was diagnosis of type 2 Diabetes Mellitus according to "American Diabetes Association (ADA)" criteria for at least 2 years. Exclusion criterias were: Previously recorded myocardial infarction, angina pectoris according to Rose questionnaire (1), stroke, coronary artery by-pass surgery, percutanous transluminal coronary angioplasty, uncontrollable hypertension (blood pressure ≥ 180/100 mmHg), medications like digitalis or drugs affecting homocysteine metabolism (including vitamins), peripheral vascular disease, smoking or alcohol consumption, diagnosis of hepatic and/or thyroid disease according to physical examination and laboratory findings, arrhythmic or ischemic changes in resting electrocardiogram (ECG), serious acute or chronic illness, serum creatinine ≥ 1.4mg/dl and proteinuria.

Thirty-eight attendants of the diabetes outpatient clinic (23 females, 15 males, mean age: 51.57±8.08 years) meeting the inclusion criterion and lacking exclusion criteria were enrolled.

Age and sex matched twenty-five healthy subjects were recruited as controls. These individuals' medical histories, physical examinations, routine biochemical and hematological tests and ECG records were in normal ranges; they were not on any medication (including vitamins) and did not report any consumption of tobacco or alcohol.

Twelve derivation ECGs were recorded and patients interpreted as having a normal ECG were included in the study. Maximal treadmill exercise test according to Bruce protocol was used as screening test in order to rule out ischemic heart disease. Cardiologists in a blinded fashion performed evaluation of exercise tests and only patients with a "negative maximal effort test" were included in the study.

Fundoscopic examination after pupillary dilatation using an ophthalmoscope and/or biomicroscope was performed at the department of ophthalmology. All patients underwent neurological examination and if necessary electromyography was obtained. Venous blood samples were taken from subjects after 12 hour fasting. The routine biochemical and hematological tests were analysed at Biochemistry and Heamatology Laboratory of Kocaeli University Hospital. Liver function tests, total cholesterol and triglyceride levels were measured by using standard commercial enzymatic kits. LDL-cholesterol concentration was calculated by Friedewald's formula. Glycated hemoglobulin was measured by high-performance liquid chromotography. Microalbuminuria was measured by nephelometry (Beckman, Milan Italy). For homocysteine measurement we used plasma obtained by addition of EDTA and centrifugated immediately after collection. Plasma samples were frozen and stored at -80°C. Homocysteine levels were deter-

mined by polarization immunoassay method using Abbott IMX analyser.

Statistical analyses of the data were performed on SPSS (Statistical Package for Social Science) for Windows version 9.0. Appropriate parametric (student t test, independent samples t test), and non-parametric (chi-square, Mann-Whitney U test) statistical tests and correlation technique were used to analyze data sets.

Results

The clinical and metabolic characteristics of the patient and the control group were shown in table 1. Three of the diabetic patients (7.8%) were under dietary control, 25 patients (65.7%) were receiving oral anti-diabetic drug therapy, 8 patients (21%) were receiving insulin and 2 patients(5.2%) were receiving combined oral anti-diabetic drug and insulin therapy. Sixteen diabetic patients (42%) were receiving anti-hypertensive therapy, 12 (31.5%) were using cholesterol-lowering agents. Ten of the diabetic patients (26.3%) had established retinopathy, 9 patients (23.6%) had neuropathy. Both conditions were present in four patients. When compared with the control group, except for fasting plasma glucose levels, there was no statistically significant difference in means of the diabetic group in terms of other variables like age, BMI, total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, vitamin B_{12} and folic acid levels.

Also, healthy controls did not differ significantly from the diabetic patients in terms of homocysteine levels (9.38±2.2 μmol/L and 9.66±3.23 μmol/L, respectively). The relationship between homocysteine levels and laboratory and clinical findings in the diabetic patient population was shown in Table 2.

