The aspirin resistance and platelets' receptors expression in chronic heart failure patients with type 2 diabetes mellitus

Zaporizhzhia State Medical University

Key words: Aspirin, Drug Resistance, Chronic Disease, Heart Failure, Platelet Activation, Type 2 Diabetes Mellitus.

Aim. Chronic heart failure and type 2 diabetes mellitus are closely inter-related conditions through different pathological mechanisms, including platelet activation. The aim of our study was to estimate the prevalence of aspirin resistance in heart failure patients with type 2 diabetes mellitus and to clarify platelets’ abnormalities according to glycemic control status.

Methods and results. We studied 65 patients with heart failure and type 2 diabetes mellitus. Platelet function was evaluated using standard light transmission aggregometry. Measurements of CD41, CD41a, CD61 and CD62P were carried out by fluorescent microscopy study. The plasma levels of soluble P-selectin and soluble CD40L were measured by ELISA assays. Among the studied patients, 23.1 % were aspirin-resistant by ADP-induced platelet aggregation test and 36.1 % after the use of epinephrine-induced platelet aggregation test. The poor glycemic control in patients with chronic heart failure was associated with high plasma P-selectin level and high CD41 and CD61 expression.

Conclusion. The study findings confirm the role of glycemic control status in prevention of thrombotic complications in chronic heart failure patients with type 2 diabetes mellitus.

It was shown, that platelet reactivity is high in CHF patients with sinus rhythm despite aspirin use [4,5]. The prognostic role of platelets’ disorders in CHF remains unclear [6]. The presence of DM in CHF patients can dramatically increase platelet dysfunction. The problem is much complicated by high level of aspirin resistance in diabetic patients [7]. The prevalence of aspirin resistance in CHF with DM has never been studied. Poor glycemic control is thought to be another important cause of increased platelet reactivity [8]. Hyperglycaemia in diabetes can cause glycation of platelets and fibrinogen [9].
The aim of this study was to estimate the prevalence of aspirin resistance in CHF patients with DM and to clarify platelet function abnormalities according to glycemic control status.

**Materials and methods**

**Study population.** The inclusion criteria for study were: a more than 1-year documented history of CHF due to coronary artery disease, sinus rhythm, type 2 diabetes mellitus, no change in medication for > 3 months, daily aspirin use 100 mg > 6 months.

All patients had previous history of myocardial infarction. Exclusion criteria were: type 1 DM, patients with type 2 DM on insulin therapy and/or thiazolidinediones, major bleedings in inclusion criteria were: type 1 DM, patients with type 2 DM on insulin therapy and/or thiazolidinediones, major bleedings in exclusion criteria were: type 1 DM, patients with type 2 DM on insulin therapy and/or thiazolidinediones, major bleedings in

All patients had previous history of myocardial infarction. Exclusion criteria were: type 1 DM, patients with type 2 DM on insulin therapy and/or thiazolidinediones, major bleedings in

All patients were Caucasian.

The study protocol was approved by the local Ethics Committee, and all subjects gave written informed consent. The study was conducted in accordance with the Helsinki Declaration.

**Laboratory tests.** Venous blood samples were taken in the morning following overnight fast and after a supine rest of ≥15 min. Blood samples were taken between 7 and 9 into plasma vacuum tubes containing 7.2 mg di-potassium EDTA. After centrifugation at 1800 x g for 10 min, plasma was collected and frozen at -70 °C until being investigated.

The plasma glucose and glycated hemoglobin (HbA1c) were measured using the Roche Cobas Integra immunoassay method (Roche, Vienna, Austria). The detection of HbA1c was certified by the National Glycohemoglobin Standardization Program (NGSP). The optimal glucose control was considered by HbA1c of less than 7%. Insulin resistance was assessed from fasting insulin and glucose levels using homeostasis model assessment (HOMA-IR), thus: HOMA-IR=fasting glucose (mmol/L) x fasting insulin (μU/mL)/22.5.

