The influence of statin monotherapy and statin-ezetimibe combined therapy on FoxP3 and IL 10 mRNA expression in patients with coronary artery disease

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2019;28(9):1243–1248

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Funding sources
The study was funded by the Medical University of Lodz grant No. 502/5-16501/502-54-168, granted to Paulina Jackowska, and grant No. 03/5-165-01/503-51-001.

Conflict of interest
None declared

Received on August 2, 2018
Reviewed on November 3, 2018
Accepted on April 18, 2019

Published online on August 20, 2019

Abstract

Background. FoxP3 is a marker of human T regulatory cells (Tregs), which are supposed to play an important role in the pathophysiology of atherosclerosis. Interleukin 10 (IL-10) is a cytokine with pleiotropic, immunoregulatory properties, produced mostly by Tregs and B regulatory cells. Due to their anti-inflammatory action, both Tregs and IL-10 are believed to inhibit plaque development and decrease atherosclerosis progression. The effect of hypolipidemic drugs – statins or ezetimibe – on FoxP3-positive Tregs and anti-inflammatory cytokines, such as IL-10, is still unclear.

Objectives. The objective of the study was to investigate the effects of 3 different therapies of equivalent hypolipidemic activity: atorvastatin, rosuvastatin, and combination therapy of atorvastatin and ezetimibe on FoxP3–Tregs transcription factor and IL-10 mRNA expression in peripheral blood mononuclear cells (PBMCs) from patients with stable coronary artery disease (CAD).

Material and methods. Sixty-five patients with diagnosed CAD participated in the study. They were randomly assigned to 3 therapeutic groups: atorvastatin at a dose of 40 mg/day (A40 group); rosuvastatin 20 mg/day (R20 group); and atorvastatin 10 mg/day combined with ezetimibe 10 mg/day (A10+E10 group). After 1 month and 6 months of therapy, the mRNA expression for FoxP3 and IL-10 in PBMCs was evaluated using real-time polymerase chain reaction (RT-PCR) and lipid parameters.

Results. An improvement in lipid parameters was observed in each of the groups studied; however, hypolipidemic treatment did not induce any change in FoxP3 and IL-10 mRNA expression. After 6 months, an increase in FoxP3 mRNA expression was noted in A40 group as compared to R20 group.

Conclusions. None of the therapies of equal hypolipidemic efficacy affected FoxP3 and IL-10 mRNA expression in patients with stable CAD.

Key words: statins, ezetimibe, FOXP3, IL-10, regulatory T cells

Cite as
Jackowska P, Chałubiński M, Łuczak E, et al. The influence of statin monotherapy and statin-ezetimibe combined therapy on FoxP3 and IL 10 mRNA expression in patients with coronary artery disease. *Adv Clin Exp Med*. 2019;28(9):1243–1248. doi:10.17219/acem/108627

DOI
10.17219/acem/108627

Introduction

Statins are well-established medications for both the primary and secondary prevention of cardiovascular disease. If a patient does not achieve low-density lipoprotein cholesterol (LDL-C) goal of <70 mg/dL during conventional statin therapy, 3 other therapeutic options are available: firstly, an increase in statin daily dose (however, the expected improvement is estimated at only 6–8%[1]); secondly, implementation of statin with more potent hypolipidemic features, such as rosuvastatin in a dose of 40 mg/day or atorvastatin in a dose of 80 mg/day; and finally, a combination of statin with ezetimibe-inhibitor of cholesterol absorption in the brush border of small intestine.[2] One should emphasize that in spite of many different statins available there is no consensus regarding which of the above therapeutic options is optimal. Not only equal hypolipidemic effect of these options, but also divergent pleiotropic and immunomodulatory properties should be considered as the reason of differences in their clinical efficacy. Cases concerning anti-inflammatory features of statins and their effect on the regulation of Th1/Th2 homeostasis are well-documented.

