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Effect of Different Doses of Atorvastatin Therapy on Endothelial Progenitor Cells and Angiogenic Factors in Patients with Ischemic Heart Disease

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Abstract **Aim.** The purpose of current research was to assess changes in endothelial progenitor cells (EPC) counts and angiogenic factors levels during atorvastatin therapy in different doses in patients with ischemic heart disease (IHD) as an independent predictor of cardiovascular morbidity and mortality. **Methods and Results.** The main group included 58 patients with IHD during atorvastatin therapy. EPC quantity (CD34+/CD133+/CD309+ phenotype) was measured by flow cytometry two times – before treatment and 3 months after. Vascular endothelial growth factor (VEGF), C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), endostatin levels and lipid profile were also measured twice. The control group consisted of 15 healthy volunteers with the same analyzes performed once. Atorvastatin therapy in IHD patients within three months of treatment caused a significant (72% on average) increase of EPC counts ($p<0.05$). Dependence of EPC gain on statin dose was not reliable ($p=0.10$), but it was higher when initial EPC counts were low ($p=0.01$). The therapy showed reliable reduction of VEGF level (by 11%, $p<0.01$), CRP – by 26% ($p<0.01$), total cholesterol (TCh) – by 30% ($p<0.01$), low density lipoprotein (LDL-C) – by 35% ($p<0.01$), triglycerides (TG) – by 18% ($p<0.01$), while endostatin, MCP-1 and high density lipoprotein (HDL-C) levels did not change. Correlations between the EPC, TCh and LDL-C changes during therapy were revealed: higher EPC counts gain was associated with higher TCh ($p=-0.37$, $r<0.01$) and LDL-C ($p=-0.41$, $r<0.01$) levels decrease. **Conclusion.** We found a significant increase of EPC counts in IHD patients when treated with atorvastatin for 3 months, without statistically reliable difference depending on dosage.

Keywords: *endothelial progenitor cells, ischemic heart disease, atorvastatin, angiogenic growth factors*

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1. Introduction

Progenitor stem cells are immune system cells that are capable to self-renew and differentiate into various cell types. These cells have the potential to regenerate damaged human tissue [1,2]. Endothelial progenitor cells (EPC) represent a heterogeneous cells population, which differentiate into endothelial cells [3]. It is considered that they are involved in the processes of endothelium recovery, new blood vessels formation, inhibiting of atherosclerosis [4,5]. EPC are involved in vasculogenesis in situ both during embryonic development and in adults [6,7,8].

In 1997 Asahara et al. demonstrated that certain bone marrow cells can be used for vascular endothelial reparation and perfusion restoration in the ischemic tissue [9]. Since then, the plurality of experimental studies proved that EPC impact on ischemic processes. However, those promising data were not fully confirmed by further clinical trials. Despite low level of circulating EPC is considered as independent risk factor for cardiovascular disease since it may reflect endothelium reparation insufficiency [10], mechanisms of growth factors and EPC involvement in damaged tissue recovery and new blood vessels formation are not completely understood until now. Furthermore, although a plurality of different EPC classes were discovered, specific phenotypes among them that are capable of differentiating exclusively in endothelial cells, and so therapeutically useful, are not well defined [11]. All of the hematopoietic stem cells represent CD34 and CD133 markers, while EPC surface expresses endothelial markers also, such as VEGFR-2 (CD309), CD31, endothelial nitric oxide synthase and vascular endothelial cadherin [12,13,14]. Endothelial cells may develop from less mature progenitors (e.g. CD133+/CD34+/CD309- phenotype) as well from more mature phenotypes. Yet many authors agree that EPC should be defined as different subpopulations of progenitor cells, mainly those that co-express these three markers in various combinations: CD133+/CD34+, CD133+/CD309+, CD34+/CD309+ or

CD133+/CD34+/ CD309+ [15,16,17,18,19]. Due to this variety of EPC classes, several kits for different phenotypes identifying are currently available.

Among factors that enhance plasma EPC titer and bring them into the damaged area, nitric oxide, estrogens, high density lipoprotein (HDL-C), erythropoietin and VEGF group are mentioned [20,21]. VEGF has many angiogenesis-related effects on endothelial cells: increased cell migration and survival, production of plasminogen activators and interstitial collagenases [22,23].

An important signal for directing EPC mobilization to the damage area is stromal chemokine SDF-1. Other chemokine, monocyte chemotactic protein-1 (MCP-1), increases mononuclear cells influx, and it also stimulates arteriogenesis. MCP-1, due to its directional cell specificity, plays a pathogenic role in different disorders characterized by mononuclear cells infiltration, including atherosclerosis and rheumatoid arthritis. Elevated MCP-1 levels have been connected with myocardial ischemia [24,25,26].

