

# Effects of atorvastatin on the HDL subpopulation profile of coronary heart disease patients

Bela F. Asztalos,<sup>1,\*</sup> Katalin V. Horvath,<sup>\*</sup> Judith R. McNamara,<sup>\*</sup> Paul S. Roheim,<sup>†</sup> Joel J. Rubinstein,<sup>§</sup> and Ernst J. Schaefer<sup>\*</sup>

Lipid Metabolism Laboratory,<sup>\*</sup> Jean Mayer USDA Human Nutrition Center on Aging at Tufts University and Division of Endocrinology, Metabolism, Diabetes, and Molecular Medicine, New England Medical Center, Boston, MA; Physiology Department,<sup>†</sup> Louisiana State University Medical Center, New Orleans, LA; and Cardiology Division,<sup>§</sup> Newton-Wellesley Hospital, Newton, MA

**Abstract** We investigated the effects of atorvastatin on the lipid and the apoA-I-containing HDL subpopulation profiles in 86 patients with established coronary heart disease (CHD). The entire drug treatment period lasted 12 weeks (4-week periods of 20 then 40, then 80 mg/day). Each dose of atorvastatin treatment resulted in significant reductions in plasma total-C, LDL-C, and triglyceride (TG), and non-significant increases in HDL-C levels compared with placebo treatment. ApoA-I levels did not change significantly during any of the treatment periods. Despite the modest increase of HDL-C (6%, 7%, 5%) and no change in apoA-I levels, the distribution of the apoA-I-containing HDL subpopulations changed significantly during each treatment period. There were significant increases in the concentrations of the large LpA-I  $\alpha$ -1 (24%, 39%, 26%) and pre $\alpha$ -1 (51%, 61%, 63%) subpopulations at the expense of the small lipoprotein LpA-I:A-II  $\alpha$ -3 subpopulations which decreased on all doses, and the decreases were significant on the 40 and 80 mg/day doses (6%, 5%). **■** Atorvastatin influences the lipid-related risk for CHD in two ways: first, it significantly decreases LDL-C and TG levels while increasing HDL-C, and second, it significantly shifts the HDL subpopulation profile of CHD patients toward that observed in subjects without CHD.—Asztalos, B. F., K. V. Horvath, J. R. McNamara, P. S. Roheim, J. J. Rubinstein, and E. J. Schaefer. Effects of atorvastatin on the HDL subpopulation profile of coronary heart disease patients. *J. Lipid Res.* 2002, 43: 1701–1707.

**Supplementary key words** CHD • lipids • lipoproteins • apolipoproteins • statin

Many prospective epidemiological studies have indicated that a low level of HDL-C is a significant independent risk factor for coronary heart disease (CHD) (1–6). HDL is composed of a heterogeneous group of particles

differing in electrophoretic mobility, size, and chemical composition as well as metabolic function. It is generally accepted that the different HDL subpopulations have different physiological function, therefore, their anti-atherogenic potential and their utility as risk markers may vary (7–11). Evidence is accumulating that the large lipoprotein LpA-I HDL particles play a more important role in reverse cholesterol transport and in the development of CHD than the smaller, LpA-I:A-II HDL particles (7–13). A variety of methods, including analytical ultracentrifugation, differential precipitation, immunoaffinity chromatography, and non-denaturing one- and two-dimensional gel electrophoresis, have been developed to separate HDL subclasses. HDL subspecies differ in apolipoprotein and lipid composition, physicochemical parameters, and have different physiological functions (8, 14–16). Based on the work of Castro and Fielding (15), we have developed a quantitative two-dimensional gel electrophoresis immunoblot image-analysis method for separating HDL subpopulations in plasma (17, 18). The majority of apolipoprotein A-I has  $\alpha$  mobility and has been classified as  $\alpha$ -1,  $\alpha$ -2, and  $\alpha$ -3 with sizes of 11.2 nm, 9.51 nm, and 7.12 nm, respectively (17). Small amounts of apoA-I are present in pre $\beta$ -1 and pre $\beta$ -2 and in the pre $\alpha$  mobility (pre $\alpha$ -1–3) subpopulations. We have demonstrated that  $\alpha$ -1, along with pre- $\beta$  and pre- $\alpha$  mobility particles, contain only apoA-I; subsequently they are LpA-I particles (18). We also published that apoA-II is present only in  $\alpha$ -2 and  $\alpha$ -3 HDL subpopulations, consequently these two subpopulations are LpA-I:A-II particles. Recently, with the use of two different labels (<sup>125</sup>I and fluorescent labels), we have been immunoprobng the same membrane for apoA-I and apoA-II. With this precise new method, we verified this observation in several hundred subjects including control

Manuscript received 22 January 2002, in revised form 17 April 2002, and in re-revised form 27 June 2002.

DOI 10.1194/jlr.M200037JLR200

Copyright © 2002 by Lipid Research, Inc.

This article is available online at <http://www.jlr.org>

Abbreviations: apo, apolipoprotein; CHD, coronary heart disease; TG, triglyceride.

<sup>1</sup> To whom correspondence should be addressed.  
e-mail: basztalos@hnrc.tufts.edu

and CHD subjects treated or untreated for lipid disorders with various drugs (unpublished data). However, in this study we immunoprobed the membranes for apoA-I only.

There is a strong positive correlation between HDL-C and the  $\alpha$ -1 HDL subpopulation (18). The HDL subpopulation profiles of CHD subjects are significantly different from those of controls even after normalizing for HDL-C (12). Namely, the concentrations of the large LpA-I  $\alpha$ -1 and pre $\alpha$ -1 HDL subpopulations are higher, while the concentration of the LpA-I:A-II  $\alpha$ -3 subpopulation is lower in controls compared with CHD patients.

It is generally accepted that the extent of the reduction in CHD events associated with the use of statin drugs can be explained largely by the reduction of LDL-C levels (19–22). The protective role of raising HDL-C might also be important but this mechanism is not completely understood (23–25). There may be value in determining which HDL subpopulation(s) might be responsible for the protection against CHD and to define which HDL subpopulation(s) are affected by statins. It is worth noting that the  $\alpha$ -1 subpopulation resembles HDL-2, as previously reported (18).