FoxP3 is a marker of human T regulatory cells (Tregs), which are supposed to play an important role in the pathophysiology of atherosclerosis not only by the inhibition of pro-atherogenic Th1 and Th17 lymphocyte-mediated immune mechanisms, but also by the activation and migration of dendritic cells towards the plaque, suppression of inflammatory macrophages and their conversion into foam cells, and finally, reduction in the activation of endothelial cells.[3]

Interleukin 10 (IL-10) is a cytokine with pleiotropic, immunoregulatory properties, produced mostly by Tregs and B regulatory cells.[4,5] Due to their anti-inflammatory properties, both Tregs and IL-10 are believed to inhibit the plaque development and decrease atherosclerosis progression.[6] The effect of 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase inhibitors on FoxP3-positive Tregs and anti-inflammatory cytokines, such as IL-10, is still unclear.[7–10]

Therefore, the aim of our study was to investigate the effects of 3 different therapies of equivalent hypolipidemic activity: atorvastatin, rosuvastatin, and combination therapy of atorvastatin and ezetimibe on both FoxP3, the Tregs transcription factor, and IL-10 mRNA expression in peripheral blood mononuclear cells (PBMCs) from patients undergoing the secondary prevention therapy. The second aim of the study was to assess the association of hypolipidemic activity of atorvastatin, rosuvastatin, and a combination of atorvastatin and ezetimibe with the mRNA expression of FoxP3 and IL-10 in PBMCs from these patients.

This project was approved by the Bioethics Committee of the Medical University of Lodz, Poland (approval No. RNN/846/12/KB).

Material and methods

Sixty-one patients aged 44–76 years participated in the study. They had undergone a myocardial infarction and/or percutaneous coronary intervention (PCI) and/or coronary artery bypass grafting (CABG) in the period over 6 months, and despite ongoing hypolipidemic treatment (simvastatin 10–40 mg/day, lovastatin 10–40 mg/day, atorvastatin 10–30 mg/day, rosuvastatin 5–15 mg/day) did not achieve the level of LDL-C < 70 mg/dL.

Exclusion criteria were the following: type 1 and type 2 diabetes mellitus, chronic kidney disease at stage IV or V (estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m^2), New York Heart Association (NYHA) class III–IV heart failure with ejection fraction (EF) <40%, severe hypertension, acute infection within the past 2 weeks, autoimmune and allergic diseases, thyroid diseases, liver diseases and impairment of hepatic functions at active stage or with persistent elevated serum aminotransferase activity (more than 3 times the upper normal limit of non-defined cause), myopathy, myalgia, the state after rhabdomyolysis, documented cases of cancer, administration of glucocorticoids and other immunomodulatory drugs, HIV infection, pregnancy and lactation, women of childbearing potential not using effective contraception, alcohol abuse, smoking during the last 6 months, hypersensitivity to statin and ezetimibe components, statin or ezetimibe intolerance, therapy with rosuvastatin at a dose ≥20 mg/day, atorvastatin ≥40 mg/day, or combination therapy with statin and ezetimibe prior to randomization.

Qualified patients were randomly assigned to 3 therapeutic groups. Twenty patients received atorvastatin 40 mg/day (A40 group); 21 patients – rosuvastatin 20 mg/day (R20 group); and 20 patients – combination therapy: atorvastatin 10 mg/day with ezetimibe 10 mg/day (A10+E10 group). The study material was collected from patients at 3 different timepoints of the treatment: before the treatment, and after 1 month and 6 months of the treatment.

Therapeutic groups were homogeneous. They did not differ in terms of lipid parameters, body mass index (BMI), liver enzymes, and glycemia. However, they differed in high-sensitivity C-reactive protein (hsCRP) – its highest significant values before the treatment were observed in A40 group (mean values did not exceed the upper limit) and lowest in R20 group (Table 1). No significant changes in the activity of liver enzymes or glycemia were observed in the therapeutic groups. There were no muscular complaints and no significant increase in creatine kinase (CK).

