

THE PLEIOTROPIC EFFECTS OF ATORVASTATIN ON STABLE ANGINA PATIENTS: EVIDENCE BY ANALYSIS OF HIGH-DENSITY LIPOPROTEIN SIZE AND SUBCLASSES, AND PLASMA mRNA

PLEJOTROPNI EFEKTI ATORVASTATINA KOD PACIJENATA SA STABILNOM ANGINOM: DOKAZI DOBIJENI ANALIZOM VELIČINE I RASPODELE SUBFRAKCIJA LIPOPROTEINA VELIKE GUSTINE I PLAZMATSKE mRNA

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Summary

Background: High-density lipoproteins (HDL) have atheroprotective biological properties: antioxidative, anti-apoptotic, anti-inflammatory, and they have the efflux capacity of cellular cholesterol. Plasma mRNA analysis can be used to investigate statin pleiotropy *in vivo* as a new analytical tool for non-invasive assessment of gene expression in vascular beds. The aim of this study was to assess the pleiotropic effects of atorvastatin in stable angina patients with high-risk values (group A) as compared with patients who had borderline and desirable HDL-cholesterol (HDL-C) values (group B).

Methods: The atorvastatin therapy (20 mg/day) was given to forty-three patients with stable angina for 10 weeks. We investigated three statin pleiotropy-targeted genes: intercellular adhesion molecule-1, chemokine (C-C motif) ligand 2 and cathepsin S and assessed by gel electrophoresis gradient the effects of atorvastatin on HDL size and subclasses.

Kratak sadržaj

Uvod: Lipoproteini velike gustine (HDL) imaju atero-protektivne biološke osobine: antioksidativne, antiapoptičke, anti-inflamatorne kao i kapacitet da izvlače kolesterol iz ćelija. Analiza plazmatske mRNA može da se koristi za ispitivanje plejotropnih efekata statina *in vivo* kao novo analitičko sredstvo za neinvazivnu procenu ekspresije gena u zidu krvnog suda. Cilj ove studije je bio da se procene plejotropni efekti atorvastatina kod pacijenata sa stabilnom anginom sa visokorizičnim vrednostima (grupa A) u odnosu na pacijente sa graničnim i poželjnim vrednostima HDL holesterola (HDL-C) (grupa B).

Metode: Četrdeset tri pacijenta sa stabilnom anginom su primala terapiju atorvastatinom (20 mg/dan) 10 nedelja. Mi smo ispitivali tri gena značajna za plejotropno delovanje statina: intracelularni adhezioni molekul-1, hemokin (C-C motiv) ligand 2 i katepsin S i procenjivali smo efekte atorvastatina na veličinu i raspodelu HDL subfrakcija pomoću elektroforeze na poliakrilamidnom gradijentu gelu.

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Abbreviations: HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CH, total cholesterol; TG, triglycerides; OxLDL, oxidized low-density lipoprotein; mRNA, messenger ribonucleic acid; CCL2, gene encoding monocyte chemoattractant protein-1; ICAM1 gene encoding intercellular adhesion molecule-1; CTSS, gene encoding cathepsin S; CRP, C-reactive protein; SAA, serum amyloid A; CVD, cardiovascular disease; SA, stable angina; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

Results: In group A, after therapy, HDL-C concentration was significantly increased but not in group B. Atorvastatin lowered plasma chemokine (C-C motif) ligand 2 and intercellular adhesion molecule-1 mRNA levels in both groups, but did not change the plasma cathepsin S mRNA levels. In group A only, baseline total bilirubin showed negative correlations with the genes of cathepsin S ($r=-0.506$; $p=0.023$) and significantly increased after therapy.

Conclusions: HDL-C and bilirubin can be promising therapeutic targets in the treatment of cardiovascular diseases. Analysis of cell-free mRNA in plasma might become a useful tool for estimating statin pleiotropy.