In the present study, we investigated the effects of atorvastatin on the apoA-I-containing HDL subpopulations and on the lipid and lipoprotein profile of CHD patients. We hypothesized that treatment with statins would normalize not only apoB-containing lipids and lipoproteins, but would also have a beneficial effect on the HDL subpopulation profile.

## METHODS

### Study population

One hundred and fourteen patients with established CHD participated in this study. Inclusion criteria included: age 21 years or older and established heart disease (post coronary artery bypass grafting, post angioplasty, post documented myocardial infarction, significant coronary artery stenosis as assessed by angiography of greater than 50%, or significantly decreased cardiac perfusion based on cardiac imaging with and without exercise). If female, the subject had to be postmenopausal or surgically sterile. All subjects were required to have LDL-C value of  $>130$  mg/dl and TGs  $<400$  mg/dl both at the time of screening and randomization. Patients had to be free of any clinical event for a period of  $\geq 6$  months before enrollment and they had to agree to discontinuation of any lipid-lowering medication for a period of 6 weeks before the beginning of the study. Exclusion criteria included: unstable/uncontrolled clinically significant disease, uncontrolled primary hypothyroidism (thyroid stimulating hormone  $> 5.5$   $\mu$ IU/ml), nephrotic syndrome, or renal dysfunction (blood urea nitrogen  $\geq 30$  mg/dl, or 10.5 mM/l; creatinine  $\geq 2.5$  mg/dl, or 220  $\mu$ M/l; or creatinine clearance  $<30$  ml/min), diabetes mellitus requiring insulin therapy, or uncontrolled diabetes (hemoglobin A1c  $>10\%$ ), a body mass index (BMI)  $>35$  kg/m<sup>2</sup>, or clinically significant clinical laboratory/ hematology abnormalities. Additional exclusion criteria were the presence of active liver disease or hepatic dysfunction, CPK levels  $>3$  times the upper limit of normal, uncontrolled hypertension, and a current or recent history of drug abuse or consumption of more than 14 alcoholic drinks per week. Patients were maintained on other medications throughout the study with no change, including calcium channel blockers,  $\beta$  blockers, diuretics, and other antihypertensive therapy. Patients

previously received instruction on a National Cholesterol Education Program Step 2 diet containing less than 30% of calories as fat, less than 7% of calories as saturated fat, and less than 200 mg of cholesterol per day. Plasma samples on each treatment (placebo, 20, 40, 80 mg/day atorvastatin) were available from 86 subjects (77 males and 9 females, mean age  $61 \pm 9$  years).

### Study design

The study was designed to compare the effects of five different statins on the lipid and the apoA-I-containing HDL subpopulation profiles of CHD patients (26). In this subgroup analysis, we further analyzed the effects of atorvastatin 20, 40, and 80 mg/day on the HDL subpopulation profiles of CHD patients compared with placebo treatment.

It was a single center, randomized, open label and two-period crossover incomplete block design study. The physician responsible for treatments and the patients were not blinded for treatment, however, our laboratory staff carrying out the analyses were blinded, since they received number coded plasma samples and the code was broken only after completion of all measurements. The study design is summarized in Fig. 1. After enrollment, there was a four-week diet run-in period to further assess a patient's qualifications for study entry. Qualified patients were randomized to receive atorvastatin or one of the other four different statins (simvastatin, pravastatin, lovastatin, fluvastatin). All treatments started at 20 mg/day and increased to 40 mg/day, and then to 80 mg/day. Each active drug treatment period lasted 12 weeks (three 4-week periods of each dose). After the first 12-week active drug treatment period, patients received placebo (washout) for 8 weeks. After this washout period, those patients receiving atorvastatin first were then placed on one of the other four statins, and patients received any of the other statins first were placed on atorvastatin. Finally, all patients received atorvastatin, placebo, and one of the other four statins. All patients were sampled after a 12 h overnight fast at the end of each study period. The study protocol was approved by the Human Investigation Review Committee of New England Medical Center. All participants of the study gave written informed consent.

### Lipid and lipoprotein analysis

Fasting blood samples (12 h) were collected into tubes containing 1.5 g/l EDTA and centrifuged at 2500 rpm for 20 min at 4°C to obtain plasma. Aliquots of each plasma sample were stored at  $-80^{\circ}\text{C}$ . Plasma total cholesterol and TG concentrations were measured in fresh plasma using automated standardized enzymatic assays, as previously described (27). HDL-C was measured in fresh plasma after precipitation of apoB-containing lipoproteins with dextran sulfate magnesium precipitation procedures (28, 29). LDL-C was measured in fresh plasma directly after immunoprecipitation of TG-rich lipoproteins and HDL, as previously described (30). ApoA-I was measured in plasma previously stored at  $-80^{\circ}\text{C}$  using immunoturbidimetric assay kits (Wako Bioproducts, Richmond, VA), as described (31). For all assays, the coefficient of variation was less than 10%.

### HDL subpopulation analysis

Two-dimensional non-denaturing agarose-polyacrylamide gel electrophoresis and image analysis for determining the apoA-I-containing HDL subpopulations were carried out on plasma previously stored at  $-80^{\circ}\text{C}$ , as described (17, 18).

In the first dimension, HDL was separated by charge, on agarose gel, into pre $\beta$ ,  $\alpha$ , and pre $\alpha$  mobility particles. Low endosmoticity 0.7% agarose (SeaKem LE, FMC Bioproducts, Rockford, ME) was cast into 3 mm thick vertical glass cassettes and electrophoresed in a Pharmacia GE 2/4 recirculating apparatus (Uppsala, Sweden). Four microliters of plasma per sample channel

