

## RETRACTED

## Short term effects of atorvastatin on leucocytes integrin expression in unstable angina

### Efecte de scurtă durată ale atorvastatinei asupra expresiei integrinelor leucocitare în angina pectorală instabilă

Minodora Dobreanu<sup>1\*</sup>, D. Dobreanu<sup>2</sup>, Catrinel Gălățeanu<sup>3</sup>, Andrea Fodor<sup>1</sup>, Anca Bacârea

<sup>1</sup> U.M.P. Tg.Mureș, Dept. of Clinical Biochemistry – Immunology,

<sup>2</sup> U.M.P. Tg.Mureș, Dept. of Physiology,

<sup>3</sup> Pfizer România

#### Summary

*Inflammatory reactions in coronary plaques play an important role in the pathogenesis of acute atherothrombotic events. The most powerful class of lipid-lowering drugs available – statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) – have additional actions, unrelated to cholesterol reduction, including anti-inflammatory and immunomodulatory properties. This study sought to determine if atorvastatin affects monocyte and lymphocyte activation in patients with unstable angina and mild primary hypercholesterolemia. Following a 4-weeks hypolipemiant-free baseline period, 22 patients -12 with unstable angina (UA) and 10 patients with stable coronary heart disease (SCHD) – were treated with Atorvastatin 20 mg/day. Lipopolysaccharide (LPS)-receptor (CD14) and HLA-DR expression on monocytes and beta-integrins (CD11b, 11c, 49d) on monocytes and lymphocytes were measured by flow cytometry before and after treatment with atorvastatin for 8 weeks. Monocyte CD11b, 11c and CD14 expression and T lymphocytes CD11b expression were significantly ( $p < 0.001$ ) higher in UA patients before treatment when compared with those in SCHD patients. In patients with UA, they decreased markedly with atorvastatin treatment. The reduction in expression of adhesion molecules on monocytes and lymphocytes and in concentrations of C reactive protein (CRP) and sICAM-1 may crucially contribute to the clinical benefit of atorvastatin in coronary artery disease, independent of cholesterol lowering effects.*

**Key words:** atherosclerosis, adhesion molecule, inflammation.

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\* **Adresa pentru corespondență:** Minodora Dobreanu, UMF Tg.Mureș, Dept. Laborator- Biochimie Clinică, Str. Gh. Marinescu 38, 540136, Tg. Mureș, România  
Tel./Fax.: 0265 217 425, E-mail: mdobreanu@yahoo.com

## Rezumat

Reacțiile inflamatorii în plăcile de aterom joacă un rol important în patogeneza evenimentelor aterotrombotice acute. Cea mai puternică clasă de hipolipemiante disponibile – statinele (inhibitori de 3-HMG coenzimă A reductază) – au, se pare, și efecte pleiotrope, independente de efectul hipocolesterolemiant (anti-inflamator și imunomodulator). Acest studiu și-a propus să analizeze dacă tratamentul de scurtă durată cu atorvastatin influențează activitatea monocitelor și limfocitelor la pacienți cu angină instabilă și hipercolesterolemie moderată. După o perioadă de 4 săptămâni fără tratament hipolipemiant, am inclus în studiu 22 de pacienți – 12 cu angină instabilă (UA) și 10 cu boală coronariană stabilă (SCHD) - având în schema terapeutică și atorvastatin 20 mg/zi. S-a analizat prin citometrie în flux expresia receptorului lipopolizaharidic (LPS R) și a HLA-DR pe suprafața monocitelor și a beta-integrinelor (CD11b, 11c, 49d) pe monocite și limfocite, înainte și după tratamentul cu Atorvastatin timp de 8 săptămâni. Înainte de inițierea tratamentului, expresia monocitară a CD11b, 11c și a CD14, precum și expresia CD11b pe suprafața limfocitelor T au fost semnificativ crescute ( $p < 0,001$ ) la pacienții cu angină instabilă, comparativ cu cei cu angină stabilă. La pacienții cu angină instabilă, tratamentul a scăzut semnificativ nivelurile acestor markeri, dar și concentrațiile Proteinei C-Reactive și a moleculei de adeziune celulară solubilă sICAM-1. Aceste efecte ar putea contribui la beneficiul clinic imediat al tratamentului cu atorvastatin la pacienții cu sindroame coronariene instabile, independent de efectul hipocolesterolemiant.