P-selectin and sCD40L were tested in duplicate using ELISA module sets obtained from Bender MedSystems (Vienna, Austria). Plasma levels of insulin and C-peptide were measured with immunoassays from DRG Diagnostics (Germany). Commercially available assay kit was used for determination of plasma brain natriuretic peptide (BNP) purchased from Bio-Medica (Austria).

Renal function was expressed as estimated glomerular filtration rate (eGFR, mL/min/1.73 m²) calculated from the Modification in Diet and renal Disease equation [10].

**Preparation of washed platelet suspension.** The method for isolation of human platelets by centrifugation and washing by Cazenave et al. was used [11]. The procedure was described in details previously [12]. Briefly, the blood from a forearm vein was collected into a conical 15-mL centrifuge tube containing 1 volume of acid-citrate-dextrose (ACD) anticoagulant for 6 volumes of blood (final pH 6.5 and citrate concentration 22 mM). Platelet-rich plasma was prepared by centrifugation at 200 g for 20 minutes. Platelets were then isolated from platelet-rich plasma by centrifugation at 1400 g for 10 minutes in the presence of 50 ng/mL prostaglandin E1 and 10% (v/v) acid citrate/dextrose, pH 4.6, and resuspended at a concentration of 4 x 10⁹ cells/mL in Tyrode’s-HEPES buffer. After three procedures of washing and centrifugation a drop of platelet rich suspension was placed on adhesive slides «Super Frost Plus» from Menzel Gmb & Co KG (Germany) and the cells were allowed to settle for 20 min at room temperature. The spread cell monolayer was fixed with 100% methanol.

**Fluorescent microscopy study.** The surface expression of platelet receptors was determined by immunofluorescent technique, using the following monoclonal antibodies against CD41 (alpha IIb integrin, platelet glycoprotein IIb), CD 41a (glycoprotein IIb/IIIa or fibrinogen receptor), CD 61 (beta 3 integrin, platelet glycoprotein IIIa) and CD 62P antigens (P-selectin).

All primary antibodies were obtained from Diaclone (France). The FITC-conjugated goat anti-mouse IgG was obtained from Sigma-Chemical (USA). The fixed adherent platelets on slides were transferred onto the primary antibody diluted in a ratio 1:1000 in phosphate-buffered saline, pH 7.4 (PBS). The samples were incubated for 24 hours at + 4°C. After that the platelets were washed multiple times in PBS. Then the samples were transferred to a goat anti-mouse IgG diluted in a ratio 1:64 in PBS. The incubation was 60 min at +37°C. After washing the samples were transferred in the medium containing 90% glycine and PBS in a ratio 9:1 for fluorescent microscopy investigation. After incubation with antibodies the samples were observed with fluorescence microscope Axioskop (Carl Zeiss, Germany). Carl Zeiss 38HE UW filter (excitation: 470/40 nm; emission: 525/50 nm) was used. The images were recorded with a high-sensitive CCD camera (model COHU-4922, COHU Inc., USA) and were analyzed with automatic digital morphology analysis system VIDAS-386 and VIDAS-2.5 software (Kontron Elektronik, Germany). Microscopic examination was done with a magnification of ×400 (objective: ×40, eye piece: ×10) and oil immersion. Totally 10 microscopic fields were analyzed. Absolute fluorescence was expressed as arbitrary units (pixel units, AU) and represented sum of fluorescence or individual adherent platelet in one defined area.

**Platelet aggregation tests.** Blood samples were collected in 3.8% sodium citrate tubes in a ratio 9:1 blood to anticoagulant. Whole-blood specimens were centrifuged for 10 min at 200 g to obtain platelet-rich plasma. Platelet-poor plasma was obtained on the remaining specimen by recentrifugation at 2,000 g for 15 min. Platelet count was measured on the platelet-rich plasma and was adjusted to between 200 x 10⁹/μL and 300 x 10⁹/μL with platelet-poor plasma. Aggregation test was performed with adenosine 5'-diphosphate (ADP) (Sigma, USA) at 10 μM using an optical aggregometer (Solar, Minsk, Belarus). Aspirin resistance was defined by previously reported criteria: ≥ 70% ADP- and ≥ 40% epinephrine-induced aggregation [13,14].