Keywords: high-density lipoprotein subclasses, cathepsin S, bilirubin, mRNA in plasma, atorvastatin

Introduction

High-density lipoproteins (HDL) have atheroprotective biological properties: antioxidative, antiapoptotic, antiinflammatory, and they have the efflux capacity of cellular cholesterol (1). These plasma HDL particles have heterogeneous physicochemical properties, metabolism, and biological activity (1). HDL is the next target in reducing cardiovascular risk in statin-treated high-risk patients, as evidenced from epidemiological data, the data based on animal studies and clinical trials (2). Prospective studies have shown that coronary heart disease (CHD) risk was increased by 3% in women and by 2% in men per decrement of 1 mmol/L in HDL-C (3). Therapeutic normalisation of attenuated antiatherogenic HDL function is the target of modern pharmacological approaches to HDL raising (1). Common cardiovascular prevention strategies emphasise therapeutic reductions in low-density lipoprotein cholesterol (LDL-C) (4), however, attention is being focused on HDL cholesterol as a secondary prevention target to address the risk of residual cardiovascular disease (CVD) (5).

Statins, the inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are drugs that effectively reduce LDL-C levels. The studies indicate that the overall benefits observed with statins can be mediated through cholesterol-independent effects, the so-called pleiotropic effects (6). Plasma mRNA analysis, as a new research tool for the noninvasive follow-up of gene expression in vascular walls, can be used to investigate statin pleiotropy *in vivo* (7).

Monocyte chemoattractant protein-1 (MCP-1) is the major chemokine responsible for attracting monocytes to sites of active inflammation, including the formation of atheromatous plaque (8). The ICAM-1 is considered a major factor when circulating monocytes adhere to the endothelium and then transmigrate into the intima (9). Macrophages, smooth muscle cells, and endothelial cells synthesise cathepsin S (CTSS) (10). When released extracellularly, CTSS exerts collagenolytic and elastolytic activities which have as effects: elastic lamina degradation, plaque rupture, and necrotic core formation (11, 12).

Rezultati: U grupi A, posle terapije, HDL-C koncentracija se značajno povećala, ali ne i u grupi B. Atorvastatin je snizio plazmatski nivo hemokin (C-C motiv) liganda 2 i intracelularnog adhezionog molekula-1 mRNA u obe grupe, ali nije promenio plazmatski nivo gena za katepsin S. Samo u grupi A, ukupni bilirubin je pokazao negativnu korelaciju sa genom za katepsin S ($r=-0,506$; $p=0,023$) pre započinjanja terapije i značajni porast nakon terapije atorvastatinom.

Zaključak: HDL-C i bilirubin mogu biti obećavajući terapijski ciljevi u lečenju kardiovaskularnih bolesti. Analiza slobodne mRNA (eng. cell-free mRNA) u plazmi može postati korisno sredstvo za procenu plejotropnog delovanja statina.

Ključne reči: subfrakcije lipoproteina velike gustine, katepsin S, bilirubin, mRNA u plazmi, atorvastatin

In this study, we assessed the effects of atorvastatin on HDL size and subclasses. In addition, the aim of this study was to assess the pleiotropic effects of atorvastatin (20 mg/day, for 10 weeks) on markers of inflammation, oxidative stress and destabilisation of atherosclerotic plaque in stable angina patients with high-risk values, as compared with patients who had borderline and desirable HDL-C values. We investigated three statin pleiotropy-targeted genes: intercellular adhesion molecule-1 (*ICAM1*), chemokine (C-C motif) ligand 2 (*CCL2*) and cathepsin S (*CTSS*).

Materials and Methods

Subjects and blood sampling

The atorvastatin therapy (20 mg/day) was given to forty-three patients with stable angina for 10 weeks. Stable angina was diagnosed by clinical assessment, electrocardiogram evaluation and laboratory findings (13). Criteria for the inclusion of patients were a stable angina diagnosis and statin free treatment for at least one year. Criteria for the exclusion of patients were (by detailed history and clinical examination): inflammatory, endocrine, thyroid, liver, neoplastic and renal diseases (serum creatinine concentration values $\geq 120 \mu\text{mol/L}$). Likewise, patients with hemolysed samples were excluded from the study.