**Cuvinte cheie:** ateroscleroză, molecule de adeziune, inflamație.

## Introduction

Atherosclerosis is clearly multifactorial and it is now universally recognised that inflammation within the lesions contributes importantly to their initiation and progression<sup>9</sup>. The immunologic response in atherosclerosis involves not only cells of the arterial wall, but also circulating lymphocytes and macrophages. A potentially important role of inflammation in the onset of acute ischemic syndromes is indicated by peripheral blood mononuclear cells (PBMCs) activation and elevated levels of various acute-phase proteins in unstable angina<sup>2,3,6</sup>. Interaction of the peripheral blood mononuclear cells adhesive receptor CD11b/CD18 (Mac-1, a  $\beta$ 2-integrin heterodimer) with intercellular adhesion molecule-1 (ICAM-1) has been involved in the adhesion to and migration across endothelium of monocytes and T lymphocytes. Patients with ischemic heart disease and peripheral arterial vascular disease have elevated levels of circulating adhesion molecules (sICAM-1 and P-selectin), compared

with asymptomatic control subjects<sup>1</sup>. The amount of soluble ICAM-1 and E-selectin released has been demonstrated to be directly correlated with the surface expression of ICAM-1 and E-selectin in endothelial cells in culture<sup>8</sup>. *Ex vivo* adhesion to endothelial cells of monocytes from hypercholesterolemic patients is greater than in normocholesterolemic persons<sup>5</sup>.

The aim of this study was to determine whether atorvastatin (one of the most effective 3HMG-CoA reductase inhibitors) affects monocyte and lymphocyte activation in patients with unstable angina and mild primary hypercholesterolemia, the levels of soluble adhesion molecules and their receptors expression on PBMCs.

## Patients and protocols

Following a 4-weeks hypolipemiant-free baseline period, 12 patients with unstable angina UA (clinical and ECG positive criteria) and 10 with stable coronary artery disease SCHD (without acute coronary event within the

past 3 months), gave their informed consent for the study. All patients had mild hypercholesterolemia ( $130 \text{ mg}\% \leq \text{LDL-Chol} \leq 160 \text{ mg}\%$ ). The criteria for involvement in the screening program were coronary heart disease (diameter coronary artery stenosis  $\geq 70\%$  in at least one major coronary artery branch), lack of infection and systemic disorders unrelated to lipid or lipoprotein metabolism. The age distribution and lipid parameters of the patients are given in *Table 1*.

Exclusion criteria were as follows: triglycerides  $\geq 400 \text{ mg}\%$ , CPK and ALAT  $\geq 3$  times upper normal limit, creatinine  $\geq 2 \text{ mg}\%$ , myopathy, nephrotic syndrome, diabetes (fasting glucose  $\geq 140 \text{ mg}\%$ ), pancreatitis, smoking and overweight persons ( $\text{BWI} \geq 30 \text{ kg/m}^2$ ). Initial CRP  $\leq 8 \text{ mg/l}$  and a marked increase after 1 month ( $\geq 25 \text{ mg/l}$ ) suggest the possibility of an acute infection and were excluded from the statistical analysis. Baseline laboratory parameters

were determined and treatment with atorvastatin 20 mg once a day was started. Laboratory tests were performed after 4 and 8 weeks.

## Methods and statistics

**Cholesterol, HDL-Cholesterol, Triglycerides and Apoproteins** (A1 and B), were measured in at least 12 hours fasting venous serum, on a COBAS Integra 400 autoanalyser, using standard methods and reagents from Roche Diagnostics. **LDL-Chol** was calculated by Friedewald formula. **Highly sensitive CRP** was measured by latex enhanced immunoturbidimetry on COBAS Integra autoanalyser (Roche Diagnostics).