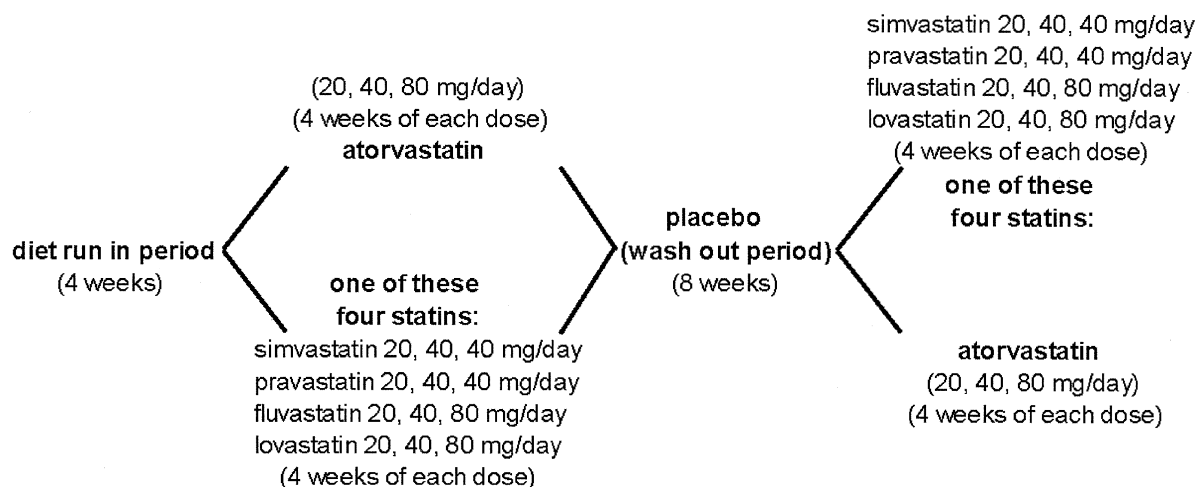


Fig. 1. Schematic representation of study design.

were electrophoresed at constant voltage (250 V) and temperature (10°C) in tris-tricine buffer (25 mM, pH 8.6) until the endogenous albumin, stained with bromophenol blue, ran 3.5 cm from the origin. Agarose was slipped out of cassettes and individual strips were cut out.

In the second dimension, each sample was further separated according to size by non-denaturing polyacrylamide gel electrophoresis. After electrophoresis was completed in the first dimension, the agarose strips were placed onto concave-gradient (3–35%) polyacrylamide gels and sealed with agarose. Electrophoresis was performed using a buffer containing 90 mM Tris, 80 mM boric acid, and 2.5 mM EDTA (pH 8.3) in a SE 600 Hoefer unit (Amersham Pharmacia, Piscataway, NJ). Gels were run at constant voltage (250 V) and temperature (10°C) for 24 h. Gels were electrotransferred using Hoefer TE 600 units (Amersham Pharmacia, Piscataway, NJ) to nitrocellulose membranes (0.2  $\mu$ m, BA-S83, Schleicher and Schuell, Keene, NH) at constant voltage (30 V) and temperature (10°C) for 24 h in buffer containing 20 mM tris and 150 mM glycine (pH 8.4). Membranes were dried at room temperature.

Prior to immunoblot for apoA-I, membranes were completely wetted in 10 mM phosphate buffered saline (PBS) followed by 10 min incubation in PBS containing 0.03% glutaraldehyde for fixing proteins on membrane. After rinsing membrane two times to remove glutaraldehyde, the free protein binding capacity was blocked by incubating membranes in PBS containing 0.05% Tween (PBST) and 5% nonfat dry milk for 10 min. ApoA-I was immunolocalized by incubation with PBST containing 5% milk and monospecific goat human apoA-I antibody for 7 h. The unbound primary antibody was then washed off by rinsing membranes in PBST three times for 3 min followed by incubation with  $^{125}$ I labeled secondary antibody (in PBST+5% milk) overnight. Signals were quantitatively determined by image analysis using a FluoroImager (Molecular Dynamics, Sunnyvale, CA) (17, 18). Pre $\beta$ 1 and pre $\beta$ 2 particles did not overlap with any other HDL particles so they were easily delineated. Designation of the  $\alpha$  mobility HDL subpopulations were based on integration of  $\alpha$ -migrating HDL. The integration curve showed three peaks. We delineated each peak area for  $\alpha$ 1-3. Pre $\alpha$  mobility particles have the same size as the  $\alpha$  mobility counterparts. Finally 10 apoA-I-containing HDL subpopulations were encircled and signals were measured in each area and used for calculating the percent distribution of the apoA-I-containing HDL subpopulations. We pooled some particles and calculated with one pre $\beta$ -1 and one

pre $\beta$ -2 HDL subpopulations. Data were expressed as pixels linearly correlated with the disintegrations per minute of the  $^{125}$ I bound to the antigen-antibody complex (32). ApoA-I concentrations of the subpopulations were calculated by multiplying percentiles by plasma total apoA-I concentrations. We had a CV of <10% for all of the  $\alpha$  mobility subpopulations and <15% for the rest of the particles.

In this study, we analyzed data of only those patients whose plasma samples were available on both arms (placebo, atorvastatin 20, 40, 80 mg/day) of the study. ANOVA was used to test the hypothesis of no difference between data obtained on placebo and after each of the atorvastatin treatments. For analysis, non-normally distributed data were logarithmically transformed.

## RESULTS

Biochemical parameters of patients ( $n = 86$ ) on placebo, on atorvastatin treatments, as well as the changes between placebo and treatments are presented in **Table 1**. As reference, we also present the same parameters from an earlier published healthy male population ( $n = 79$ ), mean age =  $53 \pm 6$  years (12). The means of the measured lipid parameters of patients on placebo represent unfavorable levels according to the recent NCEP guidelines (33). Total cholesterol, LDL-C, and TG were higher, while HDL-C was lower than the recommended values. The HDL subpopulation profiles of these patients were very similar to those of the CHD patients that were characterized earlier (12). Patients'  $\alpha$ -1 HDL subpopulations were lower by 37% and the  $\alpha$ -3 subpopulations were higher by 22% than these values in the reference healthy male subjects.

As a result of treatment, concentrations of total cholesterol, LDL-C, and TG decreased significantly, while HDL-C increased but not significantly. Plasma total apoA-I levels were practically unchanged on any treatment. The  $\alpha$ -1 HDL subpopulation, a large LpA-I particle increased significantly by 24% ( $P < 0.05$ ), 39% ( $P < 0.001$ ), and 26% ( $P < 0.01$ ) on the 20, 40, and 80 mg/day doses, respectively.