Factors that inhibit angiogenesis include thrombospondin, angiostatin and endostatin. Specifically, endostatin inhibits endothelial cell proliferation, angiogenesis, and so tumor growth. Though endostatin researches are mainly oncologic, investigations of endostatin level changes due to ischemia and treatment in IHD patients may clarify some mechanisms of coronary angiogenesis [27,28,29].

Various cardiovascular risk factors that lead to dysfunction and apoptosis of mature endothelium, also negatively influence the EPC, due to mechanical (e.g. in arterial hypertension) or metabolic (diabetes, hyperlipidemias) damage of vessel wall [30,31,32]. There is evidence that circulating EPC counts and activity are inversely related to the presence of various risk factors [15,33].

In addition to the therapy with EPC, there is another approach that is associated with an attempt to activate own EPC proliferation by drug therapy, including statins, increasing their survival and activity in the damaged area. Some studies have shown that statin therapy leads to increase of different EPC phenotypes, reducing the level of their apoptosis and increasing the capacity for regeneration of ischemic tissue, which are impaired in IHD patients [34-40]. Still there is no available data of statin dosage influence on EPC gain. For that reason we examined the effects of statin therapy in different doses on endothelial progenitor cells maintenance and relations of resulting changes to angiogenesis factors and lipid profile dynamics. Dose-dependence of EPC dynamics was analyzed in IHD patients before and after therapy with atorvastatin 10 mg or 40 mg per day. We also compared quantity of endothelial progenitor cells and angiogenesis factors concentrations in healthy volunteers and in patients with IHD.

2. Material and Methods

The study included 58 patients over 18 years old with ischemic heart disease (IHD) and with indications for statins assignment (LDL-C≥1.8 mmol/l) [41]. Patients with acute coronary syndrome, myocardial infarction less than 6 months old, hemodynamically significant heart defects, NYHA III-IV class heart failure, infectious diseases, increased transaminases levels > 2 upper limits, patients after previous (less than 6 months ago) statin therapy were excluded. All included patients were treated and followed-up on the base of Atherosclerosis Department of Russian Cardiology Research Center (RCRC, Moscow, Russian Federation) after informed consent signing. Patients were randomized into two groups: Group 1 received atorvastatin 10 mg daily (n=26), Group 2 – 40 mg (n=32). All patients underwent standard clinical laboratory (clinical and biochemical blood tests with lipids content) and instrumental examinations (ECG, cardiac US, Holter ECG monitoring; stress-tests and CAG if necessary), VEGF, CRP, MCP-1 and endostatin levels were measured. The key analysis was to determine EPC in whole blood, performed in laboratory of immunology of RCRC. Cells were isolated by magnetic separation. The main criterion for EPC selection from white blood cells pool was simultaneous expression of markers CD34, CD133 and CD309, thus undifferentiated ("young") EPC were detected. EPC quantity of CD34+/CD133+/CD309+ (VEGFR-2+) phenotype was measured in 10 ml whole blood test sample with Miltenyi Biotec GmbH set using MACS technology by flow cytometry (Cytomics FC500, Beckman Coulter). Additionally 10 ml of whole blood was used for CD309 control sample, and 200 µl for CD133 control sample. EPCs were detected in the EPC sample by staining with the cocktail containing CD34-FITC, CD133/2(293C3)-PE and CD309 (VEGFR-2/KDR)-APC for positive staining of EPCs and CD14-PE-Cy5 for exclusion of monocytes. Four-color (FITC, PE, APC, PE-Cy5) flow cytometry was performed in multiple stages: debris and platelets excluding, dead cells and monocytes excluding, identification of CD34+ cells and CD133 specificity, identification of CD34+/CD133+/CD309+ pool cells. All measurements were performed twice – before treatment and after 3 months. The control group consisted of 15 healthy volunteers in whom these tests were performed once. The second stage of EPC analysis in volunteers after placebo therapy was rejected due to its high overall costs and low benefit for the main task of the research (assessment of EPC level dynamics in IHD patients). Clinical characteristics of patients in the main group, statin dose subgroups and control group are shown in Table 1. In the main group reception frequency of the following drugs was analyzed: aspirin, β-blockers, calcium antagonists, nitrates, ACE inhibitors and diuretics, with no significant differences in all reception frequencies in the subgroups ($p>0.3$).