The diabetic group displayed no significant relationship between homocysteine levels and age, BMI, plasma glucose, urea, total cholesterol, triglyceride, HDL-cholesterol LDL-cholesterol, and HbA_1c levels. Homocysteine levels were influenced by 24h urinary albumin excretion rate (UAER) and creatinine; furthermore they displayed an inverse relationship with vit-B_{12} and folate levels, the latter being highly significant.

Four patients (10.5%) in the diabetic group had hyperhomocysteinemia, whereas this was the same in only 1 case (4%) in the control group. Hyperhomocysteinemia was more frequent in diabetic patients without cardiovascular disease compared to healthy controls. All four of the hyperhomocysteinemic patients had established microalbuminuria and hypertension (Table 3).

While there was a significant relationship between homocystein levels and hypertension according to our study result, the same could not be proven for diabetic microvascular complication like neuropathy and retinopathy (Table 4).

Table 1. The clinical and metabolic characteristics of the patient and control group.

	Patient group (n: 3 8)	Control group (n: 25)	P value
Age (years)	51.57 ± 8.08	51.76 ± 7.76	0.82
Body mass index (kg/m^2)	30.93 ± 5.7	30.08 ± 2.86	0.489
Duration of diabetes (years)	6.44 ± 4.35	_	_
Fasting plasma glucose(mg/dl)	165.3 ± 45.86	89 ± 17.6	P < 0.01
Creatinine (mg/dl)	0.95 ± 0.2	0.9 ± 0.27	0.9
Total cholesterol (mg/dl)	216 ± 48.3	198.2 ± 35.4	0.22
Triglyceride(mg/dl)	160.31 ± 79	122.8 ± 71.8	0.3
HDL-cholesterol (mg/dl)	45.5 ± 9.4	42.56 ± 7.4	0.28
LDL-cholesterol (mg/dl)	131.89 ± 39.34	124.68 ± 20.68	0.4
Homocysteine (μmol/L)	9.66 ± 3.23	9.38 ± 2.2	0.7
Vitamin B12 (pg/ml)	352.47 ± 184.11	373.28 ± 117.68	0.6
Folic acid (ng/ml)	8.95 ± 3.45	9.64 ± 2.41	0.3
Hgb A1c (%)	8.11 ± 1.4	_	_
Microalbuminuria (mgr/day)	49.07 ± 68.01	_	_

Table 2. The relationship between homocysteine levels and laboratory and clinical findings

	Relation with homocysteine (r)	P value
Age (years)	0.141	0.398
Duration of diabetes (years)	0.27	0.874
Body mass index (kg/m^2)	0.005	0.977
Fasting plasma glucose (mg/dl)	0.185	0.266
Post prandial pl. gluc.(mg/dl)	0.255	0.123
Urea (mg/dl)	0.204	0.218
Creatine (mg/dl)	0.369	0.023*
T. Cholesterol (mg/dl)	- 0.307	0.084
Triglycerid (mg/dl)	- 0.246	0.137
HDL-cholesterol (mg/dl)	- 0.104	0.533
LDL-cholesterol (mg/dl)	- 0.203	0.222
Hgb A1c (%)	0.186	0.280
Microalbuminuria (mg/day)	0.327	0.02*
Vitamin B_{12} (pg/ml)	- 0.349	0.032*
Folic acid (ng/ml)	- 0.477	0.002**

* $p < 0.05$ **: $p < 0.01$

Table 3. Clinical characteristics of patients with hyperhomocysteinemia

Case	Duration of diabetes (years)	BMI (kg/m^2)	Hb A1c (%)	Homocyst. (μmol/L)	Microalbuminuria (mg/day)	Ht	NP/RP
1	5	31	8.3	18.57	121	+	- / -
2	9	36	6.9	15.04	189.8	+	- / -
3	4	29	10.06	16.29	63	+	- / -
4	3	28	8.3	15.9	67.7	+	- / -

Table 4. The relationship between clinical findings and homocysteine levels

	Wann-Whitney U coefficient	P
Hypertension	68	0.001*
Diabetic retinopathy	100	0.768
Diabetic neuropathy	99.00	0.279

*: $p < 0.01$

When grouped according to therapy regimen, the use of insulin or oral anti-diabetic agents did not produce any significant differences concerning homocysteine levels.