The patients with stable angina from the Health Centre Laktasi (Bosnia and Herzegovina) were consecutively enrolled in our study. Blood was collected from the subjects prior to therapy and 10 weeks later. The study was approved by the National Ethics Committee. All participating subjects gave their written consent. The procedure for collecting cell-free plasma, a detailed description, has been published elsewhere (7).

mRNA quantification in plasma

RNA was isolated from 3 mL of plasma using QIAvac 24 Plus (Qiagen) and QIAamp Circulating

Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with certain modifications. The time of proteolysis was 5 min, and RNA was eluted from the column with 47 μ L of RNase-free water. Collected eluate was used for the second elution of RNA from the same column. RNA eluates were incubated in a 65 °C water bath for 5 min and then immediately placed on ice for another 5 min. The eluted mRNA was transcribed to cDNA with a Super Script VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). For more efficient mRNA transcription to cDNA we increased the incubation time from 60 min to 120 min at 42 °C. The resulting cDNA solution was stored at -80 °C until further processing.

The primers used for cDNA transcript quantification (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA, USA) were: Hs00164932_m1 for *ICAM1* (exon boundary 2-3, amplicon length 87 bp), Hs00234140_m1 for *CCL2* (exon boundary 1-2, amplicon length 101 bp), Hs00175403_m1 for *CTSS* (exon boundary 3-4, amplicon length 82 bp) and Hs99999905_m1 for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the reference gene.

Four pairs of samples were chosen for selection of the most appropriate reference gene among the 32 candidates available on the TaqMan Human Endogenous Control Plate (Applied Biosystems). The cDNA transcripts were amplified by PCR according to the manufacturer's protocol with FastStart Universal Probe Master (ROX) (Roche Applied Science, Mannheim, Germany), using 11.25 μ L diluted (1:3) of cDNA (*CCL2*, *ICAM1*); 11.25 μ L diluted (1:8) of cDNA (*CTSS*) and 10.00 μ L diluted (1:12) of cDNA (*GAPDH*) and the kit components in a total reaction volume of 25 μ L run in an ABI Prism™ SDS PCR analyser (Applied Biosystems).

The possibility of DNA interference was excluded by starting PCR reaction with samples of the subject but omitting the reverse transcription for every commercially available TaqMan Gene Expression Assay. Negative control (blank) was analysed to exclude the possibility of external mRNA contamination. The coefficient of variation (CV) of Ct was less than 2% between three averaged Ct values (14). The amount of each investigated transcript was normalised to the amount of the reference gene (15).

HDL subclasses determination

Particle size and distribution of HDL subclasses were assessed by a method of polyacrylamide gradient gel electrophoresis, introduced by Rainwater et al. (16). The procedure described in detail has been published elsewhere (17, 18). Briefly, in house made polyacrylamide gradient (3–31%) gels were processed in a Hoefer SE 600 Ruby electrophoresis

unit (Amersham Pharmacia Biotech, Vienna, Austria) for 20 h, at 8 °C using Tris (90 mmol/L)–boric acid (80 mmol/L)–Na₂EDTA (2.7 mmol/L) buffer, pH 8.35. For calibration purposes, we used Pharmacia High Molecular Weight protein standards, carboxylated polystyrene microsphere beads and standard samples of human plasma, standardized in Dr. David Rainwater's Laboratory (Southwest Foundation for Biomedical Research, San Antonio, TX, USA). Following electrophoretic separation, the gels were stained for proteins with Coomassie brilliant blue G-250 and for lipids with Sudan black. Image Scanner (Amersham Pharmacia Biotech, Vienna, Austria) with Image Quant software (version 5.2; 1999; Molecular Dynamics) was used as a device for densitometric analysis of the gels. By using the calibration curve created of migration distances of standards with known particle diameters, we calculated particle diameters corresponding to each absorbance peak in the HDL regions of gels. Dominant HDL particle diameter was assessed by estimation of the diameter of the major absorbance peak. The relative content of each HDL subclass was evaluated through determination of the areas under the peaks of densitometric scans. The relative proportion of small-sized HDL particles was assessed as the area of the densitometric scan at or below 8.8 nm (19). Increased small sized HDL particles were considered if more than 50% of total HDL absorbance was detected in the area \leq 8.8 nm.