2.1. Statistical Analysis

Due to non-gaussian distributions (by the Shapiro-Wilk test) of the majority of data compared, nonparametric statistics was used: groups are represented as median with interquartile range, correlation analysis was performed with Spearman test, medians comparison of independent groups – by Mann-Whitney test, dependent groups – by Wilcoxon test, qualitative data was analyzed with exact Fisher's test. To analyze the influence of two independent factors on parameter changes, two-factor ANOVA was used (in case of non-gaussian sample distribution – after Box-Cox normalization).

Table 1. Clinical characteristics of patients in IHD and control (healthy volunteers) groups

	IHD (n=58)			Control (n=15)	p
	Group 1 (10 mg, n=26)	Group 2 (40 mg, n=32)	p		
Males	22 (38%)			10 (67%)	ns**
	12 (46%)	10 (31%)	ns**		
Age, years	66 (55-70)			59 (42-66)	ns*
	61 (54-69)	67 (60-72)	ns*		
Body mass index, kg/m^2	29.0 (26.4-32.0)			23.7 (23.1-24.8)	<0.01*
	28.4 (26.0-31.5)	29.4 (26.7-32.3)	ns*		
Waist, cm	97 (92-104)			76 (70-82)	<0.01*
	97 (87-107)	97 (92-103)	ns*		
Systolic blood pressure, mmHg	130 (120-140)			120 (120-125)	ns*
	123 (120-130)	130 (120-143)	0.03*		
Diastolic blood pressure, mmHg	80 (70-80)			70 (65-70)	0.012*
	73 (70-80)	80 (70-90)	0.02*		
Heart rate, beats/min	68 (66-72)			67 (64-70)	ns*
	68 (66-70)	70 (66-74)	ns*		
Family history	42 (72%)			1 (7%)	<0.01**
	19 (73%)	23 (72%)	ns**		
Smoking	10 (17%)			1 (7%)	ns**
	7 (27%)	3 (9%)	ns**		
Arterial hypertension	47 (81%)			0 (0%)	<0.01**
	20 (77%)	27 (84%)	ns**		
Diabetes mellitus	6 (10%)			0 (0%)	ns**
	1 (4%)	5 (16%)	ns**		
Myocardial infarction	7 (12%)			0 (0%)	ns**
	4 (15%)	3 (9%)	ns**		
Therapy					
Aspirin	26 (100%)	32 (100%)	ns**		
β-blockers	16 (62%)	18 (56%)	ns**		
Calcium blockers	6 (23%)	4 (13%)	ns**		
Nitrates	4 (15%)	3 (9%)	ns**		
ACE inhibitors	18 (69%)	18 (56%)	ns**		
Diuretics	4 (15%)	3 (9%)	ns**		

** – Mann-Whitney test, ** – exact Fisher's tes.*

3. Results

Comparison of CD34+/CD133+/CD309+ phenotype EPC quantity, leukocytes quantity, levels of VEGF, MCP-1, CRP and endostatin, between IHD groups and control group, is shown in Table 2.

As shown in Table 2, EPC counts are reliably lower in IHD patients compared to healthy volunteers. Also in IHD group endostatin level and leukocytes counts were significantly lower, total cholesterol, LDL-C, VEGF and CRP levels were higher than in control group, with no significant differences in HDL-C, triglycerides (TGs) and MCP-1 levels. All of studied parameters did not differ reliably between Group 1 (10 mg of atorvastatin) and group 2 (40 mg).

EPC, VEGF and endostatin levels did not differ in men and women. There also were no associations of these factors with previous myocardial infarction, coronary angioplasty/bypass history or other burdened anamnesis, with body mass index. Non-smoking IHD patients had reliably higher endostatin level, than smokers – 156.9 (138.6-176.2) and 140.2 (136.9-151.1) ng/ml, respectively (p=0.043). Also smoking IHD patients had increased EPC counts and VEGF levels (p=0.053 and 0.061, respectively). Diabetes as a factor for even more reduced EPC counts (compared to IHD patients without diabetes mellitus), also almost reached the validity criteria (p=0.066). No significant relationships were revealed between EPC counts and leucocytes counts, levels of TCh, LDL-C, HDL-C, triglycerides, VEGF, endostatin, MCP-1 and CRP in IHD group before statin therapy and in control group.

