The Effect of Combined Ezetimibe/Atorvastatin Therapy vs. Atorvastatin Monotherapy on the Erythrocyte Membrane Structure in Patients with Coronary Artery Disease: A Pilot Study*

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

Abstract

Background. Erythrocytes play an important role in atherogenesis. An excessive accumulation of cholesterol in erythrocyte membranes leads to disruption of the erythrocytes.

Objectives. The aim of the study was to compare the effect of two different hypolipidemic therapies on the structure of erythrocyte membranes.

Material and Methods. The study included 18 patients with angiographic confirmed coronary artery disease who, despite at least 6 months of hypolipidemic treatment, had not achieved LDL-C < 70 mg/dL and 18 healthy individuals as the control group. The following parameters were studied: total cholesterol level and erythrocyte membrane fluidity, lipid peroxidation, SH groups in membrane protein and plasma lipids.

Results. We observed a decrease in TC (20%), LDL-C (35%), level of lipid peroxidation (25%) and total cholesterol in erythrocytes (23%), and an increase in HDL-C (8%) and erythrocyte membrane fluidity of subsurface layers (14%) after 6 months of 10 mg atorvastatin + 10 mg ezetimibe therapy, in comparison with healthy controls. In the group treated with 40 mg atorvastatin for 6 months, decreased LDL-C (23%), lipid peroxidation (37%) and membrane cholesterol concentration (18%) was noted, as well as an increase in erythrocyte membrane fluidity in the subsurface layers (12%).


Key words: coronary artery disease, statins, total cholesterol, membrane fluidity, ezetimibe.

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The first administration of statin results in the greatest reduction in LDL-C, while doubling the statin dose only entails a further reduction of LDL-C by an additional 6–8% [1]. In secondary prevention, in the case of reduced statin monotherapy efficacy or intolerance, there is the possibility of combination therapy with statins and ezetimibe – an inhibitor of cholesterol absorption from the gastrointestinal tract by the selective blocking of protein transporting sterols (NPC1L1) [2].

The pleiotropic effects of statins are well known, but little data exists concerning combination therapy with ezetimibe, except for studies demonstrating its favorable impact on lipids. Furthermore, until now, there has been no clinical proof that combination therapy improves the rheological properties of erythrocytes in patients with coronary artery disease after cardiovascular events.

Another important issue to consider in patients with dyslipidemia is a pro-antioxidant imbalance, which leads to plasma membrane damage. Free radical processes affect the structure and function of erythrocyte membranes by oxidation of polyunsaturated fatty acids and proteins [3]. An excessive accumulation of cholesterol in erythrocyte membranes is not only dangerous due to the disruption of the erythrocytes, but it can also be perceived as one of the reasons behind the development of cardiovascular disease [4]. Therefore, in this study erythrocytes were used as the plasma membrane damage model.

The aim of this study is to compare the effect of two different hypolipidemic therapies (atorvastatin 40 mg/d vs. atorvastatin + ezetimibe 10 mg/d +10 mg/d) on erythrocyte membrane structure by determining the degree of lipid peroxidation, total cholesterol level in the membranes, membrane fluidity and the assessment of the concentration of SH groups in the proteins of erythrocyte membranes in patients with confirmed angiographic coronary artery disease.

Material and Methods

Patients

The study included 18 patients aged from 53 to 77 who underwent myocardial infarction and/or percutaneous coronary intervention (PCI) and/or coronary artery bypass surgery (CABG) over a period of 6 months. These patients did not achieve LDL-C < 70 mg/dL, despite receiving hypolipidemic treatment with 10–40 mg simvastatin, 10–40 mg lovastatin, 10–30 mg atorvastatin and 5–15 mg rosuvastatin.

The exclusion criteria comprised the presence of one or more of the following: chronic heart failure type III and IV with EF < 40% according to NYHA, acute hypertension, chronic renal failure stage IV, V with eGFR ≤ 30 mL/min/1.73 m², diabetes type I or II, hyper or hypothyroidism, liver diseases and active liver dysfunction or persistent elevation of liver enzymes (aminotransferases) in serum, exceeding more than 3 times the upper normal limit, myopathy, myalgia, autoimmune or allergic diseases, infectious diseases, statin and/or ezetimibe intolerance, an acute infection experienced in the previous 2 weeks, an ongoing infection, confirmed malignancy, pregnancy and breast feeding, being a women of reproductive age with no effective contraception application, alcohol abuse, smoking, atorvastatin therapy at doses ≥ 40 mg/dL, rosuvastatin therapy at doses ≥ 20 mg/d or statin therapy with ezetimibe before randomization.