Oxidized low-density lipoprotein quantification in plasma

Oxidized low-density lipoprotein (OxLDL) was assessed by means of a solid phase dissociation enhanced lanthanide time-resolved fluorescence immunoassay (DELFIAR[®]) based on the sandwich technique (20), using the monoclonal antibody OB/04 (21), and 24 hours oxidized LDL as a standard. The plates were precoated with OB04, directed against an epitope generated on oxidized apolipoprotein B (apo B) upon oxidation. The plates were incubated with diluted plasma samples, applied in duplicate at room temperature on a plate shaker. After 2 hours the plates were washed 4 times using a plate washer (Anthos Fluido Laptec, Salzburg, Austria), to remove nonreactive plasma components. Then, the plates were incubated for 1 hour at 4 °C with a rabbit anti-apo B antibody (Behring, Mannheim, Germany), which recognized the OxLDL bound to the solid phase. The second washing step (4 times) removed unbound anti-apo B antibody. As the last step the wells were incubated with an Eu-labeled anti-rabbit IgG for 1 h at room temperature. The antibody (Sigma, St. Louis, MO, USA) was labeled with an Eu-labeling kit (Perkin Elmer, Waltham, MA, USA). The fluorescence was developed using the enhancement solution, after washing the plates for 6 times. Eu³⁺ was dissociated from the labeled antibody by a low

pH, whereafter the free lanthanide formed a fluorescent chelate with the components of the enhancement solution. Counts were measured on a Viktor™ multilabel counter (Perkin Elmer).

Lipids quantification and protein quantification in plasma

HDL-C, LDL-C, TC, triglycerides and total bilirubin were measured by reagents from Roche Diagnostics (Roche Diagnostics, Mannheim, Germany). Commercial ELISA kit (Invitrogen, Frederick, MD, USA) was used for measurement of serum amyloid A (SAA). C-reactive protein (CRP) was measured by Beckman Coulter CRP Latex reagent (Beckman Coulter, Inc. Brea, CA, USA).

Statistical analysis

Calculations were performed using SPSS v.20.0 (SPSS Inc. Chicago, IL, USA). Numerical data are shown as median and interquartile range for non-normally distributed variables. Categorical variables are presented as relative frequencies. Comparisons of the data before and after 10 weeks of atorvastatin therapy were performed by the Wilcoxon's signed-rank test for non-normally distributed variables. The Mann-Whitney U-test was used to compare data between subgroups with HDL-C \leq 1.2 mmol/L and HDL-C $>$ 1.2 mmol/L. Continuous variables are presented as mean \pm standard deviation and were compared by the Student's t-test. Categorical variables are presented as relative frequencies and were compared by the Chi-square test. The Spearman rank-order correlation test was used for determining relations between the variables.

Results

Patients were divided in two groups according to HDL-C. Patients with HDL-C \leq 1.2 mmol/L ($<$ 1.0 mmol/L for men, $<$ 1.2 mmol/L for women, $n = 20$), also known as high-risk values, were represented by group A, and patients with borderline and desirable values, i.e. $>$ 1.2 mmol/L were represented by group B ($n=23$). There were no differences between the two analysed groups (Table I). Group A comprised three smokers and group B two. Patients were taking other therapy prior to inclusion into this study (Table I). Patients took drugs before and continued with the statins without time between.