Beta-integrins (CD11b, 11c, 49d), HLA-DR and lipopolysaccharide (LPS)-receptor (CD14) expression were measured by flow cytometry as fluorescence intensity before and after treatment with atorvastatin for 4 and 8

**Table 1. Baseline characteristics and lipid profiles of patients in the study**

Group (no)	A: UA (12)	B: SCHD (10)	p A vs B
Age (years)	55,8 ± 9,87	53,5 ± 11,46	NS
Sex (Male)	11	9	NS
Hypertension	1	0	NS
BWI (kg/m <sup>2</sup> )	28,4 ± 1,64	27,8 ± 2,20	NS
Chemical diabetes	1	1	NS
Family history of CHD	5	4	NS
CHOL (mg/dl)	240,8 ± 25,33	235,7 ± 32,21	NS
TG (mg/dl)	175,9 ± 52,23	151,7 ± 55,57	NS
HDL-Chol (mg/dl)	48,5 ± 6,71	50,15 ± 11,10	NS
LDL-Chol (mg/dl)	151,0 ± 14,73	149,6 ± 19,48	NS
ApoA1 (mg/dl)	131,1 ± 23,10	146,0 ± 17,48	NS
ApoB (mg/dl)	124,9 ± 51,52	118,5 ± 21,62	NS
CHOL/HDL-C	5,0 ± 1,13	4,8 ± 1,26	NS
CRP (mg/l)	20,5 ± 4,26	3,8 ± 1,26	p < 0,001

weeks. We studied the differences in expression of integrins on monocytes and lymphocytes, as follows:

**Staining for cell surface immunofluorescence.** Sample preparation and analysis were performed within 4 hours of venipuncture. For flow-cytometric immunophenotyping 100  $\mu$ l of whole blood collected on K<sub>3</sub>EDTA were incubated for 15 minutes at room temperature (RT) in the dark with saturating concentrations of the fluorochrome-conjugated mouse anti-human monoclonal antibodies (mAb). The monoclonal antibodies: CD11b (clone ICRF44), CD11c (clone 3.9), CD49d (clone 44H6), CD14 (clone TUK-4), were obtained as FITC, R-PE or R-PE Cy5 conjugates from Immunotech. CD3 (clone SK7), anti HLA DR (L243) and IgG1 (clone X40), IgG2a (clone X39) isotype controls were obtained as FITC, R-PE or PerCP conjugates from Becton Dickinson (BD). After incubation the blood samples were treated for 10 minutes at RT in the dark, with 3 ml erythrocyte lysis solution from BD (1x FACS lysing solution), followed by two washes (5 minutes each, 500g) with 3 ml of BD Cell wash solution.

**Data acquisition and analysis:** CaliBRITE beads and FACSComp software were used for setting the photomultiplier tube voltages, the fluorescence compensation and for checking instrument sensitivity prior to use. For flowcytometric setup, acquisition and analysis CellQuest software was used on a FACScan flow-cytometer (BD). The cellular light-scatter signals and three fluorescence signals were analysed in list mode at a channel resolution of 1024, with forward scatter (FSC) as trigger parameter. The photomultiplier gains were calibrated with polychromatic fluorescent reference beads (Polysciences). Compensation was adjusted with FITC, R-PE and PerCP – coated microbeads (BD) and triple-stained (CD4, CD8 and CD3) peripheral blood lymphocytes as a biological control. Using a FSC threshold all events are acquired with at least 10.000 lym-

phocytes per sample by setting an acquisition gate on CD3+ lymphocytes in SSC/CD3 dot plot and at least 5.000 monocytes per sample by setting an acquisition gate on CD14+ monocytes in SSC/CD14 dot plot. Data is displayed as two-collor dot plots and histogram statistics to determine cell surface markers (as specific fluorescence intensity). Cellular antigen densities were calculated with the assumption that there was only one cellular binding site for each mAb on its target antigen, on the basis of calibration with reference beads that carried a defined number of anti-mouse binding sites (QuantiBRITE System, BD).