TABLE 1. Concentrations of plasma lipids, apoA-I, and HDL subspecies of coronary heart disease patients on placebo and on atorvastatin for 12 weeks (4 weeks each dose) and of a control group as reference

	Control (n = 79)	Placebo (n = 86)	20 mg/day (n = 86)	$\Delta\%$	40 mg/day (n = 86)	$\Delta\%$	80 mg/day (n = 86)	$\Delta\%$
TC	212 $\pm$ 38	269 $\pm$ 69	183 $\pm$ 48 <sup>a</sup>	-30	170 $\pm$ 44 <sup>a</sup>	-38	156 $\pm$ 37 <sup>a</sup>	-41
LDL-C	145 $\pm$ 34	175 $\pm$ 60	111 $\pm$ 41 <sup>a</sup>	-36	98 $\pm$ 42 <sup>a</sup>	-45	85 $\pm$ 35 <sup>a</sup>	-50
HDL-C	44 $\pm$ 12	37 $\pm$ 9	40 $\pm$ 10	6	41 $\pm$ 10	7	40 $\pm$ 10	5
TG	120 $\pm$ 63	185 $\pm$ 87	148 $\pm$ 77 <sup>b</sup>	-13	126 $\pm$ 63 <sup>a</sup>	-27	114 $\pm$ 57 <sup>a</sup>	-35
apoA-I	121 $\pm$ 21	119 $\pm$ 18	120 $\pm$ 19	3	120 $\pm$ 19	2	118 $\pm$ 18	0
LDL-C/HDL-C	3.0 $\pm$ 1.0	4.9 $\pm$ 2.1	3.0 $\pm$ 1.4 <sup>a</sup>	-38	2.5 $\pm$ 1.3 <sup>a</sup>	-48	2.2 $\pm$ 1.0 <sup>a</sup>	-52
pre $\beta$ -1	7.9 $\pm$ 4.9	12.6 $\pm$ 5.4	14.7 $\pm$ 6.0 <sup>c</sup>	25	10.5 $\pm$ 4.5	-3	13.5 $\pm$ 5.8	16
pre $\beta$ -2	3.8 $\pm$ 1.8	3.0 $\pm$ 1.6	2.2 $\pm$ 1.1 <sup>c</sup>	-10	3.3 $\pm$ 1.6	10	2.1 $\pm$ 1.1 <sup>c</sup>	-11
$\alpha$ -1	19.6 $\pm$ 8.2	12.4 $\pm$ 5.8	14.2 $\pm$ 6.3 <sup>c</sup>	24	15.8 $\pm$ 6.6 <sup>a</sup>	39	14.9 $\pm$ 7.1 <sup>b</sup>	26
$\alpha$ -2	40.1 $\pm$ 8.2	38.4 $\pm$ 8.3	36.7 $\pm$ 9.0	3	41.2 $\pm$ 8.7	6	36.5 $\pm$ 8.3	-1
$\alpha$ -3	34.0 $\pm$ 6.2	41.5 $\pm$ 6.4	40.9 $\pm$ 8.4	-1	39 $\pm$ 6.9 <sup>b</sup>	-6	38.5 $\pm$ 7.5 <sup>c</sup>	-5
pre $\alpha$ -1	4.8 $\pm$ 2.2	2.3 $\pm$ 1.2	3.1 $\pm$ 1.8 <sup>a</sup>	51	3.0 $\pm$ 1.6 <sup>a</sup>	61	3.6 $\pm$ 2.4 <sup>a</sup>	63
pre $\alpha$ -2	6.3 $\pm$ 2.5	4.3 $\pm$ 1.2	4.6 $\pm$ 1.7	12	4.7 $\pm$ 1.4 <sup>b</sup>	17	5.1 $\pm$ 1.9 <sup>b</sup>	20
pre $\alpha$ -3	4.2 $\pm$ 1.7	3.4 $\pm$ 1.1	3.4 $\pm$ 1.5	4	2.9 $\pm$ 0.9 <sup>c</sup>	-5	3.4 $\pm$ 1.4	4

Values are mean (mg/dl)  $\pm$  SD.  $\Delta\%$  represents the mean of individual responses for treatment (active treatment vs. placebo).

*P* values obtained by comparing values on medication to placebo:

<sup>a</sup> *P* < 0.001.

<sup>b</sup> *P* < 0.01.

<sup>c</sup> *P* < 0.05.

$\alpha$ -2, the larger LpA-I:A-II subpopulation, did not change significantly on any treatments, while  $\alpha$ -3, the smaller LpA-I:A-II subpopulation, decreased slightly but significantly (6% *P* < 0.01 and 5% *P* < 0.05) on the 40 and 80 mg doses, respectively. The larger pre- $\alpha$  mobility LpA-I particles, pre $\alpha$ -1 and pre $\alpha$ -2, increased significantly during each treatment. The smaller pre $\alpha$ -3 changed significantly (-5% *P* < 0.05) only on the 40 mg dose. Changes in the concentrations of the pre $\beta$  mobility particles were not consistent and we have no explanation for that.

We investigated whether baseline HDL-C levels interfere with the changes in the concentrations of the  $\alpha$ -1 subpopulation. We found that the two regression lines were parallel indicating that baseline HDL-C levels do not interfere with response of the  $\alpha$ -1 subpopulation for treatment (Fig. 2). We also checked whether the changes in the concentrations of the  $\alpha$ -1 subpopulation are proportional with the changes in HDL-C levels. It was found that as a result of treatment, the concentrations of the  $\alpha$ -1 subpopulation increased more than the concentrations of HDL-C, and the difference between the two regression lines were significant (*P* < 0.001). After 40 mg/day atorvastatin treatment, subjects increased the average concentration of  $\alpha$ -1 HDL by 2.71 mg/dl at any given HDL-C level.

All three doses of atorvastatin changed the apoA-I-containing HDL subpopulation profile the same way. As the changes were the most pronounced on 40 mg/day treatment, we selected data obtained on this treatment for further analyses of the effect of baseline levels (lipid levels on placebo treatment) on the response to treatment.