Peripheral blood mononuclear cell isolation

Blood collected by venipuncture in sodium heparin vacuum tubes was diluted in phosphate-buffered saline (PBS) (1:3) (Biomed, Lublin, Poland) and centrifuged on Histopaque 1077 density gradient (Sigma-Aldrich, St. Louis, USA) at 800 g.

mRNA extraction, complementary DNA (cDNA) preparation, RT-PCR

mRNA was isolated using a RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Potential genomic DNA contamination was removed with on-column DNase I digestion, and 10 µg of mRNA was reverse-transcribed with a High-Capacity cDNA Kit (Applied Biosystems, Foster City, USA). Polymerase chain reaction (PCR) was then carried out using the Applied Biosystems 9700HT Fast Real-Time PCR System (Applied Biosystems). The PCR mixture consisted of cDNA solution, SYBR-Green PCR Mastermix (Applied Biosystems) and both sense and antisense primers. The reaction was conducted as follows: 4 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Elongation factor-1α (EF1-α) was amplified as a housekeeping gene. FoxP3 and IL-10 mRNA expression was normalized to EF1-α using ΔΔCt calculation. The following primers were used:

FoxP3 forward: 5'-CGGACCATCTTCTGGATGAG-3', reverse: 5'-TTGTCGGATGATGCCACAG-3';
IL-10: forward: 5'-GTGATGCCCCAAGCTGAGA-3', reverse: 5'-CACGGCCTTGCTCTTGTTTT-3';
EF1-α: forward: 5'-CTGAACCATCCAGGCCAAAT-3', reverse: 5'-GCCGTGTGGCAATCCAAT-3'.

Laboratory tests

Complete blood count was determined using a 5diff hematology analyzer (UniCel DxH 800 Coulter Cellular Analysis System, USA). Glucose level and lipid profile (LDL-C was determined with direct method) were evaluated with the enzymatic method. The creatinine level was determined using the compensated Jaffe colorimetric method. Estimated glomerular filtration rate (eGFR) was calculated from the Modification of Diet in Renal Disease (MDRD) equation. Alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured using the kinetic method with nicotinamide adenine dinucleotide hydrogen (NADH) and Tris buffer (according to the International Federation od Clinical Chemistry and Laboratory Medicine (IFCC). Creatine kinase was determined with the N-Acetyl-Cysteine (NAC) method.

Table 1. Characteristics of the selected parameters of 3 therapeutic groups (A40, R20 and A10+E10) prior to the treatment

Parameter	A40	R20	A10+E10
Age [years]	61.80 (±7.10)	61.95 (±6.71)	63.65 (±7.39)
Men/women, n	18/2	12/9	16/4
BMI [kg/m^2]	26.66 (±2.60)	26.41 (±2.25)	25.76 (±2.18)
TC [mg/dL]	185.45 (±28.21)	181.62 (±38.20)	183.40 (±36.62)
LDL-C [mg/dL]	111.85 (±20.22)	114.52 (±27.83)	110.70 (±30.49)
HDL-C [mg/dL]	52.70 (±12.42)	54.77 (±16.69)	53.49 (±9.32)
Non-HDL-C [mg/dL]	132.75 (±28.1)	126.85 (±32.96)	129.92 (±38.26)
TG [mg/dL]	130.95 (±55.40)	134.43 (±57.19)	131.80 (±57.77)
SBP [mm Hg]	129.75 (±15.77)	127.14 (±10.79)	125.65 (±14.60)
DBP [mm Hg]	78.25 (±10.92)	80.71 (±6.76)	74.35 (±7.17)
HR [beats/min]	67.10 (±9.44)	68.38 (±9.54)	72.90 (±8.74)
AST [U/L]	24.10 (±8.77)	25.52 (±6.74)	23.05 (±6.25)
ALT [U/L]	26.95 (±15.63)	25.60 (±10.56)	28.63 (±10.52)
CK [IU/L]	123.45 (±38.85)	131.35 (±44.20)	155.00 (±96.88)
hsCRP [mg/L]	2.27 (±1.45)[a]	1.21 (±0.91)[a]	1.58 (±1.44)
Glucose [mg/dL]	93.83 (±6.66)	94.47 (±10.12)	93.88 (±8.06)
WBC [× 10^3/µL]	6.86 (±1.84)	6.34 (±1.45)	5.89 (±1.57)
RBC [× 10^6/µL]	4.84 (±0.38)	4.60 (±0.32)	4.67 (±0.37)
Hb [g/dL]	15.10 (±1.07)	14.64 (±1.00)	14.77 (±1.15)
Ht [%]	45.15 (±3.02)	43.86 (±2.76)	44.54 (±3.32)
PLT [× 10^3/µL]	181.58 (±36.86)	199.10 (±43.10)	202.61 (±38.75)
Creat [mg/dL]	0.96 (±0.12)	0.79 (±0.16)	0.92 (±0.16)
eGFR [mL/min/1.73 m^2]	83.40 (±10.31)	96.53 (±17.39)	87.36 (±15.29)