Baseline laboratory parameters and their changes after 10 weeks of atorvastatin treatment are shown in Table II, Table III and Figure 1. After therapy there was a significant difference between small-sized HDL particles and total bilirubin values between the two groups. Likewise, HDL 2b was different in group A and group B, at baseline (median 39.28 vs. 43.87, $p=0.054$) and after the therapy (median 40.92 vs. 47.13, $p=0.013$). Figure 1 represents the distribution of HDL subclasses in both groups of patients. As shown, atorvastatin treatment significantly increased HDL 2b in group B only ($p=0.004$).

There was no correlation between the degree of changes in the plasma LDL-C after 10 weeks of statin treatment and the degree of changes in the following plasma biochemistry markers: CCL2 mRNA, ICAM1 mRNA, CTSS mRNA, CRP and SAA (results not shown).

In group A, HDL-C negatively correlated with small-sized HDL at baseline ($r=-0.486$; $p=0.030$) and positively correlated with dominant particle diameters HDL ($r=0.555$; $p=0.011$). As expected, HDL-C correlated with TG ($r=-0.529$; $p=0.011$). In group

Table I Clinical characteristics of the 43 patients with stable angina.

Parameter	Patients with HDL-C \leq 1.2 mmol/L $n=20$ (group A)	Patients with HDL-C $>$ 1.2 mmol/L $n=23$ (group B)	p
Age (years)	60.95 \pm 8.62	62.13 \pm 9.74	0.678
Body mass index	27.87 \pm 6.96	28.40 \pm 3.34	0.758
Gender (female)	60.0%	82.6%	0.099
ACE inhibitors	80.0%	60.9%	0.173
Beta blockers	60.0%	34.8%	0.098
Calcium channel blockers	30.0%	34.8%	0.739
Acetylsalicylic acid	15.0%	8.7 %	0.520
Nitrate drugs	45.0%	39.1%	0.697

Continuous variables are presented as mean \pm standard deviation and were compared by the Student's t-test, whereas categorical variables are presented as relative frequencies and were compared by the Chi-square test. Abbreviations: ACE, angiotensin-converting enzyme.

Table II Lipids before and after atorvastatin treatment in the patients with stable angina.

	Patients with HDL-C \leq 1.2 mmol/L n=20 (group A)			Patients with HDL-C $>$ 1.2 mmol/L n=23 (group B)		
	Baseline	After 10 weeks	p	Baseline	After 10 weeks	p
HDL-C (mmol/L)	1.0 (0.9–0.1) ^{§,***}	1.2 (1.0–1.3) ^{§,***}	0.002	1.6 (1.4–1.8)	1.5 (1.4–1.8)	0.962
Dominant HDL diameter (nm)	9.26 (8.81–9.66)	9.60 (9.10–10.60)	0.014	9.27 (9.04–10.60)	9.8 (9.50–10.70)	0.051
Small-sized HDL particles (%)	35.56 (31.50–42.00)	33.03 (31.22–37.71) ^{§,*}	0.279	31.41 (27.93–38.94)	28.80 (23.82–37.84)	0.002
LDL-C (mmol/L)	4.9 (4.3–5.6)	2.6 (2.4–3.2)	$<$ 0.001	4.7 (4.4–5.0)	2.7 (2.4–3.0)	$<$ 0.001
OxLDL (mg/L)	17.93 (14.64–19.96)	11.02 (9.47–12.94)	0.002	15.92 (13.62–17.81)	11.00 (9.75–14.20)	0.003
TC (mmol/L)	7.1 (6.6–7.9)	4.3 (4.2–5.0)	$<$ 0.001	7.1 (6.6–7.4)	4.9 (5.5–5.2)	$<$ 0.001
TG (mmol/L)	2.2 (1.5–3.4) ^{§,**}	1.7 (1.30–2.3) ^{§,**}	0.005	1.5 (1.1–1.8)	1.2 (1.1–1.6)	0.060

The values are presented as medians (Q1–Q3) and compared by the Wilcoxon's test.