After 3 months of atorvastatin therapy EPC counts increased from 171 (77-435) to 423 (164-739) pcs in 10 ml, growth rate was 1.72 (1.24-2.64), which corresponds to 72% gain in average. In group 1 EPC counts increased

from 234 (114-434) to 466 (170-709), in group 2 – from 142 (70-443) to 382 (137-742) (Figure 1). Effects of atorvastatin therapy on all of studied parameters in both groups are summed in Table 3.

Table 2. Comparison of the studied parameters in IHD groups and control group

Parameter	IHD (whole group, n=58)			Control (n=15)	p*
	Group 1 (10 mg, n=26)	Group2 (40 mg, n=32)	p*		
EPC, pcs in 10 ml	171 (77-435)			938 (412-1778)	**<0.01**
	234 (114-434)	142 (70-443)	0.33		
Leukocytes, mln/ml	6.4 (5.6-7.2)			7.6 (6.5-8.1)	**0.013**
	6.4 (5.9-7.1)	6.3 (5.6-7.5)	0.81		
TCh, mmol/l	6.77 (6.35-7.56)			4.68 (4.03-4.86)	**<0.01**
	6.55 (6.10-6.98)	6.95 (6.42-7.84)	0.07		
TCh>5.2 mmol/l	57 (98%)			0 (0%)	**<0.01**
LDL-C, mmol/l	4.45 (4.26-5.19)			2.60 (1.90-2.97)	**<0.01**
	4.34 (3.96-4.63)	4.59 (4.14-5.28)	0.09		
HDL-C, mmol/l	1.31 (1.10-1.66)			1.15 (0.91-1.38)	0.08
	1.20 (1.09-1.66)	1.37 (1.11-1.66)	0.67		
TGs, mmol/l	1.67 (1.25-2.43)			1.46 (0.98-1.97)	0.49
	1.38 (1.11-2.16)	1.86 (1.36-2.64)	0.10		
VEGF, pg/ml	383.7 (244.8-454.7)			259.5 (144.9-310.3)	**0.016**
	375.3 (244.8-462.3)	388.4 (253.6-448.4)	0.94		
Endostatin, ng/ml	154.6 (138.1-170.0)			182.1 (154.5-187.6)	**0.012**
	150.9 (139.1-160.8)	156.4 (137.5-179.3)	0.32		
MCP-1, pg/ml	211.6 (175.0-275.8)			200.7 (168.9-251.2)	0.57
	219.9 (175.0-278.0)	209.6 (175.0-247.1)	0.65		
CRP, mg/dl	0.27 (0.14-0.47)			0.13 (0.03-0.25)	**0.017**
	0.22 (0.10-0.39)	0.31 (0.18-0.49)	0.07		

* – Mann-Whitney test. IHD, ischemic heart disease; EPC, endothelial progenitor cells; TCh, total cholesterol; LDL-C, low density lipoprotein; HDL-C, high density lipoprotein; TG, triglycerides; VEGF, vascular endothelial growth factor; CRP, C-reactive protein; MCP-1, monocyte chemotactic protein-1.

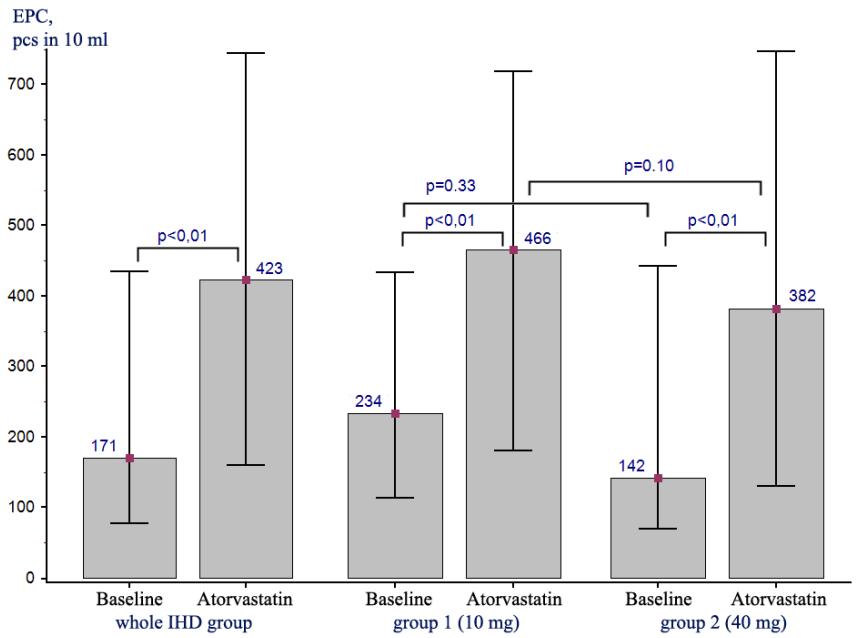

Figure 1. Changes in EPC counts in IHD patients after 10 mg or 40 mg atorvastatin therapy. EPC, endothelial progenitor cells; IHD, ischemic heart disease