**Statistical methods.** Statistical analysis was performed using standard commercial software Statistica (Statsoft, Tulsa, USA). All continuous variables were tested for a normal distribution using the Shapiro-Wilk’s W test. Continuous variables are presented as mean±standard deviation or median (inter-quartile range) if non-normally distributed. Categorical variables are presented as counts and proportions. All normally distributed parameters were compared using a one-way ANOVA, followed, in case of significance, by a two-side Tukey test for multiple comparisons. Differences in non-normally distributed variables between groups were assessed by Kruskal-Wallis test with post hoc Mann-Whitney test. Correlation analysis was performed using Spearman rank correlation.
Results and discussion

Patients’ characteristics. During the study period, we prospectively recruited 65 patients with CHF and DM and 35 patients with CHF without DM, whose baseline characteristics are shown in table 1.

<table>
<thead>
<tr>
<th>Baseline characteristics of study population related to diabetes</th>
<th>Patients with CHF and DM with HbA1c &gt; 7 % (n=41)</th>
<th>Patients with CHF and DM with HbA1c &lt; 7 % (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes duration (years; median and range)</td>
<td>3 (1.5–8)</td>
<td>3 (1–5.5)</td>
</tr>
<tr>
<td>Laboratory findings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPG (mmol/L; median and range)</td>
<td>9.6 (6.4–12.0)</td>
<td>6.1 (4.8–6.8)**</td>
</tr>
<tr>
<td>HbA1c (%; median and range)</td>
<td>9.1 (8.0–10.0)</td>
<td>6.1 (5.4–6.3)**</td>
</tr>
<tr>
<td>Insulin (mU/mL)</td>
<td>14.5±6.1</td>
<td>14.7±6.4</td>
</tr>
<tr>
<td>HOMA-IR (median and range)</td>
<td>5.4 (3.8–7.7)</td>
<td>3.7 (2.5–6.3)*</td>
</tr>
<tr>
<td>C-peptide (ng/mL; median and range)</td>
<td>8.8 (5.2–16.3)</td>
<td>5.1 (4.2–10.3)</td>
</tr>
</tbody>
</table>

NB: CHF, chronic heart failure; DM, type 2 diabetes mellitus; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin A1c; HOMA-IR, Homeostasis Model Assessment – insulin resistance index. Values presented are mean ± SD unless otherwise stated. *P < 0.05; **P < 0.01; ***P < 0.0001.

Patients with CHF and decompensated DM had higher fasting plasma glucose, HbA1c, and HOMA-IR levels. The duration of the diabetes was similar. Glucose lowering treatment on admission was comparable between groups (data not shown).

Aspirin resistance rate. The rate of aspirin resistance was tend to be higher in patients with CHF and DM (by ADP-induced aggregation: 23.1% in diabetic patients vs. 14.3% in non-diabetic patients, P=0.43; by epinephrine-induced aggregation: 36.9% in diabetic patients vs. 31.4% in non-diabetic patients, P=0.66), but the difference was not statistically significant. The compensation of DM didn’t influence on aspirin resistance rate also.

Platelet activation markers in CHF patients according to glycemic status. The platelet data are presented in table 3. No significant changes in platelets’ parameters were observed between CHF patients without DM and patients with CHF and compensated DM.