[§] Significantly different from patients with HDL-C $>$ 1.2 mmol/L at the same time point, according to Mann-Whitney U test. * p $<$ 0.05; ** p $<$ 0.01; *** p $<$ 0.001.

Abbreviations: HDL-C, high density cholesterol; LDL-C, low-density lipoprotein cholesterol; OxLDL, oxidized low-density lipoprotein; TC, total cholesterol; TG, triglycerides; p, level of significance.

Table III Laboratory characteristics of stable angina patients at baseline and after 10 weeks of atorvastatin therapy.

	Patients with HDL-C \leq 1.2 mmol/L n=20 (group A)			Patients with HDL-C $>$ 1.2 mmol/L n=23 (group B)		
	Baseline	After 10 weeks	p	Baseline	After 10 weeks	p
CCL2 mRNA	9.50 (6.00–11.17)	5.67 (5.00–7.83)	0.028	8.25 (5.67–10.41)	5.86 (4.71–6.92)	0.042
ICAM1 mRNA	63.37 (46.36–76.45)	46.92 (34.60–48.66)	0.051	66.34 (35.95–83.36)	39.71 (26.87–48.47)	$<$ 0.001
CTSS mRNA	29.40 (17.40–39.11)	33.76 (26.30–39.38)	0.970	36.51 (26.51–44.48)	29.62 (25.63–34.90)	0.465
Total bilirubin (μ mol/L)	6.8 (6.4–10.10)	9.4 (7.6–12.4) ^{§,*}	$<$ 0.001	7.0 (5.3–10.3)	6.7 (5.4–9.7)	0.080
CRP (mg/L)	1.9 (1.1–3.6)	1.5 (1.0–3.1)	0.332	2.8 (1.7–5.1)	1.9 (1.2–3.7)	0.011
SAA (mg/L)	22.11 (12–31.25)	15.0 (7.60–18.83)	0.011	23.74 (13.27–33.38)	13.85 (10.53–25.27)	0.004

The values are presented as medians (Q1–Q3).

CCL2 mRNA, ICAM1, CTSS mRNA levels were standardised to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase as a reference gene.

Data are compared by Wilcoxon's test. [§]Significantly different from patients with HDL-C $>$ 1.2 mmol/L at the same time point, according to Mann-Whitney U test. * p $<$ 0.05.

Abbreviations: CCL2, gene encoding monocyte chemoattractant protein-1; CTSS, gene encoding cathepsin S; ICAM1, gene encoding intercellular adhesion molecule-1; CRP, C-reactive protein; SAA, serum amyloid A; p, level of significance.