Data is presented as mean ± standard deviation (SD); p-value <0.05 is considered statistically significant; [a] $p < 0.05$; BMI – body mass index; TC – total cholesterol; LDL-C – low-density lipoprotein cholesterol; HDL-C – high-density lipoprotein cholesterol; Non-HDL-C – non-high-density lipoprotein cholesterol; TG – triglycerides; SBP – systolic blood pressure; DBP – diastolic blood pressure; HR – heart rate; ALT – alanine aminotransferase; AST – aspartate transaminase; CK – creatine kinase; hsCRP – high sensitivity C-reactive protein; WBC – white blood cells; RBC – red blood cells; Hb – hemoglobine; Ht – hematocrit; PLT – platelets; Creat – creatinine; eGFR – estimated glomerular filtration rate.

Statistical analysis

The results obtained were subject to statistical analysis using STATISTICA PL v. 7.1. (StatSoft Polska, Kraków, Poland) and PQSTAT v. 1.6.2. (PQStat Software, Poznań, Poland). For comparison of quantitative variables measured at subsequent time intervals, the Student's t-test was used for repeated measurements if the condition of normality of distributions (comparing the results of 2 measurements) was fulfilled. The Wilcoxon signed-rank test (for 2 measurements) and the nonparametric Friedman's analysis with the Dunn–Bonferroni post-hoc test (for 3 measurements) were used to compare repeated results in the absence of normal distribution. For comparison of patient groups, when the assumption of normality of distributions was fulfilled, the analysis of variance was used (equality of variances was determined with Levene's test) and, in the case of rejecting the hypothesis of equality of variances, the modified Brown–Forsythe's test was applied. In the absence of normal distribution, the non-parametric Mann–Whitney U test (for 2 groups) and the Kruskal–Wallis test with the Dunn–Bonferroni POST-HOC test (for 3 groups) were used.

Correlations were tested with Spearman's tests. In all tests, a p-value lower than 0.05 was considered statistically significant.

Results

The effect of atorvastatin, rosuvastatin and combination therapy: atorvastatin with ezetimibe on the FoxP3 and IL-10 mRNA expression in PBMCs

The value 1 was regarded as the level of the FoxP3 and IL-10 mRNA expression before the treatment. Relative changes related to the value before the treatment, as well as after 1 month and 6 months of therapy, were defined. No statistically significant differences in FoxP3 and IL-10 mRNA (Fig. 1) expression induced by hypolipidemic therapy were detected in any study group and in any patient after 1 month and 6 months of the treatment. While comparing all the groups with each other at the same timepoints, the statistically significant differences were noted between groups A40, R20 and A10+E10 for FoxP3 mRNA expression after 6 months of therapy (p = 0.015). The comparison of the groups in pairs revealed statistically significant differences only between A40 group and R20 group. An increase in FoxP3 mRNA expression in A40 group after 6 months of therapy was significantly greater as compared to R20 group (p = 0.012); however, it did not significantly differ from FoxP3 mRNA expression in A10+E10 group (p = 0.466) (Fig. 1).

The effect of atorvastatin, rosuvastatin and combination therapy

In each therapeutic group, a significant improvement in lipid parameters was observed. In A40 group, after 6 months of therapy, the level of LDL-C decreased by 21% (p = 0.006) and non-high-density lipoprotein cholesterol (non-HDL-C) by 22% (p = 0.003); in R20 group, a reduction by 24% (p < 0.001) and 21% (p = 0.001), respectively, was found; and in A10+E10 group, the LDL-C level decreased by 28% (p < 0.001) and non-HDL-C by 29% (p < 0.001) (Fig. 2). There were no statistically significant differences between the levels of lipids (TC, LDL-C, HDL-C, non-HDL-C, TG) in particular groups of patients at defined timepoints (prior to the treatment, after 1 month and 6 months of the treatment).