When the data were sorted by HDL-C levels on placebo and grouped into "low-HDL-C" (HDL-C  $\leq$  40 mg/dl, mean HDL-C 32 mg/dl) and "normal-HDL-C" (HDL-C > 40 mg/dl, mean HDL-C 48 mg/dl) groups, we found differences in response to the treatment between the low

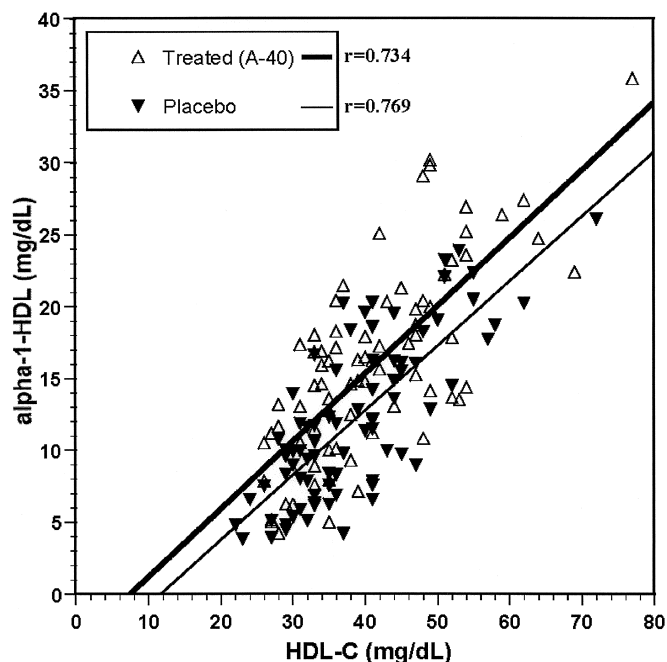


Fig. 2. Plots and calculated regression lines of  $\alpha$ -1-HDL subpopulations versus HDL-C. Because every subject was measured both on 40 mg/day atorvastatin and on placebo treatment, the expected difference in  $\alpha$ -1 levels for a given level of HDL-C was estimated as the intercept in the linear regression of the difference in  $\alpha$ -1 on atorvastatin and on placebo on the difference in HDL-C on atorvastatin and on placebo. That is, on atorvastatin, the relation between  $\alpha$ -1 and HDL-C was assumed to be  $\alpha_{1a} = \beta + \delta \text{HDL-C}_a$ , while on placebo it was assumed to be  $\alpha_{1p} = \mu + \delta \text{HDL-C}_p$ . Therefore,  $\alpha_{1a} - \alpha_{1p} = \beta - \mu + \delta \text{HDL-C}_a - \text{HDL-C}_p$ , and the intercept of this regression line estimates the difference between the intercepts of the individual regression lines.

TABLE 2. Biochemical parameters of coronary heart disease patients on placebo and on atorvastatin (40 mg/day for 4 weeks) grouped by HDL-C levels on placebo

	HDL-C < 40 mg/dl (n = 46)			HDL-C > 40 mg/dl (n = 40)		
	Placebo	Atorvastatin	Δ%	Placebo	Atorvastatin	Δ%
TC	289 ± 91	168 ± 50 <sup>a</sup>	-41	265 ± 42	173 ± 36 <sup>a</sup>	-34
LDL-C	189 ± 76	101 ± 46 <sup>a</sup>	-46	175 ± 46	94 ± 37 <sup>a</sup>	-44
HDL-C	32 ± 4	35 ± 5 <sup>b</sup>	10	48 ± 8	50 ± 9	5
TG	216 ± 97	138 ± 67 <sup>a</sup>	-33	141 ± 47	109 ± 53 <sup>b</sup>	-19
apoA-I	108 ± 13	110 ± 12	3	133 ± 14	135 ± 16	2
LDL-C/HDL-C	6 ± 3	3 ± 1 <sup>a</sup>	-50	4 ± 1	2 ± 1 <sup>a</sup>	-45
preβ-1	11 ± 5	10 ± 4	-2	14 ± 5	12 ± 5	-11
preβ-2	3 ± 1	3 ± 2	26	3 ± 2	3 ± 2	4
α-1	9 ± 4	13 ± 5 <sup>a</sup>	46	16 ± 5	20 ± 5 <sup>b</sup>	31
α-2	35 ± 6	37 ± 6	7	46 ± 7	48 ± 8	5
α-3	41 ± 7	38 ± 6 <sup>b</sup>	-8	43 ± 6	41 ± 8	-4
preα-1	2 ± 1	3 ± 1 <sup>a</sup>	80	3 ± 1	4 ± 1 <sup>c</sup>	32
preα-2	4 ± 1	4 ± 1 <sup>c</sup>	18	5 ± 1	5 ± 1	15
preα-3	3 ± 1	3 ± 1 <sup>c</sup>	-8	3 ± 1	3 ± 1	-8

Values are mean (mg/dl) ± SD. Δ% represents the mean of individual responses for treatment (active treatment vs. placebo). TG values were logarithmically transferred for analyses.

P values obtained by comparing values on medication to placebo:

<sup>a</sup> P < 0.001.

<sup>b</sup> P < 0.01.

<sup>c</sup> P < 0.05.

and normal groups (Table 2). On placebo, the group with low HDL-C level had higher concentrations of total-C, LDL-C, and TG levels, and lower concentrations of apoA-I. Similar to the difference in HDL-C levels, the mean concentration of the α-1 subpopulation was 44% lower in the low-HDL-C group. α-2 was also lower (24%) in the low-HDL-C group with no notable difference in the concentrations of α-3 compared with the normal-HDL-C group. Concentrations of TC, LDL-C, and TG decreased more while concentrations of HDL-C and the α-1 HDL particles increased more in this group.

In Table 3, data were sorted by TG levels on placebo and subjects were divided into two groups at the median. The mean TG level in the <median group (120 mg/dl) was below and that of the > median group (254 mg/dl) was above the recommended ≤150 mg/dl level. There were no significant differences between the two groups in terms of total cholesterol, LDL-C, and apoA-I concentrations, while the HDL-C concentration was 17% lower in the group with higher TG level on placebo. The differences in the HDL subpopulation profiles were similar to the differences in HDL-C levels. The mean concentrations of the α-1 and preα-1 subpopulations were lower by 29% and 33%, respectively, in the higher TG group. No major differences were found in the concentrations of the other HDL subpopulations. When the responses to the treatment were compared, we found larger increases in the α-1 and preα-1 HDL subpopulations in the group with higher TG levels compared with the group with lower TG level.