To determine serum concentration of **soluble Intercellular Adhesion Molecule (sICAM-1)** a Colorimetric Sandwich ELISA from R&D Systems has been used: fasting venous blood was obtained after nontraumatic venipuncture and was allowed to clot at room temperature (RT) for 30 minutes. Serum was separated after centrifugation for 15 minutes at 3000 rpm and was stored at  $-70^{\circ}\text{C}$ . In a blinded manner, ELISAs were used to determine serum concentration of sICAM (Colorimetric Sandwich ELISA from R&D Systems). Standard curves based on six reference concentrations were created according to the manufacturer's recommendations. According to the commercial suppliers, no cross-reactivity of sICAM-1/ELISA with sVCAM-1, sE-Selectin, ICAM-2 or ICAM-3 was observed.

**Statistical analysis.** All data were expressed as mean value  $\pm$  SD, when a normal distribution was assumed. For patient characteristics the Mann-Whitney test was performed for continuous data and Fisher's exact test for categorical data. Comparison between control subjects and patients and between patients before and after treatment were analyzed by Student's t test. Pearson and Spearman Rank correlation were performed to examine associations between parameters tested. Values of  $p < 0,05$  were considered statistically significant.

**Results and Discussion**

In mild primary hypercholesterolemic CHD patients after 4 weeks of treatment, 20 mg Atorvastatin reduced CHOL by 33% ( $p < 0,001$ ) and LDL-Chol by 47% ( $p < 0,001$ ) and after 8 weeks of treatment by 36% and 49% respectively. ApoB was reduced with 32% ( $p < 0,001$ ) after 1 month and by 35% after 2 months of treatment, in all subjects. HDL-Chol was almost unchanged (1,5% increased in both groups) and also ApoA1 (3,2% and 2,5% increased). Triglycerides decreased by 10% ( $p < 0,05$ ).

After gating for CD14+ (LPS R) mono-

cytes (Figure 1a), specific mean fluorescence intensity (sMFI) for CD11b (Figure 1b), CD11c, CD49d, CD14 and HLA-DR were recorded.

*Ex vivo* monocyte surface expressions of  $\beta 2$  (CD11b -  $\beta 2\alpha M =$  anti C3biR, CD11c -  $\beta 2\alpha X =$  anti CR4) and  $\beta 1$  (CD49d -  $\beta 1\alpha 4 =$  VLA-4) integrins, LPS R (CD14) and HLA-DR were significantly greater in UA patients comparatively to SCHD patients. Data expressed in channels as fluorescence intensity (mean value  $\pm$  SD) are shown in Table 2.

Vedder et al<sup>12</sup> demonstrated that surface expression of CD11b is dissociated from its adhesive function. We found a linear (Pearson) correlation between sICAM-1 levels and CD11b ( $r=0.66$ ,  $p=0.0128$ ), respectively CD11c ( $r=0.76$ ,  $p=0.0043$ ), expression on monocytes (Figure 2).

In our study, treatment with atorvastatin 20 mg/day for 4 and 8 weeks, significantly reduced *ex vivo* monocyte CD11b, CD11c integrin expression and LPS R already after 4 weeks and CD49d, HLA-DR after 8 weeks of treatment (Table 2 and Figure 3).

Statins have been shown to inhibit pro-inflammatory cytokine formation in leucocytes (TNF $\alpha$  and IFN $\gamma$ ), adhesion molecule expression in endothelial cells<sup>7</sup> and down-regulate cy-

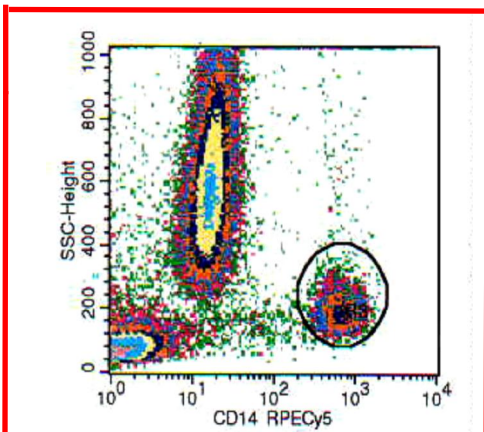


Figure 1a.