The same applied for the comparison between diabetic patients taking metformine and those who did not. However, although still in the normal range, vitamin B_{12} levels were lower in patients receiving metformine compared with those who did not, but the difference in means was not significant.

Discussion

Cardiovascular disease is 2-4 times more prevalent in type 2 diabetics when compared with healthy individuals. Researches have claimed that this remarkable increase in cardiovascular risk of diabetic patients cannot sufficiently be explained with classical risk factor theories. Therefore intense efforts have been directed towards investigating the possible role of new risk factors.

Mc Cully was the first to draw attention to the relationship between homocysteine levels and cardiovascular disease in 1967. Ever since, this topic has been subject to many studies. Presently plasma homocysteine levels are widely accepted as an independent risk factor for cardiovascular disease. Results of studies investigating the relationship between plasma homocysteine levels and cardiovascular disease in type 2 diabetic patients are controversial. While some studies report slightly elevated homocysteine level as a car-

diovascular risk factor in diabetic patients (2,3), some other studies particularly the ones conducted in type 1 diabetic patients, have revealed low or normal homocysteine levels, which led to the conclusion that elevated homocysteine levels have no influence whatsoever on the frequency of cardiovascular disease in diabetic patients (3-5). Hoogeven et al. (3) have shown that, when compared to non-diabetics, hyperhomocysteinemia in type 2 diabetic patients is a 1.6 fold more powerful risk factor in terms of cardiovascular disease. Buysschaert et al (6) have shown that patients with established macroangiopathy had higher homocysteine levels, which led to the conclusion that, also in type 2 diabetic patients, homocysteine levels are an independent risk factor for cardiovascular disease.

Endothelial cells, once having been exposed to advanced glycosylated end products (AGE), start to increase thrombomodulin secretion when they get in contact with homocysteine. This mechanism is used to explain the theory of how homocysteine induces thrombosis by damaging endothelial cells with prior AGE exposure (7). This hypothesis might be an explanation for the study results claiming that hyperhomocysteinemia is a more potent cardiovascular disease risk factor in diabetic patients than it is in normal population.

Some studies suggest that, particularly in type 2 diabetes mellitus, hyperhomocysteinemia is an independent risk factor for cardiovascular disease. However, these studies mostly were featuring diabetic patients who had already established cardiovascular disease, where it is not possible anymore to relate cardiovascular disease solely to the presence of diabetes mellitus. It is for this reason that, in this study, we have chosen to assess plasma homocysteine levels and the relationship between homocysteine and various metabolic variables in a patient population that is devoid of factors influencing homocysteine metabolism, like ischemic heart disease, smoking, overt nephropathy and vitamin use.