## DISCUSSION

A negative association between HDL-C and CHD has been verified in many epidemiological and interventional studies (3–6, 34, 35). HDL represents a heterogeneous

group of particles differing in their anti-atherogenic potential. Most data indicate that the large LpA-I HDL particles carry a significant portion of the anti-atherogenic properties of HDL. We demonstrated earlier that male CHD patients had significantly lower concentrations of the large LpA-I α-1 and preα-1-3 subpopulations, and significantly higher concentrations of the small LpA-I:α-II α-3 subpopulations when compared with healthy male control individuals (12). We have also documented that Tangier patients with defective reverse cholesterol transport have only preβ-1 HDL subpopulations in the homozygous

TABLE 3. Biochemical parameters of coronary heart disease patients on placebo and on atorvastatin (40 mg/day for 4 weeks) sorted by TG levels on placebo and segregated at the median

	<Median (n = 43)			>Median (n = 43)		
	Placebo	Atorvastatin	Δ%	Placebo	Atorvastatin	Δ%
TC	264 ± 61	162 ± 42 <sup>a</sup>	-38	295 ± 85	179 ± 46 <sup>a</sup>	-38
LDL-C	179 ± 60	91 ± 40 <sup>a</sup>	-47	187 ± 70	105 ± 44 <sup>a</sup>	-43
HDL-C	42 ± 10	43 ± 11	3	35 ± 7	39 ± 9 <sup>c</sup>	12
TG	120 ± 29	100 ± 40 <sup>c</sup>	-17	254 ± 76	154 ± 76 <sup>a</sup>	-38
apoA-I	121 ± 19	124 ± 20	3	115 ± 16	117 ± 16	2
LDL-C/HDL-C	4 ± 2	2 ± 1 <sup>a</sup>	-48	6 ± 3	3 ± 1 <sup>a</sup>	-48
preβ-1	12 ± 5	10 ± 5	-5	12 ± 5	11 ± 4	-6
preβ-2	3 ± 2	3 ± 2	13	3 ± 1	3 ± 2	21
α-1	14 ± 5	18 ± 6 <sup>b</sup>	33	10 ± 5	14 ± 6 <sup>b</sup>	47
α-2	41 ± 9	43 ± 9	6	37 ± 7	39 ± 7	7
α-3	41 ± 6	39 ± 8	-4	43 ± 6	39 ± 6 <sup>b</sup>	-9
preα-1	3 ± 1	3 ± 1 <sup>c</sup>	41	2 ± 1	3 ± 2 <sup>b</sup>	81
preα-2	4 ± 1	5 ± 1	13	4 ± 1	5 ± 1 <sup>c</sup>	20
preα-3	3 ± 1	3 ± 1	-6	4 ± 1	3 ± 1	-5

Values are mean (mg/dl) ± SD. Δ% represents the mean of individual responses for treatment (active treatment vs. placebo).

P values obtained by comparing values on medication to placebo:

<sup>a</sup> P < 0.001.

<sup>b</sup> P < 0.01.

<sup>c</sup> P < 0.05.

state and markedly depleted large HDL subpopulations ( $\alpha$ -1,  $\alpha$ -2 and pre $\alpha$ -1, pre $\alpha$ -2) in the heterozygous state (36). Without cholesterol efflux from peripheral cells, there is no maturation of the phospholipid and apoA-I containing pre $\beta$ -1 into larger HDL particles. We hypothesize that  $\alpha$ -1 and pre $\alpha$ -1, the large LpA-I subpopulations, are the most anti-atherogenic particles of HDL.

Assessing HDL subpopulation profiles by two-dimensional non-denaturing gel-electrophoresis and image-analysis might improve our ability to predict risk for CHD beyond what can be estimated by traditional risk factors. It has to be noted that this technique measures the apoA-I concentrations of the HDL subpopulations.

The objective of the present study was to examine the effects of atorvastatin treatment (20, 40, and 80 mg/day for 4 weeks each dose) on the lipid, lipoprotein, and HDL subpopulation profiles of CHD patients. We investigated any carryover effects by comparing lipid data obtained at 4 weeks and 8 weeks of the placebo (wash-out) period to data obtained after the 4-weeks diet run-in period. Results obtained after 4 and 8 weeks on placebo were not significantly different from those obtained after the diet run-in period, indicating no carryover effects among the different drugs used in the whole study (data not shown).

Despite only a small increase in the mean level of HDL-C (6%, 7%, 5%) and no change in the mean apoA-I concentration, the HDL-subpopulation profile changed significantly on each dose of atorvastatin. There were significant increases in the large LpA-I  $\alpha$ -1 (24%, 39%, 26%) and pre $\alpha$ -1 (51%, 61%, 63%) subpopulations, while the small LpA-I:A-II  $\alpha$ -3 particles were slightly but significantly decreased by 6%, 5% on the two higher doses of the drug. Previously, we demonstrated that in normolipidemic, healthy male subjects the concentrations of the  $\alpha$ -1 HDL subpopulations and HDL-C were highly correlated ( $r = 0.846$   $P < 0.001$ ) (18). We also found that in CHD subjects the HDL-C/ $\alpha$ -1 ratio was 36% higher compared with matched controls due to 35% lower  $\alpha$ -1 and only 14% lower HDL-C in the CHD group compared with controls. These results indicated that in CHD patients the concentrations of the large LpA-I  $\alpha$ -1 subpopulation are disproportionately lower than in controls. The present data confirm earlier observations and clearly show that in this group of CHD patients, atorvastatin treatment in all administered doses not only normalized lipids but also significantly changed the HDL subpopulation profiles in the direction of that seen in normal individuals.

There is controversy in the literature about which HDL subpopulation(s) are anti-atherogenic and hence the best targets for pharmacological intervention in individuals with a high risk for CHD. In this study, we demonstrated that as a response to atorvastatin treatment, the concentration of the large LpA-I  $\alpha$ -1 HDL subpopulation increased at the expense of the small LpA-I:A-II  $\alpha$ -3 subpopulation. The concentration of the also LpA-I:A-II  $\alpha$ -2 particle was practically unaffected by the treatments.

