Homocysteine as a non-classical risk factor for atherosclerosis in relation to pharmacotherapy of type 2 diabetes mellitus

Magdalena Borowska¹², Marzena Dworacka¹, Hanna Winiarska¹ and Ewa Krzyżagórka²

¹Department of Pharmacology, University of Medical Sciences, Poznań, Poland; ²Poznan Specialist Center of Medical Care, Diabetology Outpatient Clinic, Poznań, Poland

INTRODUCTION

Numerous recent studies have clearly indicated that homocysteine can promote inflammation and atherosclerosis and thus increases the risk of cardiovascular disease and death. This relation seems to be stronger in subjects with diabetes, as compared to the general population (Becker et al., 2003; Guthikonda et al., 2006). Therefore, modern therapy regimens of diabetes should be effective both in optimizing glucose control and in the prevention of vascular complications through the modification of atherosclerosis risk factors including elevated homocysteine level. Serum homocysteine concentrations in healthy subjects are within the range of 5–15 μmol/L, a concentration exceeding the threshold for hyperhomocysteinemia may have an adverse effect on vascular endothelium (Baszczuk et al., 2014). Homocysteine-induced endothelium injury is due to the increased proliferation of vascular smooth muscle cells, as well as the increase in collagen synthesis and its deposition in the vascular wall (Pushpakumar et al., 2014). The vascular toxicity of homocysteine has been linked to the impaired endothelial production of nitric oxide, and the overproduction of oxidative radicals which induces intimal injury (Signorello et al., 2009). In addition, homocysteine occurring as a thiolactone, a highly reactive product of homocysteine oxidation, modulates gene expression in pathways important for vascular homeostasis and pathways linked to HHcy-induced endothelial dysfunction and the vascular disease (Gurda et al., 2015). Homocysteine auto-oxidation has been shown to generate the superoxide anion radicals that support the oxidation of low-density lipoproteins (Seo et al., 2010). In view of this pro-atherogenic effect, hyperhomocysteinemia is considered a very important modifiable risk factor for macroangiopathy (Becker et al., 2003).

Although the results of recent intervention studies have not proved any specific therapy to be advantageous or disadvantageous in terms of the impact on the incidence of major cardiovascular complications (U.K. Prospective Diabetes Study 1998, ADVANCED Trial 2008, Duckworth et al., 2009), it is known that metformin and insulin may secondarily influence the total serum homocysteine level in type 2 diabetes mellitus (Wile et al., 2010; Gallistl et al., 2000). The link between hyperhomocysteinemia and sulfonylureas therapy remains unknown. Therefore, the aim of this study was to compare the effect of the standard used anti-diabetic drugs, such as metformin, insulin in combination with metformin, sulfonylureas and conventional insulin therapy, on the cardiovascular risk factor such as hyperhomocysteinemia.

MATERIALS AND METHODS

Subjects. The study involved 182 people with type 2 diabetes treated for at least 6 months with one of the following treatment regimens: 89 patients treated with metformin (at a dose of 500–2550 mg daily), 31 patients treated with insulin in combination with metformin (35–60 units insulin a day + metformin at a dose of 500–2550 mg daily), 31 patients treated with one of sulfonylureas such as gliclazide (at a dose of 30 mg daily) or glimepiride (at a dose of 2–6 mg daily), 31 patients treated conventionally with insulin (a ready to use mixture of sulfonylureas such as glimepiride (at a dose of 2–6 mg daily), 31 patients treated conventionally with insulin (a ready to use mixture of sulfonylureas such as gliclazide (at a dose of 30 mg daily). Each patient included in this study was also treated with statin (atorvastatin at a dose...
of 20 mg daily or simvastatin 10-40 mg daily) and ace-
tylosalicylic acid at antiplatelet doses, an angiotensin-con-
verting enzyme inhibitor or sartan and with a selective
beta-blocker. All subjects were Caucasian. The partici-
pants were identified as the outpatients of general prac-
titioners in Poznan (Poland). The clinical and metabolic
characteristics of all subjects are shown in Table 1. Be-
fore the participants were eligible for the study, a com-
plete physical and laboratory evaluation was performed.
Type 2 diabetes was diagnosed according to the ADA
and ESC/EASD criteria (American Diabetes Associa-
tion 2007; European Society of Cardiology and of the
European Association for the Study of Diabetes 2007).
Patients with a history of infections, severe inflammatory
diseases, renal failure, liver failure, heart failure, thyroid
dysfunction, anaemia and patients receiving folic acid, vi-
tamin B12 and vitamin B6 were excluded from this study.

Measurements. All blood samples for the measure-
ment of various general and metabolic factors were ob-
tained after 10 hours of overnight fasting and before ad-
ministration of the morning dose(s) of medication.
The biological materials analyzed were whole blood, plasma
and serum.

