Original Article

Interrelated Cathepsin S-Lowering and LDL Subclass Profile Improvements Induced by Atorvastatin in the Plasma of Stable Angina Patients

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***Aim*: We hypothesized that, in stable angina patients, atorvastatin therapy lowers the cathepsin S (CTSS) concentrations, as assessed non-invasively according to a plasma analysis. In addition, the low-density lipoprotein (LDL) and high-density lipoprotein (HDL) size and subclasses in the plasma were analysed to establish the association between CTSS and lipoprotein metabolism and determine whether this association is atorvastatin-sensitive.**

***Methods*: A total of 43 patients with stable angina received atorvastatin therapy (20 mg/day, 10 weeks). The plasma *CTSS* mRNA levels, CTSS protein concentrations and CTSS activity, as well as LDL and HDL size and subclasses, were analysed before and after treatment.**

***Results*: Atorvastatin treatment did not change the plasma *CTSS* mRNA levels, although it lowered the plasma CTSS concentrations and activity. An increased plasma CTSS concentration and activity were found to be associated with a more atherogenic LDL subclass profile (a decreased dominant LDL size and increased percentage of small, dense LDL particles). The atorvastatin-induced CTSS-lowering effect was concomitant with an improvement in the LDL subclass profile, and the changes were found to be interrelated. Concomitant, interrelated changes in the CTSS levels and LDL subclass profiles were found in the LDL phenotype B patients only (a dominant LDL diameter of ≤ 25.5 nm at the start of the study). In this subgroup, lowering of the plasma *CTSS* mRNA level also correlated with lowering of the proportion of small, dense LDL particles.**

***Conclusions*: Atorvastatin-induced CTSS-lowering and LDL subclass profile improvements in the plasma of LDL phenotype B patients with stable angina are concomitant and interrelated.**

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Key words: Atorvastatin, Cathepsin S, LDL subclasses, LDL phenotype

Abbreviations; cDNA, complementary deoxyribonucleic acid; CRP, C-reactive protein; CTSS, cathepsin S; *CTSS*, gene encoding CTSS; EDTA, ethylenediaminetetraacetic acid; *GAPDH*, gene encoding glyceraldehyde-3-phosphate dehydrogenase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; mRNA, messenger ribonucleic acid; SAA, serum amyloid A; TC, total cholesterol; TG, triglycerides

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Introduction

Cathepsin S (CTSS; EC 3.4.22.27) is a cysteine protease involved in autophagocytosis, damaged mitochondria clearance, major histocompatibility complex

class II antigen presentation and atherogenesis[1, 2]. As to the CTSS-mediated effects observed in the setting of atherogenesis, the *CTSS* mRNA and protein levels are increased in human and animal atheromas, but not in non-atherosclerotic arteries[3, 4]. CTSS is synthesized by macrophages, smooth muscle cells and endothelial cells[5] and, when released extracellularly, it exerts elastolytic and collagenolytic effects, leading to elastic lamina degradation[6, 7], plaque rupture[8] and necrotic core development[7]. Some studies have reported higher concentrations of CTSS in the plasma of patients with cardiovascular disease[9, 10]. Studies of animals also indicate that CTSS increases apoptosis in atherosclerotic lesions[7], although the exact mechanisms remain unknown. Current data on the effects of statins on CTSS are scarce. One study conducted in humans indicated that statin treatment does not change the CTSS activity in the wall of abdominal aortic aneurysms[11], while another study revealed that the expression of *CTSS* mRNA and protein was significantly increased in the myocardium of apoE$^{-/-}$ mice fed a Western-style diet and that simvastatin decreases the levels of *CTSS* mRNA and protein after 32 weeks of treatment[12]. In our previous study[13], a plasma mRNA analysis revealed that patients with coronary atherosclerosis have a much higher *CTSS* expression than control subjects, despite receiving statin treatment.