The screened participants were randomized into 2 groups. Ten patients received 40 mg of atorvastatin (A40 group), and 8 patients received combination therapy: 10 mg atorvastatin with 10 mg ezetimibe (A10 + E10 group). The control group consisted of healthy individuals of appropriate age, eligible for the study in accordance with above exclusion criteria.

Study specimens were obtained from patients at 3 different time intervals during ongoing therapy: Prior to the treatment, and 1 and 6 months post-treatment.

Plasma Lipids

TC – total cholesterol, TG – triglycerides, LDL-C – low density lipoprotein cholesterol, HDL-C – high density lipoprotein cholesterol. All are determined by an enzymatic method. Test results are expressed as mg/dL.

Isolation of Erythrocytes

Peripheral blood collected into tubes containing an ACD solution (23 mM citric acid, 45.1 mM sodium citrate and 45 mM glucose) was centrifuged at 600 × g at 4°C for 10 min. After removal of plasma and leukocytes, the remaining erythrocytes were washed three times with NaCl solution and suspended in a final hematocrit of 50%.

Determination of Lipid Peroxidation

The lipid peroxidation in the erythrocytes was determined as the concentration of substances reacting with thiobarbituric acid (TBA) according to Stocks and Dormandy [5]. The erythrocyte suspension in a hematocrit of 50% was incubated in the presence of 20% TCA at 4°C for 1 h, then cen-
trifuged at 1000 × g for 5 min. The obtained supernatant was added to 0.26 M TBA and heated at 100°C for 15 min. Absorbance measurement was performed at λ = 532 nm, the concentration of TBARS was calculated using a molar absorbance coefficient for malondialdehyde (MDA). The result was expressed in µmol MDA/g Hb.

**Isolation of Erythrocyte Membranes**

Plasma membranes were isolated using hypotonic hemolysis according to Dodge [6], with some modifications. The isolated erythrocytes were suspended in a hemolysis buffer (20 mM Tris buffer, pH = 7.4, cooled to 4°C, in a ratio of 1 : 5 erythrocyte to buffer volume). After incubation in an ice bath for about 20 min, the samples were subjected to repeated centrifugation cycles (12000 × g, 4°C, 5 min) and washes (20 mmol/L TRIS buffer at pH 7.4) until white, purified, erythrocyte membranes were obtained.

**Concentration of SH Groups**

The concentration of thiol groups in the membranes was performed by using the method by Ellman [7]. Briefly, the solution of isolated erythrocyte membranes was diluted in 10% SDS and incubated in the presence of 0.3 M Na₂HPO₄ and 4 mmol 1⁻¹ 5,5'-ditiobis-(2-nitrobenzoic acid) DTNB at room temperature for 30 min. Absorbance measurement was performed at λ = 412 nm. The results were shown as -SH µmol/mg proteins.

**Concentration of Hemoglobin**

Concentration of hemoglobin was performed using the method described by Drabkin [8]. Erythrocyte lysate obtained from the hematocrit of 50% was centrifuged for 5 min. The obtained supernatant was incubated at room temperature in the presence of Drabkin reagent for 15 min. Absorbance was measured at λ = 540 nm.

**Concentration of Protein**

The determination of protein concentration in isolated erythrocyte membranes was performed according to Lowry et al. [9]. Bovine serum albumin was used as the standard.

**Total Cholesterol**

Lipids were extracted according to Rodriguez-Vico et al. [10] using solvents with low toxicity. Lipids were dissolved in a mixture of ethanol and chloroform. Cholesterol level was determined on the basis of the Liebermann-Burchard reaction. Absorbance was measured at λ = 660 nm. The results were expressed as mg of cholesterol per mg of total membrane proteins (mg chol/mg proteins).

**Erythrocyte Membrane Fluidity**

Erythrocyte membrane fluidity was determined using fluorescent probes according to Shinitzky and Barenholz [11] using a Perkin Elmer LS 50B spectrofluorometer. 1,6-diphenylo-1,3,5-hexatrien (DPH) and 1-(4-trimetyloaminofenylo)-6-phe- nyl-1,3,5-heksatrien p-toluenesulfonate (TMA-DPH) were used to determine the microviscosity of the lipid bilayer at a depth of the 4th and < 4th carbons in the alkyl chains of the membrane phospholipids. After excitation at λ = 348 nm, fluorescence anisotropy was read at λ = 426 nm.