Blood samples were collected from each patient and the
fasting glucose levels in plasma, lipids in the serum and
glycosylated hemoglobin (HbA1c) in whole blood were
determined immediately after collection. Serum samples
for homocysteine, resistin, insulin, C-peptide and 1,5-AG
levels were collected and stored at −80°C
until assay.

The total homocysteine levels in the serum were as-
sayed using commercial kits (Axis® Human Homo-
cysteine ELISA Kit, IBL). The reference range was
5–15 μmol/L.

The plasma concentrations of 1,5-Anhydro-D-glucitol
(1,5-AG) were measured using a modified-column en-
zymatic method (Yabuuchi et al., 1984). The reference
range was 14.4–30.2 mg/l (Dworacka et al., 2005).

The fasting serum insulin levels and concentrations of
C-peptide were determined with commercially available
immunoenzymatic ELISA kits.

Statistical analysis. Results are expressed as median
and mean ± S.E.M. A comparison of variables between
the examined groups was performed using ANOVA
(parametric distribution) or with the Kruskal-Wallis test
(non-parametric distribution). To exclude the influence
of selected factors on the differences between the ex-
amined groups, a multivariate analysis of covariance was
used (ANCOVA). In this analysis, serum homocysteine
concentration was the dependent variable, while the di-
abetes duration, waist circumference, HbA1c, 1,5-AG,
fasting glycaemia and C-peptide were used as covariates.
The relationships between variables were analyzed by cal-
culating Spearman’s p statistic. All statistical analyses
were performed using Statistica 6.0 (StatSoft, Inc.). A p-
value ≤0.05 was considered to be statistically significant.

RESULTS

Table 1 shows the characteristics of the subjects with
type 2 diabetes. All patients were similar in terms of age
and lipid profiles. Patients treated with metformin differed
in the duration of diabetes and had a lower HbA1c than
other therapeutic groups. Fasting glycaemia was significantly

Table 1. The clinical characteristics of patients with type 2 diabetes varying in the type of pharmacotherapy (Kruskal-Wallis)

<table>
<thead>
<tr>
<th></th>
<th>Metformin Median/mean ±S.D.</th>
<th>Insulin + metformin Median/mean ±S.D.</th>
<th>Sulfonylurea Median/mean ±S.D.</th>
<th>Insulin Median/mean ±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F/M</td>
<td>47F/42M</td>
<td>13F/18M</td>
<td>14F/17M</td>
<td>15F/16M</td>
</tr>
<tr>
<td>Age [years]</td>
<td>62/61.2±8.9</td>
<td>60/61.4±8.8</td>
<td>68/67.1±12.1</td>
<td>63/65.0±10.0</td>
</tr>
<tr>
<td>Diabetes duration [years]</td>
<td>4/5.0±5.0</td>
<td>9/10.8±8.7</td>
<td>7/9.7±7.5</td>
<td>9/11.2±7.9</td>
</tr>
<tr>
<td>BMI [kg/m²]</td>
<td>30.6/31.3±5.3</td>
<td>32.8/34.9±6.9</td>
<td>30.5/31.8±7.6</td>
<td>32/35.4±11.1</td>
</tr>
<tr>
<td>Waist circumference [cm]</td>
<td>103/103.7±10.8</td>
<td>109/110.1±9.2</td>
<td>118/116.9±12.4</td>
<td>117/115.0±13.9</td>
</tr>
<tr>
<td>Fasting glycaemia (mg/dl)</td>
<td>126.0/129.6±28.3</td>
<td>137.0/148.4±45.5</td>
<td>148.0/160.1±48.5</td>
<td>157.5/176.3±60.3</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>201.0/201.7±46.3</td>
<td>188.0/190.0±47.6</td>
<td>226.0/217.8±53.2</td>
<td>196.8/200.4±53.0</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dl)</td>
<td>119.0/118.8±37.3</td>
<td>110.0/110.6±39.6</td>
<td>120.0/119.6±39.6</td>
<td>104.3/105.6±42.3</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dl)</td>
<td>47.0/50.2±14.4</td>
<td>44.0/44.3±9.6</td>
<td>54.0/53.4±13.5</td>
<td>42.0/47.4±14.7</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>150.0/173.9±94.5</td>
<td>164.7/190.2±90.1</td>
<td>147.0/206.3±154.7</td>
<td>162.0/188.5±137.7</td>
</tr>
<tr>
<td>HbA1c (%) [mmol/mol]</td>
<td>6.6/6.7±1.1</td>
<td>7.5/7.7±1.4</td>
<td>7.3/7.6±1.8</td>
<td>8.0/8.3±1.2</td>
</tr>
<tr>
<td>1,5-AG (mg/L)</td>
<td>16.4/15.6±5.9</td>
<td>12.4/12.5±6.1</td>
<td>13.5/14.3±6.7</td>
<td>8.5/9.4±4.2</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.1/2.9±2.4</td>
<td>2.7/3.1±2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>39.5/53.0±38.2</td>
<td>42.5/47.6±33.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-peptide (ng/ml)</td>
<td>2.6/2.8±1.3</td>
<td>2.0/2.2±1.5</td>
<td>1.87/2.2±1.8</td>
<td>1.2/ 1.4±0.9</td>
</tr>
</tbody>
</table>