In contrast, patients with CHF and decompensated DM presented higher platelet surface CD62P, CD41, CD41a and CD61 expression in comparison to CHF patients without DM. Patients with CHF and HbA1c of more than 7% showed also

<table>
<thead>
<tr>
<th>Platelet function profile according to glycemic control status</th>
<th>Patients with CHF and without DM (n=35)</th>
<th>Patients with CHF and compensated DM (n=24)</th>
<th>Patients with CHF and decompensated DM (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin (ng/ml)</td>
<td>69±35.4</td>
<td>62.5±28.4</td>
<td>82.3±24.4*</td>
</tr>
<tr>
<td>sCD40L (ng/mL; median and range)</td>
<td>0.53 (0.18–0.86)</td>
<td>0.46 (0.2–1.13)</td>
<td>0.29 (0.18–0.81)</td>
</tr>
<tr>
<td>Platelet surface P-selectin expression (arbitrary units, median and range)</td>
<td>1.66 (0.59–2.13)</td>
<td>1.35 (1.11–2.54)</td>
<td>1.88 (1.59–2.63)</td>
</tr>
<tr>
<td>Platelet surface CD41 expression (arbitrary units, median and range)</td>
<td>1.46 (0.49–2.7)</td>
<td>1.26 (1.0–2.25)</td>
<td>2.45 (1.75–3.08)*</td>
</tr>
<tr>
<td>Platelet surface CD61 expression (arbitrary units, median and range)</td>
<td>1.41 (0.53–2.23)</td>
<td>1.53 (1.11–2.01)</td>
<td>2.3 (1.53–3.12)</td>
</tr>
<tr>
<td>Platelet surface CD41a expression (arbitrary units, median and range)</td>
<td>1.64 (0.61–2.19)</td>
<td>1.76 (1.47–2.19)</td>
<td>2.3 (1.44–2.64)</td>
</tr>
<tr>
<td>20 μmol/L ADP–LTA (%)</td>
<td>71 ±15</td>
<td>73±13</td>
<td>73±13</td>
</tr>
<tr>
<td>5 μmol/L epinephrine–LTA (%)</td>
<td>20 (14–34)</td>
<td>27 (16–52)</td>
<td>23 (13–32)</td>
</tr>
</tbody>
</table>

NB: CHF, chronic heart failure; DM, type 2 diabetes mellitus; sCD40L, soluble CD40 ligand; ADP, adenosine diphosphate; LTA, light transmittance aggregometry. Values presented are mean ± SD unless otherwise stated. *P < 0.05 patients with CHF and compensated DM vs. patients with CHF without DM; †P < 0.05 patients with CHF and decompensated DM vs. patients with CHF and compensated DM.

© M. Yu. Kolesnyk, V. V. Syvolap, 2014
The results showed that in patients with CHF and decompensated DM the platelet activity is high despite aspirin use. Previously it was reported, that patients with CHF have increased platelet activity. In PLUTO-CHF trial 57 % of patients with CHF on aspirin therapy presented high GP IIb/IIIa and P-selectin expression [4]. Gurbel et al. found that platelet activity is heightened in 22% of outpatients with stable heart failure symptoms and is not affected by antecedent aspirin therapy [6].

In recent meta-analysis Krasopoulos et al., who included 2930 patients in 20 studies, it was shown that overall prevalence of aspirin resistance in cardiovascular diseases was approximately 28% and the resistant patients were at a greater risk of clinically important cardiovascular morbidity [15]. In Fateh-Moghadam S. et al. study the prevalence of aspirin resistance was 21.5 % in diabetic patients [7]. The results of our study showed that near one third of CHF patients with DM were aspirin-resistant.

The influence of DM on platelets is multifactorial and complex. The results of BAR1 2D substudy suggested that obesity and insulin resistance may influence platelet reactivity in type 2 DM [16]. Increased ADP-stimulated platelet P-selectin expression despite aspirin use was shown in the studied patients. The relationship between insulin resistance and platelet reactivity was of similar magnitude in patients regardless of aspirin use or nonuse. These results confirmed the limited efficacy of aspirin in patients with diabetes. In G.Y. Lip et al. study the plasma level of P-selectin was significantly higher in diabetic patients with cardiovascular disease on aspirin therapy compared to healthy individuals [20]. The reactivity was increased in obese and insulin resistant patients. Potential mechanisms of aspirin resistance in CHF and DM are follow: decreased endothelial nitric oxide production, increased platelet turnover, altered platelet structure as a result of dyslipidaemia and disproportionate increase in intra-platelet calcium concentration [21]. Another possible cause is hypersensitivity of platelets to yADP, which was shown in vitro studies [22]. Hyperglycaemia in diabetes can cause glycation of platelets increasing their reactivity [9].