As shown in Table 3, TCh, LDL-C, TGs, VEGF and CRP levels decreased reliably in both groups. It must be noted that none of those effects were dose-dependent, with an exception that TCh and LDL-C levels expectedly decreased more in group 2. For instance, EPC counts gained in both groups with unreliable difference between

groups (p=0.1). So we tried to reveal other factors that could influence EPC growth rate. In particular, we analyzed the dependence of TCh, LDL-C and EPC changes on the initial values of these parameters. Groups 1 and 2 were split into two equal subgroups according to baseline values of TCh, LDL-C and EPC – below the median and above the median, and then two-factor ANOVA was performed to those four subgroups. The results are shown in Table 4.

Table 3. Effects of atorvastatin therapy on studied parameters

	IHD (n=58)		Group 1 (10 mg, n=26)		Group 2 (40 mg, n=32)		1-2
	k[1]	p[2]	k[1]	p[2]	k[1]	p[2]	p[3]
EPC	1.72 (1.24-2.64)	**<0.01**	1.35 (1.14-2.33)	**<0.01**	1.98 (1.38-3.50)	**<0.01**	0.10
Leukocytes	0.99 (0.92-1.07)	0.68	1.00 (0.94-1.06)	0.89	0.99 (0.89-1.08)	0.56	0.69
TCh	0.70 (0.65-0.78)	**<0.01**	0.78 (0.70-0.85)	**<0.01**	0.66 (0.61-0.71)	**<0.01**	**<0.01**
LDL-C	0.65 (0.55-0.75)	**<0.01**	0.72 (0.66-0.85)	**<0.01**	0.58 (0.53-0.66)	**<0.01**	**<0.01**
HDL-C	0.99 (0.91-1.06)	0.31	1.03 (0.94-1.06)	0.85	0.98 (0.88-1.04)	0.15	0.19
TGs	0.82 (0.62-0.96)	**<0.01**	0.84 (0.74-1.00)	**<0.01**	0.76 (0.56-0.92)	**<0.01**	0.14
VEGF	0.89 (0.80-1.01)	**<0.01**	0.89 (0.83-0.96)	**<0.01**	0.88 (0.78-1.03)	**0.016**	0.99
Endostatin	0.97 (0.90-1.06)	0.22	1.00 (0.92-1.09)	0.97	0.96 (0.86-1.03)	0.11	0.28
MCP-1	0.99 (0.89-1.10)	0.42	0.97 (0.87-1.08)	0.32	1.01 (0.91-1.10)	0.81	0.45
CRP	0.74 (0.53-1.00)	**<0.01**	0.84 (0.61-1.00)	0.057	0.72 (0.50-0.95)	**<0.01**	0.31

[1] k – change coefficient (e.g. 1.72 means increase by 72%, 0.63 – decrease by 37% etc.). [2] Wilcoxon test [3] Mann-Whitney test (comparison of k between groups 1 and 2). IHD, ischemic heart disease; EPC, endothelial progenitor cells; TCh, total cholesterol; LDL-C, low density lipoprotein; HDL-C, high density lipoprotein; TG, triglycerides; VEGF, vascular endothelial growth factor; CRP, C-reactive protein; MCP-1, monocyte chemotactic protein-1

Table 4. Effect of statin dose and baseline EPC, TCh and LDL-C levels on their dynamics

Hypothesis: dynamics of the parameter depends on	**Parameter**					
	TCh		LDL-C		EPC	
	p	Result	p	Result	p	Result
Baseline parameter level	0.99	False	0.43	False	0.01	True
Statin dosage	<0.01	True	<0.01	True	0.38	False

EPC, endothelial progenitor cells; TCh, total cholesterol; LDL-C, low density lipoprotein.

As shown in Table 4, baseline TCh and LDL-C levels did not influence their decrease rate. At the same time EPC gain rate did not depend on statin dose, but did depend on their baseline counts: the lower were baseline EPC counts, the higher was their gain rate due to therapy. Those results allowed us to perform correlation analysis of the relationship between the EPC gain and TCh/LDL-C decrease, as well as HDL-C/TGs dynamics. EPC gain was inversely proportional to the LDL-C/TCh level increase (i.e. directly proportional to their level decrease), the results shown in Table 5.