Identical Figure

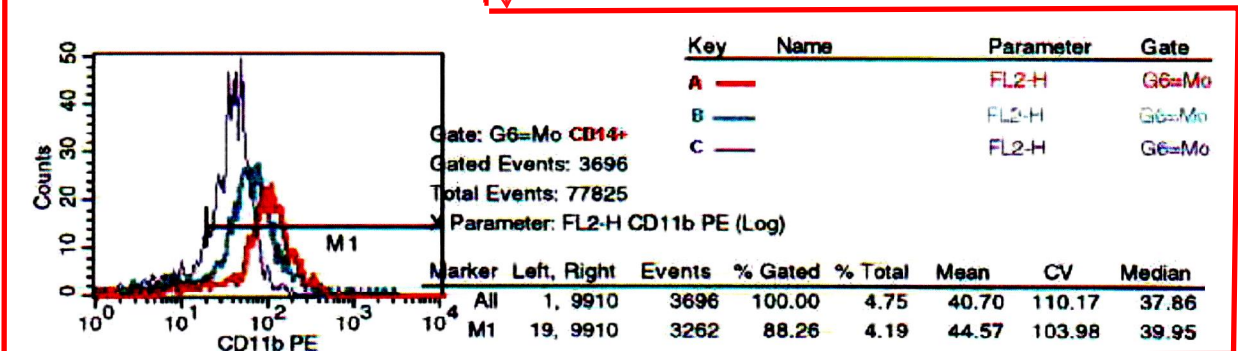


Figure 1b.

**Figure 1a).** Gating for CD14+ monocytes in SSC/CD14 dot plot and **1b)** histogram statistics to determine surface mean fluorescence intensities (sMFI) of cell surface markers

**Table 2. Effect of atorvastatin 20 mg/day, on monocyte integrin, LPSR and HLA-DR expression, with regard to the distribution at baseline (mean±SD)**

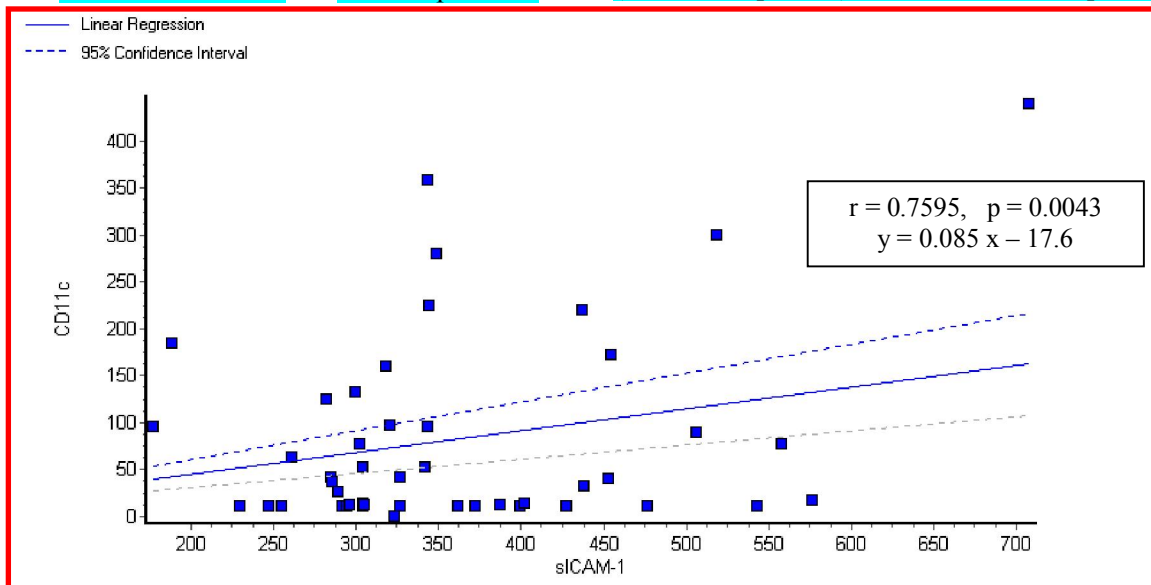
Parameter/sMFI	A: Baseline	B: 1 month	C: 2 months	p B vs A	p C vs A
CD11b in SCHD	70 ± 5	68 ± 7	71 ± 8	NS	NS
CD11b in UA	187 ± 13	155 ± 10	141 ± 8	p < 0,05	p < 0,05
CD11c in SCHD	68 ± 8	70 ± 4	65 ± 6	NS	NS
CD11c in UA	138 ± 10	118 ± 7	112 ± 5	p < 0,05	p < 0,05
CD49d in SCHD	97 ± 11	87 ± 10	92 ± 13	NS	NS
CD49d in UA	128 ± 14	119 ± 19	101 ± 15	NS	p < 0,05
CD14 in SCHD	73 ± 7	78 ± 4	69 ± 9	NS	NS
CD14 in UA	160 ± 18	128 ± 20	120 ± 17	p < 0,05	p < 0,01
HLA-DR in SCHD	156 ± 14	148 ± 18	160 ± 15	NS	NS
HLA-DR in UA	247 ± 25	229 ± 31	201 ± 20	NS	p < 0,05