**Statistical Analysis**

The normality of variables was checked using the Shapiro-Wilk normality test. The ANOVA analysis for independent or dependent samples, was used to compare the control group values with the patient values prior to treatment, and to compare the results of the therapy at different time intervals. Statistical analyses were performed with STATISTICA PL 9.0. The data in the tables is shown as averages ± SD. In the Results section, the data is shown as percentage differences. A p-value of < 0.05 was considered statistically significant.

The project was performed in cooperation with the Department of Environment Pollution Biophysics, Faculty of Biology and Environmental Protection, University of Lodz.

This project was approved by the Bioethics Committee of the Medical University of Łódź, No. RNN/846/12/KB.

**Results**

In the atorvastatin group (A40), all qualified patients finished one-month therapy, whereas eight individuals, two of whom were not taking the medication systematically, completed six-month therapy. After the 6-month therapy target, LDL-C concentration < 70 mg/dL was reached in 3 out of the 8 patients.

While all patients included in the group treated with combination therapy (A10 + E10) completed the 1-month treatment, only five completed the 6-month therapy: two subjects did not take the medication regularly, while one patient had to stop the treatment due to bleeding from the gas-
trotintestinal tract. Target LDL-C concentration < 70 mg/dL was reached in 3 out of 5 patients.

Tolerance to both therapies was good. Only two patients reported discomfort during atorvastatin therapy: the first patient complained of transient pruritus, but it resolved itself spontaneously. The second reported muscle pain, but no increase was noted in creatine kinase (CK) level in blood serum.

Table 1 shows the mean values of lipids and erythrocyte membrane structure parameters determined in patients with coronary artery disease before modification by lipid-lowering therapy. Patients with coronary artery disease demonstrated significantly higher concentrations of Total Cholesterol (TC) (16%, $p < 0.02$), LDL-C (33%, $p < 0.005$), TG (26%, $p < 0.05$), and significantly lower HDL cholesterol concentration 24% ($p < 0.05$) as compared with the group of healthy individuals. They also demonstrated a 16% increase in lipid peroxidation ($p < 0.001$), 13% higher concentrations of erythrocyte membrane cholesterol ($p < 0.001$), as well as increased erythrocyte membrane rigidity in the subsurface layers (15% higher, $p < 0.001$), compared to the control group.

In comparison to baseline values, after a 1-month treatment in the A10 + E10 group (Table 2), LDL-cholesterol (LDL-C) decreased by 26% ($p < 0.05$), lipid peroxidation by 14% ($p < 0.05$) and total cholesterol in erythrocytes by 11% ($p < 0.05$). After 6 months of treatment, TC concentration was seen to decrease by 20% ($p < 0.05$), LDL-C by 35% ($p < 0.05$), lipid peroxidation by 25% ($p < 0.01$) and a 23% reduction in total cholesterol in erythrocytes was observed ($p < 0.001$). In addition, HDL-C was seen to increase by 8% ($p < 0.05$) and the erythrocyte membrane fluidity of the subsurface layers by 14% ($p < 0.01$). A statistically significant drop in the following parameters can be seen in the six-month observation compared to those observed at the end of the first month (Table 2): The degree of lipid peroxidation (13% lower after 6 months, $p < 0.01$), membrane cholesterol concentration (14% lower after 6 months, $p < 0.01$) and an increase in the membrane fluidity of the subsurface layers (10% lower after 6 months, $p < 0.01$).

In atorvastatin-treated patients (A40), a 22% decrease of lipid peroxidation ($p < 0.05$) was demonstrated in comparison to baseline values after one month of therapy. Parameters such as total cholesterol in erythrocytes and fluidity of erythrocyte membranes were not altered in a statistically significant way. However, after 6 months of the therapy, a 23% reduction of LDL-C concentration ($p < 0.05$) was recorded. Other lipid fractions (HDL-C, TC, TG) were not altered in a statistically significant way. After 6 months, further significant decreases were recorded: lipid peroxidation by 37% ($p < 0.005$), and membrane cholesterol concentration by 18% ($p < 0.01$). A 12% increase in the erythrocyte membrane fluidity of subsurface layers ($p < 0.005$) was also revealed.