M, males; F, females. *statistically significant difference compared to every other group for p≤0.05. **statistically significant difference compared to the sulfonylurea treated group for p≤0.05. ***statistically significant difference compared to the insulin treated group for p≤0.05.
lower in the metformin-treated group than in patients receiving sulfonylurea or the patients treated with insulin in monotherapy. The plasma 1,5-AG concentrations were significantly higher in the subjects treated with metformin and in those treated with sulfonylurea than in those treated with insulin in monotherapy, whereas the fasting serum C-peptide levels were higher than in the insulin-treated group only in the patients receiving metformin.

When analyzing the differences in the homocysteine serum level among the therapeutic groups of diabetic patients, it was found that the concentrations of the homocysteine level in patients treated with insulin in monotherapy were significantly higher than those observed for every other therapeutic group ($p \leq 0.05$), which did not vary among themselves in this respect (Table 2).

The analysis of covariance confirmed the inter-group differences in the homocysteine concentrations ($p \leq 0.05$) (Fig. 1) and revealed that these differences were affected not only by the method of treatment [F=14.5, $p \leq 0.001$] but also by the waist circumference [F=5.1, $p=0.03$] and the C-peptide levels [F=18.4, $p=0.01$].

The most significant positive correlation was observed between the serum concentrations of homocysteine and waist circumference [r=0.30; $p \leq 0.05$].

**DISCUSSION**

The results of many studies clearly point to the participation of hyperhomocysteinemia in the progression of atherosclerotic changes in type 2 diabetes patients. Previous reports on the effects of anti-diabetic drugs on homocysteine levels refer only to metformin and insulin, analyzing these therapeutic groups separately (Wile et al., 2010; Jager et al., 2010; Mohammadi et al., 2009; Björck et al., 2006; Gallistl et al., 2000). Therefore, the aim of our study was to carry out a comparative analysis of different therapeutic groups in terms of homocysteine concentrations in the serum. This study showed that the use of conventional insulin therapy was related to higher homocysteine levels in the treated patients than in patients receiving oral antidiabetic drugs. At first glance, these results are surprising in the light of current knowledge, especially as far as metformin is concerned. It has been confirmed that metformin has the ability to induce high levels of homocysteine and may be a causative factor for hyperhomocysteinemia (Refsum et al., 2004; Sato et al., 2013). The relationship between hyperhomocysteinemia and metformin has been documented in numerous studies, but the strength of this relation was different in different experiments (Refsum et al., 2004; Sato et al., 2013; Dierkes et al., 2005; Valdés-Ramos et al., 2015). It is known that metformin promotes hyperhomocysteinemia primarily by inhibiting the absorption of vitamin B12 in the intestines (Valdés-Ramos et al., 2015). Unexpectedly low levels of homocysteine in the examined group of patients receiving metformin could result from the fact that the resources of endogenous vitamin B$_{12}$ were sufficient to ensure the proper conduct of the remethylation of homocysteine to methionine, despite the negative impact of metformin on the absorption of vitamin B$_{12}$. The patients in the treatment group received metformin according to the research protocol for at least six months prior to testing, but for most of them, the total duration of treatment with metformin was not longer than six months and perhaps it was too short a period for the depletion of endogenous vitamin B$_{12}$. Similar conditions accompanied the use of metformin in patients treated with insulin in combination with metformin. The development of vitamin B$_{12}$ deficiency over a relatively short period of time is highly unlikely, and perhaps, to some extent, it explains the lack of adverse effects of metformin in the study presented here. At the same time, it would be wrong to state that metformin has a particularly positive effect on the levels of homocysteine in the blood.