tokine-inducible nitric oxide synthase expression in endothelial cells<sup>13</sup>.

There are studies suggesting that statins selectively block leukocyte function antigen –1 (LFA-1), a β2 integrin (ICAM-1 receptor), via binding a novel allosteric site within LFA-1<sup>15</sup>.

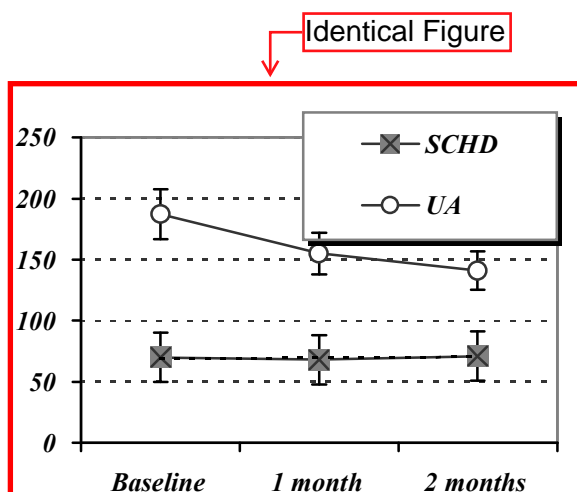
Rothe and Schmitz<sup>10,11</sup> found a positive

correlation between serum LDL-Chol levels and expression of CD45RA+ on CD14+CD16+ subpopulation, considering CD45RA as an indicator of PBMCs activation in the presence of atherogenic lipoproteins. In our study, CD45RA on CD14+ monocytes decreased significantly (with 20%, p<0,05) after treatment, especially



**Figure 2. Correlation between sICAM-1 levels and CD11c expression on monocytes**

Identical Figure



**Figure 3. Effect of atorvastatin 20 mg/day, on *ex vivo* monocyte CD11b expression in unstable angina (UA) and stable coronary heart disease (SCHD) patients**

in CD14+CD16+ subpopulation which is associated with marked expression of  $\beta 2$  integrins (data not shown).

Not only monocyte, but also lymphocyte integrins expression appears reduced by statin therapy. In our study, treatment with atorvastatin 20mg once a day for 4 weeks significantly reduced also *ex vivo* CD11b -  $\beta 2$  integrin expression on CD3+ T lymphocytes in UA patients, but no CD49d -  $\beta 1$  integrin expression (Table 3).

CRP could also reflect a state of arterial inflammation. A new idea in cardiology is that patients with acute coronary syndromes may have underlying, diffuse atherothrombosis of

the coronary arteries, precipitated by the infiltration of atheroma by macrophages, the secretion of proteases, and the erosion or rupture of plaque. This inflammatory reaction, initiated in response to the retention of atherogenic lipoproteins in the artery wall, could also lead to the release of cytokines and production of CRP by hepatocytes. There are studies suggesting that statins could reduce CRP by a number of mechanisms. One possibility is by reduction of inflammation within the artery, presumably by reducing the amount of LDL available for oxidative metabolism<sup>14</sup>. In a previous study we found that treatment with atorvastatin significantly suppresses serum levels of lipid peroxides in hypercholesterolemic patients<sup>4</sup>. A second mechanism could be by decreasing the production and/or circulation of mediators of CRP production, e.g. TNF- $\alpha$  or IL-6.