With regard to atorvastatin therapy (Table 2), statistically significant changes were observed in the following parameters after six months of treat-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Health controls (n = 18)</th>
<th>Patients with CAD before randomization (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>151 ± 27</td>
<td>180 ± 32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>69 ± 15</td>
<td>103 ± 25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>62 ± 13</td>
<td>47 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>116 ± 30</td>
<td>157 ± 64&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>TBAR-s (nmolTBARS/g Hb)</td>
<td>0.392 ± 0.122</td>
<td>0.455 ± 0.078&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>SH group in membrane protein (μmol/mg protein)</td>
<td>0.23 ± 0.04</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Cholesterol in erythrocytes (mg chol/mg membrane proteins)</td>
<td>0.107 ± 0.009</td>
<td>0.122 ± 0.014&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Membrane fluidity of erythrocytes (fluorescent anisotropy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMA-DPH</td>
<td>0.303 ± 0.006</td>
<td>0.358 ± 0.007&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPH</td>
<td>0.269 ± 0.003</td>
<td>0.283 ± 0.006</td>
</tr>
</tbody>
</table>

Values are means ± SD; <sup>a</sup> – $p < 0.05$; <sup>b</sup> – $p < 0.02$; <sup>c</sup> – $p < 0.005$; <sup>d</sup> – $p < 0.001$; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TG – triglycerides.
Combined Ezetimibe/Atorvastatin Therapy

ment compared to the values obtained after one month: a 19% decrease in the degree of lipid peroxidation ($p < 0.05$), a 12% decrease in the membrane cholesterol concentration ($p < 0.01$), as well as a 7% increase in the fluidity of the membranes of subsurface layers ($p < 0.01$) after six months. No statistically significant change in the concentration of SH groups was observed in either group (A40 or A10 + E10) after 6 months of therapy.

Irrespective of the type of lipid-lowering therapy, after 6 months of observation, all parameters associated with the erythrocyte membrane structure which had changed prior to randomization reached values close to those in the control group under the influence of intensified lipid lowering treatment (Table 1; Table 2).

Discussion

It should be emphasized that patients treated unsuccessfully with statin exhibit disorders in red blood cell membranes, which may be reflected in their future prognosis. The patients who did not achieve a target LDL-C concentration of less than 70 mg/dL, despite undergoing a course of statin treatment of at least 6 months, demonstrated disorders of the blood parameters. Although these results are based on a relatively small study group, they are nevertheless consistent with those given in an earlier study conducted on a larger group of patients [12]. Our previous studies on patients with type II hypercholesterolemia reveal decreases in membrane cholesterol concentration after only 4 weeks of treatment with atorvastatin [13], and after 8 weeks of treatment with atorvastatin or simvastatin [14]. Reduced cholesterol concentrations are also reported in other studies on statins. Zhong et al. report that, in patients with coronary artery disease, rosuvastatin reduces membrane concentrations of cholesterol in erythrocyte membranes to 28% and 33% of baseline values when given at doses of 5 mg/d and 10 mg/d, respectively [15]. However, no studies describe the impact of ezetimibe, a drug whose hypolipidemic mechanism differs from that of statins, on reducing membrane cholesterol.

The concentration of cholesterol in the erythrocyte membrane affects its fluidity. Cholesterol incorporated between the phospholipids of the membrane mainly stiffens the surface layers.

Table 2. Comparison of plasma lipid and erythrocyte membrane structure parameters in patients with coronary artery disease (CAD) at 3 different time intervals of an ongoing atorvastatin vs. atorvastatin-ezetimibe combination therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Atorvastatin</th>
<th>Atorvastatin + ezetimibe</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>before therapy (n = 10)</td>
<td>after 1 month (n = 10)</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>178 ± 22</td>
<td>–</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>101 ± 15</td>
<td>85 ± 26</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>46 ± 7</td>
<td>–</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>167 ± 73</td>
<td>–</td>
</tr>
<tr>
<td>TBARs (nmolTBARS/g Hb)</td>
<td>0.469 ± 0.067</td>
<td>0.368 ± 0.121a</td>
</tr>
<tr>
<td>SH group in membrane protein (µmol/mg proteins)</td>
<td>0.23 ± 0.03</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Cholesterol in erythrocytes (mgcholesterol/mg membrane proteins)</td>
<td>0.121 ± 0.014</td>
<td>0.112 ± 0.009</td>
</tr>
<tr>
<td>Membrane fluidity of erythrocytes (fluorescent anisotropy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMA-DPH</td>
<td>0.357 ± 0.004</td>
<td>0.334 ± 0.025</td>
</tr>
<tr>
<td>DPH</td>
<td>0.283 ± 0.005</td>
<td>0.283 ± 0.004</td>
</tr>
</tbody>
</table>