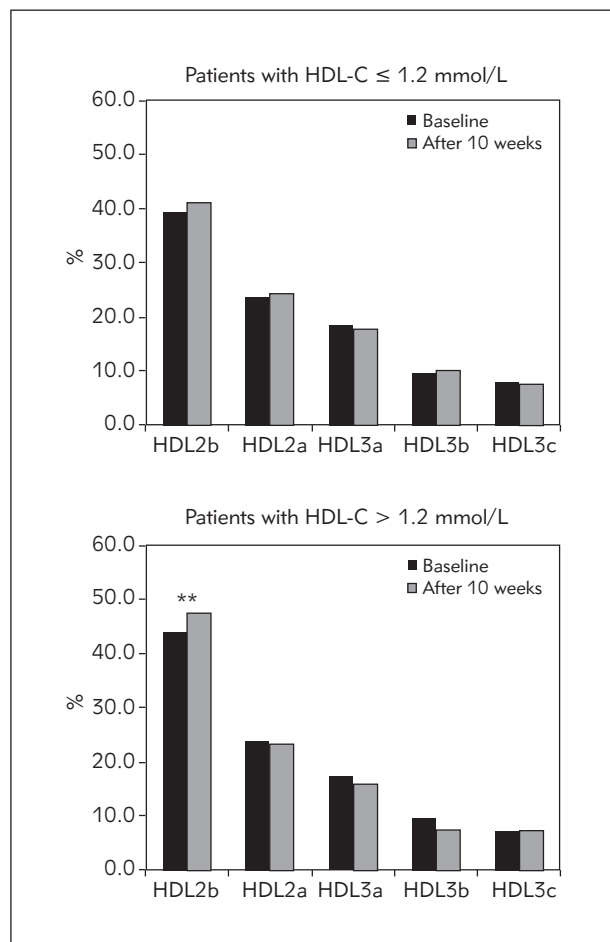


Figure 1 Distributions of HDL subclasses in patients with HDL-C ≤ 1.2 mmol/L and patients with HDL-C > 1.2 mmol/L. **p<0.01.

B, HDL-C positively correlated with HDL dominant particle diameter at baseline ($r=0.433$; $p=0.039$) and, as expected, HDL-C negatively correlated with TG ($r=-0.514$; $p=0.012$).

In group A only, baseline correlation was impressive: total bilirubin showed correlation with CTSS mRNA ($r=-0.506$; $p=0.023$).

Discussion

Atorvastatin (20 mg/day, 10 weeks) was more effective in patients with high-risk values (group A) than in the group with borderline and desirable HDL-C values (group B). In group A, after therapy, HDL-C was significantly increased but not in group B (Table II). A drug induced increase of HDL-C levels is associated with a mild reduction of CV risk (22). The antiatherogenic function of HDL relates to their ability to promote the efflux of cholesterol from cells, so as to their antiinflammatory and antioxidant properties (1). However, the atheroprotective function of

HDL is related not only to the plasma concentration of HDL-C, but also to the distribution and functional characteristics of HDL particles. It is known that altered small-sized HDL particles have a diminished ability to protect LDL from oxidation, a reduced reverse cholesterol transport capacity and antiinflammatory properties (1).

Small-sized HDL particles were more prevalent in group A than in group B, reaching statistically significant difference after therapy (Table II). A relatively high baseline proportion of small-sized HDL particles in group A (Table II) could not be significantly reduced by atorvastatin, unlike in group B. In this study, atorvastatin's beneficial effect on the HDL 2b subclasses was present in both groups, but significantly more so in group B (Figure 1).

Results of some studies indicated that HDL 2b, which is highly correlated with HDL-C, is strongly inversely related to CHD risk (23, 24). In our study there was a difference in HDL 2b between group A and group B, at baseline and after therapy. HDL 2b subclasses were more prevalent in group B where the patients had a more favourable risk profile. In both groups, the treatment shifted HDL subclasses distribution toward larger particles which is in agreement with the study by Asztalos et al. (25). In addition, our finding of significantly increased dominant HDL diameter after atorvastatin therapy in group A (Table II) is in line with the studies (26, 27) which showed beneficial effects of statins through the increase of large HDL particles that have considerably stronger antiatherogenic properties. Although the literature is indicative of HDL-C antioxidative, antiapoptotic and antiinflammatory activities, in the study there were no correlations of HDL-C with bilirubin and HDL-C with genes that are significant for the aforementioned effects (results not shown). The reason for this may be the limitations in our study, such as the small number of samples, the short treatment time of 10 weeks and the monitoring of pleiotropic effects only once, after 10 weeks of therapy (a one-time assessment).

This study indicates that atorvastatin in stable angina patients lowered the plasma mRNA levels of statin pleiotropy-targeted genes *ICAM1* and *CCL2* in both groups. This reduction was obviously independent from the LDL-C reduction (results not shown). This paper, and the previous paper (7), shows that short-term atorvastatin treatment results in anti-inflammatory effects, presumably by different mechanisms, including the inhibition of nuclear factor kappa B (NF- κ B) (28) and the interference with the prenylation of small G proteins (such as Rho and Ras) (29).