In our study, serum levels of CRP and sICAM-1 in patients with UA were significantly higher ( $p < 0,05$ ) than in SCHD patients (Table 4). Treatment with atorvastatin (20mg, once a day) decreased significantly ( $p < 0,05$ ) after 4 weeks the levels of CRP and sICAM-1 in UA patients, but not in SCHD patients.

We observed no relationship between classical plasmatic lipid parameters and monocyte integrin expression. Changes in integrin expression and in CRP concentrations were not correlated with those in LDL-Chol and ApoB levels: the effect of atorvastatin on CRP seems independent of its effect on LDL-Chol.

**Table 3. Effect of atorvastatin 20 mg/day, on T lymphocyte integrins expression, with regard to the distribution at baseline (mean $\pm$ SD)**

Parameter	A: Baseline	B: 1 month	C: 2 months	p B vs A	p C vs A
CD11b in SCHD	52 $\pm$ 10	55 $\pm$ 12	50 $\pm$ 14	NS	NS
CD11b in UA	94 $\pm$ 12	64 $\pm$ 14	55 $\pm$ 13	$p < 0,05$	$p < 0,01$
CD49d in SCHD	84 $\pm$ 18	88 $\pm$ 17	81 $\pm$ 14	NS	NS
CD49d in UA	107 $\pm$ 14	95 $\pm$ 12	98 $\pm$ 14	NS	NS

**Table 4. The course of CRP and sICAM-1 (mean±SD) in CHD patients during atorvastatin therapy with regard to the distribution at baseline**

Parameter	A: Baseline	B: 1 month	C: 2 months	p Bvs A	p C vs A
CRP in SCHD (mg.l <sup>-1</sup> )	3,8 ± 1,26	3,3± 0,86	3,1± 0,95	NS	NS
CRP in UA (mg.l <sup>-1</sup> )	20,5± 4,26	15,8± 2,16	11,2± 3,23	p < 0,05	p < 0,01
sICAM-1 in SCHD (ng.ml <sup>-1</sup> )	281±41,5	290±51,7	288±69,5	NS	NS
sICAM-1 in UA (ng.ml <sup>-1</sup> )	379 ± 129,1	306 ± 141,1	300 ± 99,02	p < 0,05	p < 0,05

A variety of immunologic processes seem to be influenced by atorvastatin: the levels of sICAM-1 and their receptor expression on PBMCs (integrins) and also the seric levels of CRP.

The reduction of CRP and of monocyte and lymphocyte integrin expression by HMG-CoA reductase inhibitors provides a novel mechanism of action of these compounds (other than cholesterol lowering) and may explain their beneficial effects early after treatment initiation. The administration of a statin in the setting of an acute coronary syndrome may have a role as powerful as that of early treatment with aspirin, a  $\beta$ -blocker or an angiotensin conversion inhibitor (ACE) inhibitor. The early CRP-lowering effect of statins, independent of effects on other acute-phase reactants and lipids, could change the way physicians manage all patients at risk for CHD, both in the early setting and in the prevention of long-term events.

### Conclusions

1. *Ex vivo* monocyte and lymphocyte surface expressions of  $\beta$ 2 and  $\beta$ 1 integrins were significantly greater in unstable angina comparatively with stable coronary heart disease patients.
2. There are linear correlations between sICAM-1 levels and CD11b, respectively CD11c, expression on monocytes.
3. Treatment with atorvastatin significantly reduced *ex vivo* CD11b, CD11c integrin and LPS R expression on PBMCs.

4. Unstable angina patients have significantly increased serum levels of CRP and sICAM-1 comparatively with stable coronary heart disease patients.

5. Short term treatment with atorvastatin decreased significantly the levels of CRP and sICAM-1 in patients with unstable angina.

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