We tested the influence of baseline lipid levels (lipid levels obtained on placebo treatment) on the response to treatment. Subjects were grouped according to HDL-C

levels into low (HDL-C  $\leq 40$  mg/dl) and normal (HDL-C  $> 40$  mg/dl) groups, or stratified and divided (at the median) into lower-LDL-C (mean 139 mg/dl) and higher-LDL-C (mean 229 mg/dl) groups (data not shown), and similarly by plasma TG levels into lower-TG (mean 120 mg/dl) and higher-TG (mean 254 mg/dl) groups. Subjects in the lower HDL-C, higher TG, and higher LDL-C groups had the most unfavorable HDL subpopulation profiles (lowest  $\alpha$ -1 and highest  $\alpha$ -3) on placebo. Moreover, subjects with the most unfavorable baseline lipid profiles benefited most from the treatment by decreasing total-C, LDL-C, and TG levels while increasing the concentrations of  $\alpha$ -1 HDL subpopulation more than subjects with better lipid values.

As the major effect of statins is reducing LDL-C, we investigated how changes in LDL-C influence changes in HDL. The high responder group, with an average 58% decrease in LDL-C, increased HDL-C by 4% and  $\alpha$ -1 by 33%, while the low responder group, with an average 31% decrease in LDL-C, increased HDL-C by 11% and  $\alpha$ -1 by 47%.

Data were also sorted by  $\Delta$ TG and separated at the median into low-responders with a mean decrease of 4% and high-responders with a mean decrease of 49%. HDL-C increased 4% and 11% in low and high responders, respectively. Interestingly, there were no differences in the responses of  $\alpha$ -1 HDL subpopulations between the two groups. The concentrations of  $\alpha$ -1 increased 40% in both groups on the treatment.

These data show that both lipid levels on placebo and lipid responses for treatment, except for the TG response, influenced the response of the large LpA-I HDL subpopulations ( $\alpha$ -1 and pre $\alpha$ -1) to treatment. Our interpretation of these results is that decreased cholesterol synthesis but not lipolysis was responsible for the changes in the HDL subpopulation profile of these patients. Decreased CETP activity after treatment with atorvastatin, probably due to decreased concentrations of TG-rich particles, has been reported (37). As a result of decreased CETP activity, both the concentration and the size of HDL increase (unpublished data).

In conclusion, atorvastatin treatment, at all administered doses, restored a normal LDL-C level, significantly decreased the concentration of plasma TG, and tended to increase HDL-C concentration. Atorvastatin also changed the HDL-subpopulation profile significantly in a beneficial way. The responses of HDL-C and HDL subpopulation profile were not strongly dose-dependent. The magnitude of the response of HDL-C and the HDL subpopulation profile was dependent rather on baseline lipid profiles. Those with the worst baseline lipid profiles benefited the most from atorvastatin treatment, and that was generally true for all of the measured parameters. **■**

This study was supported by Parke Davis/Pfizer (contract# 719-8469) New York, NY 10017; and by the National Institutes of Health/National Heart, Lung, and Blood Institute (HL-64738). The authors wish to thank Dr. Julian Marsh for his crit-

ical review of the manuscript and Gerard Dallal for helping in statistical calculations.