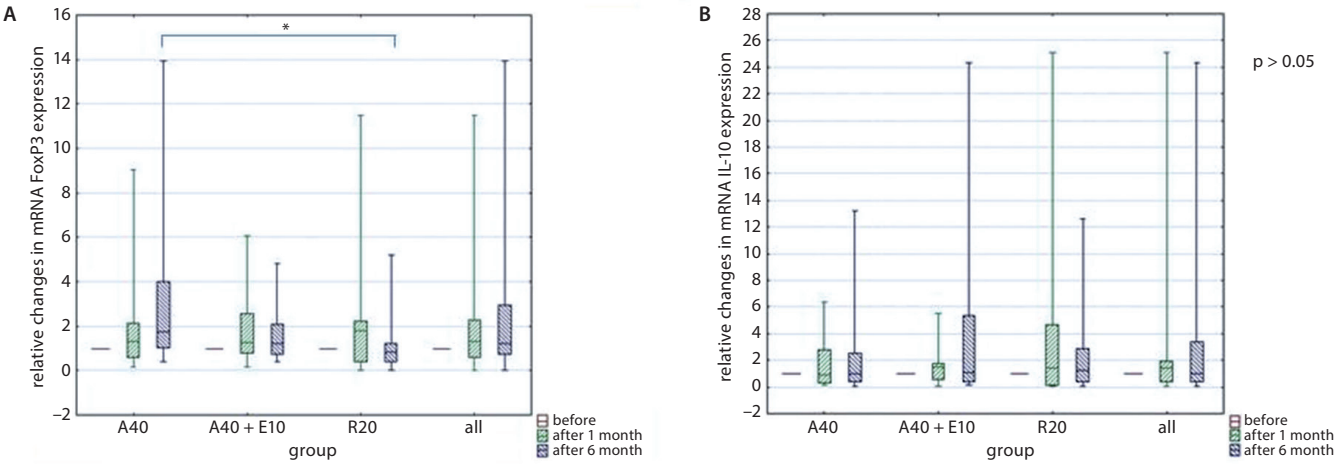

Fig. 1. A – relative changes in FoxP3 mRNA expression for particular groups (A40, A10+E10 and R20) and all patients after 1 and 6 months of therapy (p < 0.05); B – relative changes in IL-10 mRNA expression for particular groups (A40, A10+E10 and R20) and all patients after 1 and 6 months of therapy

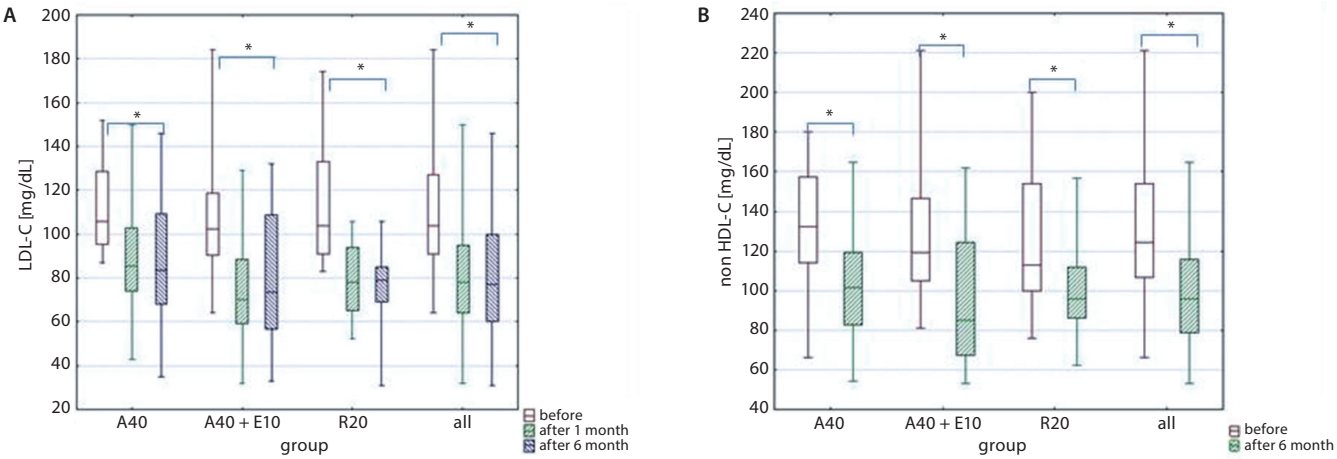

Fig. 2. A – comparison of the LDL-C level for particular study groups (A40, A10+E10 and R20) and in all patients after 1 and 6 months of therapy (p < 0.05). B – comparison of the non-HDL-C level for particular study groups (A40, A10+E10 and R20) and in all patients after 1 and 6 months of therapy (p < 0.05)