Current studies suggest that statins increase CTSS in the plasma, by statin-induced apoptosis of vascular cells (30). However, this suggestion has limitations because statin treatment did not change the activity of CTSS in the wall of abdominal aortic aneurysms in one small case-control study (31) and

CTSS deficiency by itself reduces apoptosis of vascular macrophages (11). In our study atorvastatin did not change the plasma CTSS mRNA level in both groups. An alternative explanation could be that atorvastatin in our patients downregulated inflammation and oxidative stress, which further lowered CTSS expression, or atorvastatin in our study improved the balance between CTSS activity and cystatin C (a potent endogenous CTSS inhibitor), but again, this suggestion has limitations because we could not recognise any change in the plasma CTSS mRNA level. However, it is significant that in the group A subjects, the plasma total bilirubin concentration was associated with the baseline plasma CTSS mRNA. Bilirubin is recognised as a potent antioxidant (32) and a lower total bilirubin could mean higher oxidative stress. In the development of vascular disease, oxidative stress plays a critical role. The subjects with elevated bilirubin levels had diminished risk of atherosclerosis (33). The apoptotic cell death is caused by cellular depletion of bilirubin (34) and consequently increases CTSS in the plasma. A negative correlation between bilirubin and CTSS mRNA in our clinical study (in line with *in vitro* studies) is significant since it leads to novel therapy directives aimed at preventing the clinical progression of atheroma. Bilirubin correlation with CTSS mRNA raises the question as to whether lower values of bilirubin could be a therapeutic target in the field of atherosclerosis. Some studies suggest that cathepsin S is involved in the complex pathways of cardiovascular disease and cancer. The study by Jobs et al. (35) revealed higher serum cathepsin S levels in elderly individuals in 2 independent groups were associated with an increased mortality risk. It is evident that increased bilirubin will cause lower expression of the CTSS mRNA, the gene associated with a higher mortality risk.

Thus, it is not easy to form an exact image of the mechanism of negative correlation between bilirubin and CTSS mRNA in the group A patients, and we have to propose that more detailed studies are needed to elucidate this issue.

Our study shows that, in stable angina patients, atorvastatin increased the plasma total bilirubin concentrations in group A only. These results (Table III) are in line with the abovementioned increase of dominant HDL diameter and large HDL 2b subclasses (Table II). Wallner M et al. (36) showed that elevated circulating bilirubin is associated with a reduced concentration of proatherogenic lipids and inflammation biomarkers. Statins increase total bilirubin that pro-

tects LDL from oxidation (37), which then reduces oxidative stress. On the other hand, a more favourable oxidative status after 10 weeks of therapy (lower concentration of Ox LDL) could lead to savings of bilirubin, a potent antioxidant, which is reflected in a higher concentration of bilirubin in the plasma.

Altogether, our results indicated favourable effects of statins on two compounds of the antioxidative defence system: bilirubin and large HDL 2b subclasses. The fact that elevations of both the concentration of bilirubin and the proportion of HDL 2b were more prominent in group A, which is more vulnerable in terms of antiatherogenic protection, is especially important. These findings suggest that low HDL-C concentration may also be a target for beneficial effects of statins. Results from Table III raise the question about the necessary length of therapy before evaluating the antioxidant activity of statins. Likewise, our results suggest that a significant decrease of SAA and CRP in plasma is not due to the atorvastatin effect on LDL cholesterol, but is a consequence of the pleiotropic effects of atorvastatin. Atorvastatin exerts a number of pleiotropic effects, which include anti-inflammatory and antioxidative activities. However, further, well planned studies are needed to evaluate our currently observed results.

Conclusions

The results suggest that routine care in stable angina patients could be complemented with new therapeutic approaches to raise HDL-C. HDL-C may become a therapeutic target equal to LDL-C in the field of CVD and atherosclerosis. The beneficial effect of atorvastatin on bilirubin also suggests that bilirubin can be a new therapeutic target in CVD. Analysis of cell-free mRNA in plasma could be a new tool in non-invasive assessment of statin pleiotropy.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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