## REFERENCES

1. Gordon, T., W. P. Castelli, M. C. Hjortland, W. E. Kannel, and T. R. Dawber. 1977. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am. J. Med.* **62**: 707–714.
2. Jacobs, D. R. (for Lipid Research Clinics Follow-up Study). 1985. High-density lipoprotein cholesterol and coronary heart disease cardiovascular disease and all-cause mortality (abstr.) *Circulation*. **72** (suppl. III): 111–185.
3. Miller N. E., O. H. Forde, D. S. Thelle, and O. D. Mjos. 1977. The Tromso Heart Study. High-density lipoprotein and coronary heart disease: a prospective case-control study. *Lancet*. **1**: 965–968.
4. Enger, S. C., I. Hjermann, O. P. Foss, A. Helgeland, J. Holme, P. Leren, and K. R. Norum. 1979. High-density lipoprotein cholesterol and myocardial infarction or sudden coronary death: a prospective case-control study in middle-aged men of the Oslo study. *Artery*. **5**: 170–181.
5. Wilson, P. W. F., R. B. D'Agostino, D. Levy, A. M. Belanger, H. Shillershatz, and W. B. Kannel. 1998. Prediction of coronary heart disease using risk factor categories. *Circulation*. **97**: 1837–1847.
6. Goldburt, U., S. Yaari, and J. H. Medalie. 1997. Isolated low HDL cholesterol as a risk factor for coronary heart disease mortality: a 21-year follow-up of 8000 men. *Arterioscler. Thromb. Vasc. Biol.* **17**: 107–113.
7. Cheung, M. C., B. G. Brown, A. C. Wolf, and J. J. Albers. 1991. Altered particle size distribution of apolipoprotein A-I containing lipoproteins in subjects with coronary heart disease. *J. Lipid Res.* **32**: 383–394.
8. von Eckardstein, A., Y. Huang, and G. Assmann. 1994. Physiological role and clinical relevance of high density lipoprotein subclasses. *Curr. Opin. Lipidol.* **5**: 404–416.
9. Puchois, P., A. Kandoussi, P. Fievet, J. L. Fourrier, M. Bertrand, E. Koren, and J. C. Fruchart. 1987. Apolipoprotein A-I containing lipoproteins in coronary artery disease. *Atherosclerosis*. **68**: 35–40.
10. Campos, H., G. O. Roederer, S. Lussier-Cacan, J. Davignon, and R. M. Krauss. 1995. Predominance of large LDL and reduced HDL-2 cholesterol in normolipidemic men with coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* **15**: 1043–1048.
11. Sweetnam, P. M., C. H. Bolton, I. W. Yarnel, D. Bainton, I. A. Baker, P. C. Elwood, and N. E. Miller. 1994. Associations of HDL2 and HDL3 cholesterol subfractions with the development of ischemic heart disease in British men. *Circulation*. **90**: 769–774.
12. Asztalos, B. F., P. S. Roheim, R. L. Milani, M. Lefevre, J. R. McNamara, K. V. Horvath, and E. J. Schaefer. 2000. Distribution of ApoA-I-containing HDL subpopulations in patients with Coronary Heart Disease. *Arterioscler. Thromb. Vasc. Biol.* **20**: 2670–2676.
13. Fruchart, J. C., and G. Ailhaud. 1992. Apolipoprotein A-containing lipoprotein particles: Physiological role, Quantification, and clinical significance. *Clin. Chem.* **38**: 793–797.
14. Mowri, H. O., W. Patsch, L. C. Smith, A. M. Gotto, and J. R. Patsch. 1992. Different reactivities of high-density lipoprotein2 subfractions with hepatic lipase. *J. Lipid Res.* **33**: 1269–1279.
15. Castro, G. R., and C. J. Fielding. 1988. Early incorporation of cell-derived cholesterol into pre- $\beta$ -migrating high-density lipoprotein. *Biochemistry*. **27**: 25–29.
16. Miida, T., M. Kawano, C. J. Fielding, and P. E. Fielding. 1992. Regulation of the concentration of pre $\beta$  high-density lipoprotein in normal plasma by cell membranes and lecithin-cholesterol acyltransferase activity. *Biochemistry*. **31**: 11112–11117.
17. Asztalos, B. F., C. H. Sloop, L. Wong, and P. S. Roheim. 1993. Two-dimensional electrophoresis of plasma lipoproteins: recognition of new apo A-I-containing subpopulations. *Biochim. Biophys. Acta*. **1169**: 291–300.
18. Asztalos, B. F., M. Lefevre, T. A. Foster, R. Tulley, M. Widhauser, L. Wong, and P. S. Roheim. 1997. Normolipidemic subjects with low HDL cholesterol levels have altered HDL subpopulations. *Arterioscler. Thromb. Vasc. Biol.* **17**: 1885–1893.
19. Thompson, G. R. 1997. What targets should lipid-modulating therapy achieve to optimize the prevention of coronary heart disease? *Atherosclerosis*. **131**: 1–5.
20. Gould, A. L., J. E. Rossouw, N. C. Santanello, J. F. Heyse, and C. D. Furberg. 1998. Cholesterol reduction yields clinical benefit: impact of statin trials. *Circulation*. **97**: 946–952.
21. Pedersen, T. R. 2001. Pro and Con: Low-density lipoprotein cholesterol lowering is and will be the key to the future of lipid management. *Am. J. Cardiol.* **87**(suppl): 8B–12B.
22. Olsson, A. G. 2001. Statin therapy and reduction in low-density lipoprotein cholesterol: initial clinical data on the potent new statin rosuvastatin. *Am. J. Cardiol.* **87**(suppl): 33B–36B.
23. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. 1993. Summary of the second report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation and treatment of high blood cholesterol in adults (Adult Treatment Panel II). *J. Am. Med. Assoc.* **269**: 3015–3023.
24. Prevention of coronary heart disease in clinical practice: recommendations of the Second Joint Task Force of European and Other Societies on Coronary Prevention. 1998. *Eur. Heart J.* **9**: 1434–1503.
25. Person, T. A., W. E. Boden. 2000. The imperative to raise low levels of high density lipoprotein cholesterol - a better clinical strategy in the prevention and treatment of coronary artery disease. *Am. J. Cardiol.* **86**: 1L–4L.
26. Asztalos, B. F., K. V. Horvath, J. R. McNamara, P. S. Roheim, J. J. Rubinstein, E. J. Schaefer. 2002. Comparing the effects of five different statins on the HDL subpopulation profiles of coronary heart disease patients. *Atherosclerosis*. **164**: 361–369.
27. McNamara, J. R., and E. J. Schaefer. 1987. Automated enzymatic standardized lipid analyses for plasma and lipoprotein fractions. *Clin. Chim. Acta.* **166**: 1–8.
28. Warnick G. R., J. Benderson, and J. J. Albers. 1982. Dextran sulfate-Mg procedure for quantitation of high density lipoprotein cholesterol. *Clin. Chem.* **28**: 1379–1388.
29. Nguyen, T., and G. R. Warnick. 1989. Improved methods for separation of total HDL and subclasses. *Clin. Chem.* **35**: 1086–1090.
30. McNamara, J. R., T. G. Cole, J. H. Contois, C. A. Ferguson, J. M. Ordoval, and E. J. Schaefer. 1995. Immunoseparation method for measuring low-density lipoprotein cholesterol directly from serum evaluated. *Clin. Chem.* **41**: 232–240.
31. Contois, J. H., J. R. McNamara, C. J. Lammi-Keefe, P. W. F. Wilson, T. Massov, and E. J. Schaefer. 1996. Reference intervals for plasma apolipoprotein A-I determined with a standardized commercial immunoturbidometric assay: results from the Framingham Offspring Study. *Clin. Chem.* **42**: 515–523.
32. Johnson, R. F., S. C. Picket, and B. L. Barker. 1990. Autoradiography using storage phosphor technology. *Electrophoresis*. **11**: 355–360.
33. Executive summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. 2001. *J. Am. Med. Assoc.* **285**: 2486–2497.
34. The Expert Panel. 1988. Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Cholesterol in Adults. *Arch. Intern. Med.* **148**: 36–69.
35. Gordon, D. J., J. L. Probstfield, R. J. Garrison, J. D. Neaton, W. P. Castelli, J. D. Knoke, D. R. Jacobs, Jr., S. Bangdiwala, H. A. Tyroler. 1989. High density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation*. **79**: 8–15.
36. Asztalos, B. F., M. E. Brousseau, J. R. McNamara, K. V. Horvath, P. S. Roheim, and E. J. Schaefer. 2001. Subpopulations of high density lipoproteins in homozygous and heterozygous Tangier disease. *Arterioscler. Thromb. Vasc. Biol.* **156**: 217–225.
37. Guerin, M., T. S. Lassel, W. Le Goff, M. Farnier, and M. J. Chapman. 2000. Action of Atorvastatin in Combined Hyperlipidemia: Preferential Reduction of Cholesterol Ester Transfer from HDL to VLDL1 Particles. *Arterioscler. Thromb. Vasc. Biol.* **20**: 189–197.