The explanation as to why, among the compared methods of therapy, a conventional insulin therapy has resulted in the least beneficial serum homocysteine levels and, what is more, why only this type of pharmacotherapy is related to the occurrence of hyperhomocysteinemia, should be sought in the type of metabolic transformations in which homocysteine and insulin take part. Results that are similar to the ones presented here were previously presented by Jacobs who tested animal diabetes and has shown that the application of human ultralente insulin analogue leads to an increase in the blood ho-

<table>
<thead>
<tr>
<th>F/M</th>
<th>Homocysteine [μmol/L]</th>
<th>Median/mean ± S.D.</th>
<th>Median/mean ± S.D.</th>
<th>Median/mean ± S.D.</th>
<th>Median/mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>47/42</td>
<td>10.9/12.8±7.4</td>
<td>14.5/13.8±6.9</td>
<td>14.7/14.8±6.9</td>
<td>21.4/21.1±9.4*</td>
</tr>
<tr>
<td>Insulin</td>
<td>13/18</td>
<td>14.7/14.8±6.9</td>
<td>2013; Dierkes</td>
<td>2004; Sato</td>
<td>2005; Valdés-Ramos</td>
</tr>
<tr>
<td>Sulfonylurea</td>
<td>14/17</td>
<td>10.9/12.8±7.4</td>
<td>14.5/13.8±6.9</td>
<td>14.7/14.8±6.9</td>
<td>21.4/21.1±9.4*</td>
</tr>
<tr>
<td>Insulin</td>
<td>15/16</td>
<td>14.7/14.8±6.9</td>
<td>2013; Dierkes</td>
<td>2004; Sato</td>
<td>2005; Valdés-Ramos</td>
</tr>
</tbody>
</table>

F, females; M, males. *statistically significant difference compared to every other group for $p \leq 0.05$.
mocysteine level (Jacobs et al., 1998). The author claims that the reason for such change was the influence of insulin on the decrease of cystathionin-beta synthase activity which is responsible for the transsulfuration process in the liver which eventually leads to an increase in the homocysteine level. However, Ratman and coworkers (2002) had demonstrated that, in the course of diabetes, homocysteine catabolism in the liver is more intensified and its decreased level is therefore obvious. It is only exposure to exogenous insulin that reverses this phenomenon, i.e. stops homocysteine catabolic transformations resulting in a decrease in catabolic enzymes’ activity, favoring at the same time an increase in the amount of homocysteine and its blood level. Hence, it should be concluded that the modeling of insulin-dependent Hey levels is a function of insulin concentration in the blood. This fact may explain the lack of influence of sulfonlurea derivatives on the homocysteine concentrations in our study. Although the drugs in this group exacerbate insulin secretion by beta cells, it can be expected that their effect should include inhibiting homocysteine catabolism and increasing its blood concentration. However, it seems that insulinemia thus obtained is insufficient to induce changes in the expression of cystathionin-beta synthase and consequently to make changes in the concentrations of the Hey. The results of the covariance analysis in our study indicate that along with pharmacotherapy, it is also waist circumference and C-peptide levels that determine the differences in homocysteine concentrations between the treatment groups. Similar results were obtained by Vavry and coworkers (2012) in a study where increased Hey levels were associated mostly with the abdominal obesity and with insulin resistance. The mechanism explaining the link between hyperhomocysteinemia and obesity is not clear, but there is evidence that obesity may be accompanied by a deficiency in folate that is the source of methyl group necessary for the remethylation of homocysteine to methionine (Nakazato et al., 2011; Mojtabai 2004). In this way, folate deficiency in obesity contribute to the inhibition of homocysteine catabolism leading to an increase in its concentration. In turn, other interesting scientific studies underline the genetic polymorphism of MTHFR as a predisposing factor for hyperhomocysteinemia and obesity (Bazazz et al., 2010). The Chakraborty study (2015) highlighted the relationship between the polymorphism of the MTHFR gene and homocysteine homogeneity in patients with type 2 diabetes and demonstrated that the increase in homocysteine level was determined by an individual genotype. The genetic polymorphism of MTHFR may also be a factor responsible for the occurrence of serious vascular complications in type 2 diabetes (Fekih-Missa et al., 2017; Chehadeh et al., 2015). Although the genetic aspect of hyperhomocysteinemia was not the subject of our study, the possible occurrence of MTHFR genetic polymorphism as a factor modulating homocysteine levels should be taken into consideration.

In conclusion, we observed that among the studied therapeutic regimens only conventional insulin therapy was the unfavorable treatment method in terms of its impact on the homocysteine levels. Nevertheless, the homocysteine concentrations may also be affected by other factors like β cell function and the presence of abdominal obesity. This is why we can speculate that the level of homocysteine needs to be analyzed as a final result of the influence of several factors, including pharmacotherapy, inflammatory process, anthropometric alterations and genetic factors.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Ethical standard**

The Ethics Committee of the University of Medical Sciences approved the protocol of this study.

**Acknowledgements**

This study was supported by grant No 402357638 from the Polish Ministry of Science and Higher Education, Warsaw, Poland.

**REFERENCES**