Table 5. The relationships between EPC gain rate (k_{EPC}) and lipid profile dynamics during atorvastatin therapy

	IHD (n=58)		Group 1 (10 mg, n=26)		Group 2 (40 mg, n=32)	
	r	p	r	p	r	p
TCh	**-0.37**	**<0.01**	**-0.52**	**<0.01**	-0.21	0.26
LDL-C	**-0.41**	**<0.01**	**-0.44**	**0.03**	-0.26	0.15
HDL-C	0.00	1.00	0.06	0.78	-0.03	0.87
TGs	0.00	0.98	-0.09	0.67	0.09	0.63

r – Spearman's rank correlation coefficient; p – significance level for r.
EPC, endothelial progenitor cells; IHD, ischemic heart disease; TCh, total cholesterol; LDL-C, low density lipoprotein; HDL-C, high density lipoprotein; TG, triglycerides.

4. Discussion

The mechanisms underlying statin effects on increase of different EPC phenotypes are not fully understood. Nevertheless, some clinical studies already attempt to use it. For instance, ARMIDA study demonstrated positive effects of intensive statin therapy before percutaneous coronary intervention [42]. As a further step, HIPOCRATES study investigates high-dose statin therapy effect on stent endothelialization [43]. The authors emphasize that the positive pleiotropic effects of statins, not connected directly with lipid levels lowering, but still improving revascularization outcomes, are not fully understood. The authors suggested that higher EPC counts are able to reverse endothelium damage, which occurs during stent implantation.

It remained unclear whether stimulating statin effects on EPC are dose-dependent. This aspect was our main aim of investigation. Beside this, our study showed significant differences of a number of parameters in IHD patients compared to healthy volunteers. EPC counts in IHD patients were on average four times lower (p<0.01), VEGF level by 52% higher (p<0.01), endostatin level by 13% lower (p<0.05), compared to the control group. 3-month atorvastatin treatment led to EPC counts gain on average by 72% (p<0.05). EPC gain rate did not depend on statin dose (p=0.38), but it was higher when baseline EPC counts were low (p=0.01). Besides expected decrease of TCh/LDL-C levels during the therapy, there was 11% decrease of VEGF level (p<0.05), endostatin level has not changed significantly (p=0.22). There was a relationship between the dynamics of EPC, TCh and LDL-C during therapy: higher EPC gain rate corresponded to greater reduction of TCh (r = -0.37, p<0.01) and LDL-C (r = -0.41, p<0.01).

Discussing the obtained results, importance of EPC measurement method must be noted, as various sets of expressed cell markers lead to determination of the same cells at different stages of development. The kit we used allowed us to identify a rather rare phenotype of "young" EPC, but it required enhancement of cytometry sensitivity, which led to a large spread of EPC counts both in main group and in control group: results from 100 to 2000 cells per 10 ml of blood were received. This variation may be a cause of statistically insignificant results in groups that in other circumstances might reveal significant differences and dependences. For instance, EPC gain rate dose-independence had Mann-Whitney confidence level (p) equal to 0.10, elevated EPC counts in the smokers subgroup – 0.053, reduced EPC counts in diabetes subgroup – 0.066. Smoking IHD patients showed elevated EPC counts and VEGF level, and decreased endostatin level. These results are contrary to some published data and require further study. Such an increase of pro-angiogenic factors may perhaps be a temporary involvement of reserves of the organism, which occurs in response to smoking as a vascular system damaging factor.

The most important result of our study is that the dependence of EPC gain rate on atorvastatin dose was unreliable. This to some extent corresponds to the results of clinical trials (TNT, IDEAL), which claimed that the clinical benefit of higher doses of statins to moderate doses is on the verge of reliability [44,45]. On the other hand, in a study by Antonio et al., dedicated to different doses of statin therapy in patients with myocardial infarction within 3 months, a higher EPC counts gain in the intensive statin therapy group was detected [46]. This result can be explained by another patient contingent, as well as another cell phenotype in that study. Our results confirmed a study by Pesaro et.al, where patients with stable coronary artery disease did not show further EPC count growth when increasing simvastatin dose from 20 to 80 mg or addition of ezetimibe [47].

The main clinical result of our work may be a suggestion that pleiotropic statin effects are activated even at low doses of statins. This confirms the known fact that intensity of lipid-lowering therapy must be guided only by the target level of LDL-C. More specific clinical applications of received information about the effects of different doses of statins on EPCs requires further study.

Conflict of Interest

None declared.

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