Values are means ± SD; a – $p < 0.05$; b – $p < 0.01$; c – $p < 0.005$; d – $p < 0.001$ comparison of results before therapy with values either after 1-month therapy or after 6-month therapy; e – $p < 0.05$; f – $p < 0.01$ comparison of results after 6-month therapy vs. after 1-month therapy; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TG – triglycerides.
thereby restricting the mobility of phospholipid hydrocarbon chains. It appears that lipid-lowering medications, by reducing the concentration of cholesterol in plasma and affecting the membrane cholesterol, may also have an effect on improving the fluidity of the erythrocyte membrane. The results of the present study confirm this hypothesis. A previous study based on the use of EPR (Electron Paramagnetic Resonance) and spin labels also indicated increased erythrocyte membrane fluidity during atorvastatin therapy [16]. Reduced erythrocyte membrane stiffness was also observed by Coccia et al. in patients undergoing cardiac surgery with extracorporeal circulation, who had received 40 mg/d simvastatin for 3 weeks prior to surgery [17]. There is no evidence, however, regarding the effects of ezetimibe or combination ezetimibe and statin therapy on the discussed parameter of erythrocyte membranes structure.

An important factor that may affect the structure of cell membranes is oxidative stress. One indication of its occurrence is an increase in lipid peroxidation, which causes the oxidation of polyunsaturated fatty acids present in the cell membrane. This process may also result in the damage of cell membrane proteins, thus changing the dynamics and function of the cell membranes themselves.

In the present study, an increased level of TBARS concentration was demonstrated in patients with CAD. Furthermore, earlier studies have also revealed an increase in lipid peroxidation, a type of free radical process, in patients with type II hypercholesterolemia [3]. Marinez-Sanchez et al. [18] note increased concentrations of MDA (malondialdehyde), the main end-product of lipid peroxidation, in patients with hypercholesterolemia, while Walter et al. [19] demonstrate a correlation between the concentration of TBARS and the incidence of cardiovascular events. Uydu et al. [20] report similar results following the use of atorvastatin in patients with hypercholesterolemia or hyperlipidemia: a decrease in the concentration of TBARS was seen after 12-week therapy at a dose of 10 mg/day.

The present study assesses the concentration of thiol groups (-SH) in membrane proteins. These groups are sensitive to changes in membrane structure, and to the effects of free radicals occurring under conditions of oxidative stress. Similar to our results in CAD patients, Aydin et al. [21] observed no changes in the concentration of SH groups after a 4-week treatment with atorvastatin in hypercholesterolemic rabbits.

It has been demonstrated in some observations that combination therapy of statin with ezetimibe exhibits a stronger anti-inflammatory effect, and higher ox LDL and sICAM levels as compared with statin monotherapy [22]. However, it remains unclear whether the inclusion of ezetimibe with statin therapy can improve the prognosis of patients in high and very high cardiovascular risk groups. Imaging examinations such as ENHANCE, despite identifying a strong favorable impact on lipids, have detected no regression of atherosclerotic lesions: intima-media thickness was even increased [23]. On the other hand, the results of the recently completed IMPROVE-IT trial indicate that, in patients post high-risk acute coronary syndrome, the risk of future cardiovascular events was lower in patients treated with ezetimibe 10 mg/simvastatin 40 mg in comparison with patients treated with 40 mg of simvastatin only [24].

In conclusion, the results of our pilot study indicate that the replacement of an existing lipid-lowering therapy with a more aggressive one, regardless of its type (high-dose statin monotherapy or lower-dose ezetimibe-statim combination therapy) resulted in a significant improvement in membrane structure parameters in all patients, even though target LDL-C concentration < 70 mg/dL was not reached in all patients. Both treatment with 40 mg/d atorvastatin, as well as with 10 mg ezetimibe and 10 mg/d atorvastatin, not only result in similar decreases in TC and LDL-C, but also improve the erythrocyte membrane structure parameters in patients with coronary artery disease to a similar degree, returning them to the values observed in healthy subjects.

References


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