FoxP3 and IL-10 mRNA expression in patients achieving and not achieving the expected LDL-C level goal of <70 mg/dL after 1 and 6 months of therapy

In A40 group after 1 month of therapy, the level of LDL-C <70 mg/dL was achieved by 4 of 20 patients, and after 6 months by 6 of 20 patients. In R20 group, 7 of 21 and 6 of 21 patients achieved the goal, respectively, and in A10+E10 group – 10 of 20 and 9 of 20 patients, respectively. Due to the small number of patients with LDL-C <70 mg/dL, the analysis was carried out in all therapeutic groups. Table 2 shows the changes in FoxP3 and IL-10 mRNA expression, taking into consideration the division of patients in relation to LDL-C level. There were no statistically significant changes in FoxP3 and IL-10 mRNA expression in patients with achieved and non-achieved LDL-C goal <70 mg/dL, both after 1 month and 6 months of hypolipidemic treatment ($p > 0.05$).

Correlations between the decrease in the LDL-C level and changes in FoxP3 and IL-10 mRNA expression for particular groups and all patients after 1 month and 6 months of therapy

No correlations were observed between the reduction in the LDL-C level and changes in FoxP3 and IL-10 mRNA expression related to hypolipidemic therapy in particular groups as well as in all patients studied.

Positive correlations were recorded between changes in FoxP3 and IL-10 mRNA expression for R20 group ($r = 0.47$, $p = 0.031$) after 1 month of therapy and for A40 group ($r = 0.582$, $p = 0.007$) and R20 group ($r = 0.48$, $p = 0.026$) after 6 months. There were no such relationships observed for A10+E10 group. A significant positive correlation for all studied population occurred between those parameters after 1 month ($r = 0.395$, $p = 0.002$) and 6 months ($r = 0.269$, $p = 0.036$) (Table 3).

Table 3. The association between relative changes of FoxP3 mRNA and IL-10 mRNA expression for A40, A10+E10 and R20 groups and all studied population after 1 month and 6 months of therapy

Period	Group	R Spearman's	p-value
After 1 month	A40	0.438	0.054
	A10+E10	0.130	0.586
	R20	0.471	**0.031**
	all	0.394	**0.002**
After 6 months	A40	0.582	**0.007**
	A10+E10	–0.125	0.600
	R20	0.485	**0.026**
	all	0.269	**0.036**

Spearman's R – Spearman's correlation; IL-10 – interleukin 10; statistically significant p-values are marked in bold.

Discussion

Apart from hypolipidemic features, statins were shown to have anti-inflammatory properties, which may vary depending on the particular medicine. However, their effect on immunomodulatory mechanisms regarding Tregs and Treg-driven mechanisms is still not clear. Interestingly, decreased activity of Tregs and a lower production of IL-10 is believed to be engaged in the atherosclerosis development, leading to the domination of pro-inflammatory Th1-related immune mechanisms. Therefore, it is hypothesized that statins might exert an anti-atherosclerotic effect not only by lowering cholesterol levels, but also through the induction of Tregs-dependent immunosuppressive responses.

In our study, neither rosuvastatin nor atorvastatin influenced FoxP3 and IL-10 mRNA expression in PBMCs from patients with stable coronary artery disease (CAD) upon 1 month and 6 months of therapy. Similarly, ezetimibe added to atorvastatin did not trigger any additional effect. Our results are in line with the data indicating that systemic administration of atorvastatin and pravastatin to mice did not affect FoxP3 mRNA expression and population of Tregs.[8] In contrast, in Rodriguez-Perea et al. 1-month study treatment with atorvastatin (20 mg/day) or lovastatin (40 mg/day) increased both FoxP3-positive cell number and FoxP3 mRNA expression.[9] Moreover,

Table 2. Characteristics of relative changes in the FoxP3 and IL-10 mRNA expression of patients with LDL-C ≥ 70 mg/dL and LDL-C < 70 mg/dL after 1 and 6 months of therapy