Although plasma homocysteine levels in this patient population were found to be slightly elevated compared to healthy controls, the difference was not significant. Hoogeven et al (3) could not detect any difference in plasma homocysteine levels between type 2 diabetic patients without cardiovascular disease and healthy controls. Accordingly the studies of Stabler and Munshi (5,8) did not reveal any significant differences in homocysteine levels between type 2 diabetic patients without cardiovascular disease and their healthy controls. A weak point of Munshi's study is that the absence of vascular disease is only determined by history. Recent studies have suggested that elevated homocysteine levels are not the cause but the consequence of macroangiopathy (9,10). It is remarkable, that all hyperhomocysteinemic patients in our study group had established microalbuminuria and hypertension. It is questioned whether in these patients, hyperhomocysteinemia, as an independent risk factor led to the development of complications, or whether prior established macroangiopathy and renal disease, which had not been diagnosed clinically, have led to elevation in homocysteine levels. Unfortunately it is not possible to give a clear answer to this question with our findings as yet. Further prospective designed long term outcome studies will help to elucidate the issue whether high homocysteine levels in diabetic patients are a primary independent risk factor or an early finding of arteriosclerosis. In our study, this particular group of type 2 diabetic patients, homocysteine levels displayed no significant relationship with age, duration of diabetes, BMI, fasting plasma glucose, urea, cholesterol profile and HbA_1c. This finding is in concordance with the results of Munshi et al (8), who also found no relationship between elevated homocysteine levels and age, duration of diabetes cholesterolemia and HbA_1c in their diabetic patients without macroangiopathy. Several other studies' findings are supporting our results (11,12). Our findings enforce the thesis that homocysteine levels and lipid disorders are two independent risk factors, accordingly, there is evidence that the coexistence of these two risk factors has a synergistic effect on risk increase (13). Renal insufficiency is known to cause hyperhomocysteinemia. Many researchers have found a direct relationship between homocysteine levels and microalbuminuria and plasma creatinine levels (5,14,15). Especially in type 2 diabetes mellitus, nephropathy is the most important reason for fasting hyperhomocysteinemia (2). In our patient group, there was a significant relationship between microalbuminuria, plasma creatinine and homocysteine levels. Most interestingly,

in our study, all 4 patients with hyperhomocysteinemia (>15 μmol/L) had established microalbuminuria and hypertension.

The relationship between hypertension and homocysteinemia has been shown in many studies in diabetic as well as non-diabetic patients (17-19). The mechanism responsible for this relation is said to be a damage caused to elastin fibers in the arterial wall due to hyperhomocysteinemia, leading to an increase in arterial stiffness (18). In our study there was also a significant relationship between hypertension and homocysteine levels, with all four hyperhomocysteinemic patients also being hypertensive.

The study results, like many others, revealed no significant relationship between homocysteine levels and diabetic retinopathy and neuropathy (6, 20). Interestingly Stabler et al. have found elevated homocysteine levels in two type 2 diabetic patients with neuropathy (5).

The findings of our study suggest that neither use of insulin nor oral anti diabetic agents has an influence on homocysteine levels of type 2 diabetic patients. There are publications claiming that high doses of metformin, due to accumulation in the gastrointestinal system wall, inhibit vitamin B_{12} absorption (21,22). Although still within normal range, the metformin recipients in our study population (n=16) showed decreasing vitamin B_{12} levels, but their homocystein levels were no different than the ones of patients who did not take metformin. Buysschoert et al. have obtained similar results in their study (6). It is a well known fact that folic acid, vitamin B_6 and vitamin B_{12} play an important role in homocysteine metabolism. Plasma folic acid concentrations are the most important determinant of fasting homocysteine levels (23). Accordingly, our patient data showed a highly significant ($p< 0.01$) relationship between plasma folic acid and homocysteine levels. Although not as significant as with folic acid, the same applied for vitamin B_{12} levels ($p<0.05$). There was an inverse relationship between plasma homocysteine and plasma folat and vitamin B_{12} levels, but plasma values of vitamins were all within normal range. It is still argued why plasma folat levels are low in diabetic patients with hyperhomocysteinemia. The consumption of alcohol and caffeine can affect plasma folat levels. But our study as well as control group were devoid of such an exposure. Both of the groups consisted of non-smokers. Numerous studies have shown that dietary supplementation of folic acid and/or vitamin B_{12} can be of benefit especially to hyperhomocysteinemic diabetic patients with marco-angiopathy (24,25).

We have come to the conclusion that type 2 diabetes mellitus and microalbuminuria increase the cardio-vascular risk significantly so in these patients, plasma homocysteine levels must be measured and if hyperhomocysteinemia was established it must be treated appropiately in order to decrease the risk. Further studies with greater sample sizes and longer duration are needed to elucidate this issue in the future.

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