Use of aspirin in CHF remains complex and controversial problem. Coronary artery disease (CAD) is known to be the primary etiological reason in the majority of patients with CHF. It would be reasonable to expect that aspirin would be beneficial in CHD patients with underlying CAD. Instead, there are some evidence that aspirin is not useful and even possibly harmful. The Warfarin/Aspirin Study in Heart failure (WASH) provides no evidence that aspirin is effective in patients with heart failure compared to the placebo group [17]. In the WATCH study, there was no difference in mortality in aspirin, clopidogrel, and warfarin use [18]. Conversely, the meta-analysis of two studies suggests more hospitalizations for CHF in aspirin group [19]. The potential mechanism is unfavourable interaction between ACE-inhibitors and aspirin.

So, aspirin is not ideal medication for CHF patients with DM and sinus rhythm. From one hand, the results of previous and our studies demonstrated that platelet reactivity remains high in patients on aspirin therapy. From another hand, the WASH and WATCH trials demonstrated that the risk of HF hospitalization was significantly greater in aspirin-treated patients. The use of another antiplatelet agents (clopidogrel) looks potentially beneficial but needs confirmation in clinical trials.

Another finding of our study was the influence of glycemic control on the platelet reactivity. Hyperglycemia accelerates the formation of advanced glycation end products, which are known to cause endothelial dysfunction and thus may be linked to platelet activation in diabetes. Advanced glycation end products induce tissue factor production in human monocytes in vitro and may enhance platelet reactivity [23]. It was reported previously that activation of platelet glycoprotein IIb/IIIa and P-selectin expression was increased similarly after addition of isosmotic concentrations of glucose and mannitol in whole blood samples from diabetic patients [24]. The lower blood thrombogenicity in patients with better glycemic control may have contributed to a reduction in cardiovascular events in these patients. According to the last ESC guidelines for the treatment of CHD metformin should be considered as a first-line agent in overweight patients with type 2 DM and without significant renal dysfunction. Systematic evaluation of the effect of metformin treatment on platelet function is lacking and results are controversial. Decreased sensitivity to platelet-aggregating agents during metformin treatment was demonstrated in Collier et al. study [25]. Metformin...
may have platelet stabilizing effects as shown by reduced platelet density and β-thromboglobulin [26]. However, others observed no effect of metformin on spontaneous or ADP-induced platelet aggregation in a placebo-controlled study [27].

**Study limitations.** A larger sample size may be needed to demonstrate statistically significant difference in aspirin resistance rate between diabetic and non-diabetic CHF patients. The arachidonic acid-induced platelet aggregation was not performed in our study. Finally, urine metabolites of thromboxane were not measured.

**Conclusions**

Laboratory findings of aspirin resistance are present in CHF patients with DM. The rate of aspirin resistance varies from 23 to 37% depending on tests used. Poor glycemic control of DM is associated with increased platelet activity in CHF patients.

**The perspectives for future research.** Antithrombotic therapy with thienopyridines can be alternative strategy for the heart failure patients with type 2 diabetes mellitus. The clinical perspective of this strategy need to be evaluated in future studies.

**References**


**Information about authors:**

Kolesnyk M.Yu., PhD, Zaporizhzhia State Medical University, Department of Family Medicine, Associate Professor, e-mail: zsmumk@gmail.com.

Syvolap V.V., PhD, Zaporizhzhia State Medical University, Department of Preventive Medicine, Chair.