Time period	FoxP3 mRNA expression			IL-10 mRNA expression		
	LDL-C < 70	LDL-C ≥ 70	p-value	LDL < 70	LDL ≥ 70	p-value
After 1 month	1.49 (±1.32)	2.05 (±2.35)	NS	2.97 (±6.39)	2.56 (±3.66)	NS
After 6 months	1.42 (±1.32)	2.24 (±2.49)	NS	4.04 (±5.72)	2.61 (±4.34)	NS

Data is presented as mean (±SD); p-value <0.05 is considered statistically significant; LDL-C – low-density lipoprotein cholesterol; IL-10 – interleukin 10; NS – not significant.

Mausner-Fainberg et al. evidenced that after a 8-week course of simvastatin (20 mg/day) or pravastatin (10–40 mg/day) of hypercholesterolemic patients, Tregs number and FoxP3 mRNA expression were increased as compared to the timepoint before implementation of therapy.[8] One should consider that those patients were not receiving any statins prior to the inclusion into the study. Similarly, if compared to placebo, atorvastatin was also shown to increase the number of Tregs, FoxP3 mRNA expression and IL-10 serum levels in patients with a history of myocardial infarction.[10–12]

Why do our results differ from the clinical data? Firstly, in our study, only patients who were already receiving statins before being included to the study were analyzed, but still did not achieve proper LDL-C level (<70 mg/dL) and, therefore, they needed intensification of treatment. One may assume that previous therapy with statins might have already affected FoxP3 and IL-10 mRNA expression. Secondly, we could not establish placebo arm, as statin-based hypolipidemic therapy is obligatory for the secondary prevention of cardiovascular events according to all current guidelines.[2] This may be the cause why we did not observe any statistically significant effect of atorvastatin or rosuvastatin on immunological parameters in our study. Our data revealed higher FoxP3 mRNA expression in the group treated with atorvastatin for 6 months, as compared to the group receiving rosuvastatin. All this data taken together suggests that the effect of statins on FoxP3 and IL-10 mRNA expression and Tregs population may depend on the type and dose of statin as well as the period of the treatment. One should underline that there was a strong positive correlation between FoxP3 and IL-10 mRNA expression, which indicates that IL-10 is produced mainly by FoxP3-positive Tregs in PBMCs.

The effect of hypolipidemic therapy on FoxP3 and IL-10 mRNA expression was analyzed with regard to its influence on lowering of LDL-C in our study. After 6 months, LDL-C level significantly decreased by 21% in atorvastatin group, in rosuvastatin group by 24% and in patients receiving atorvastatin with ezetimibe by 28%. In spite of significant mean hypolipidemic effect in each group, there were patients who did not achieve cholesterol level below 70 mg/dL. However, there was no effect of cholesterol lowering therapy on FoxP3 and IL-10 mRNA expression, independently on the drugs received, in the group with LDL-C concentrations below 70 mg/dL achieved, and in the group with LDL-C above 70 mg/dL. Furthermore, we did not observe any correlation between relative changes in LDL-C concentrations and FoxP3 or IL-10 mRNA expression. Our data is consistent with studies of other authors, showing no association between the lipid profile with changes in FoxP3 and IL-10 mRNA expression in patients receiving statins.[11] This indicates that cholesterol levels are not associated with the mRNA expression of FoxP3 and IL-10.

In summary, none of the therapies of equal hypolipidemic efficacy, such as atorvastatin at a dose of 40 mg/day, rosuvastatin 20 mg/day, and atorvastatin 10 mg/day combined with ezetimibe 10 mg/day, affected FoxP3 and IL-10 mRNA expression in patients with history of CAD. However, therapy with atorvastatin seems to determine higher expression of these immunoregulatory parameters as compared to rosuvastatin. As most patients had already been on cholesterol-lowering therapy before inclusion to the study and they received average doses of statins during our study, one cannot exclude that maximal doses of statins used in lipid-lowering therapy (i.e., atorvastatin 80 mg, rosuvastatin 40 mg) might affect both FoxP3 and IL-10 mRNA expression in PBMCs of CAD patients. Therefore, further investigations are required.

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