In vitro studies also indicate that CTSS may have an impact on lipoproteins metabolism. The incubation of HDL3 (high-density lipoprotein isolated via sequential ultracentrifugation from the plasma within a density range of 1.125 to 1.210 g/mL) with cathepsin S leads to the rapid loss of preβ-HDL and reduced cholesterol efflux from macrophages[14], and cathepsins in general (e.g. D, F, S and K) modify apolipoprotein B in LDL and subsequently induce foam cell formation[15]. Moreover, a study of catK^{-}/apoE^{-} mice showed a trend toward increased serum cholesterol and LDL cholesterol levels[16].

Aim

We hypothesized that, in stable angina patients, atorvastatin therapy (20 mg/day, for 10 weeks) decreases the *CTSS* expression and activity in the vascular bed, as assessed non-invasively according to the *CTSS* mRNA level, CTSS protein concentration and enzyme activity in the plasma. In addition, LDL and HDL size and subclasses analyses in the plasma were performed to establish the association between CTSS and lipoprotein metabolism and determine whether this association is atorvastatin-sensitive and/or beneficial.

Methods

Subjects and Blood Sampling

Forty-three patients were consecutively enrolled from among those with stable angina treated at the Health Centre Laktasi (Bosnia and Herzegovina). The patients were given 20 mg of atorvastatin daily for 10 weeks. Blood was collected from the subjects prior to the administration of therapy and 10 weeks later. Stable angina was diagnosed based on a clinical assessment, electrocardiogram evaluation and laboratory findings[17]. Patients with endocrine diseases, as well as inflammatory, thyroid, liver, neoplastic or renal (serum creatinine concentration ≥ 120 μmol/L) diseases, were excluded based on a detailed history and clinical examination. This study was approved by the National ethics committee. Written informed consent was obtained from all participating subjects. A detailed description of the procedure for collecting cell-free plasma has been published elsewhere[18]. Patients with haemolysed samples were excluded at the start of the study.

CTSS mRNA Quantification in the Plasma

RNA was isolated from 3 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and QIAvac 24 Plus (Qiagen) according to the manufacturer's instructions, with some modifications[18]. The following primers were used for cDNA transcript quantification (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA, USA): Hs00175403_ml for CTSS (exon boundary 3-4, amplicon lengh 82 bp) and Hs99999905_ml for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene. Four pairs of samples (obtained before and after statin therapy) were chosen to select the most appropriate reference gene among the 32 candidates available on the TaqMan Human Endogenous Control Plate (Applied Biosystems). The cDNA transcripts were amplified via PCR according to the manufacturer's protocol using the FastStart Universal Probe Master (ROX) (Roche Applied Science, Mannheim, Germany) with 11.25 μL of diluted (1:8) cDNA (*CTSS*) or 10.00 μL of diluted (1:12) cDNA (*GAPDH*). The kit components were run in a total reaction volume of 25 μL on an ABI Prism™ SDS PCR analyser (Applied Biosystems).

The possibility of DNA interference in the TaqMan Gene Expression Assay was excluded by running the PCR reaction with subject samples while omitting reverse transcription. In order to exclude the possibil-

ity of external mRNA contamination, a negative control (blank) was analysed. The between-run imprecision for the entire analytical procedure (mRNA isolation from pooled plasma, reversed transcribed into cDNA and transcript quantification as the average Ct assessment performed in triplicate - all conducted for three independent runs, thus giving three average Ct values), expressed as the coefficient of variation (CV) of Ct was less than 2%[19]. The amount of each investigated transcript was normalized to the amount of the reference gene[20]. Samples from the same patient (obtained before and after therapy) were processed in the same run.

CTSS Protein Quantification and CTSS Activity Estimation in the Plasma

Quantitative CTSS ELISA with a combination of 1E3 MAb and 2B4 Mabs (Krka, d.d., Ljubljana, Slovenia) was used as previously described[21]. The CTSS activity was determined using fluorogenic substrate Z-VVR-AMC (Biomol International, Hamburg, Germany). Substrate (final concentration: 10 μM) and serum (dilution: 1:3 in assay buffer [100 mM of phosphate buffer, pH 6.5 containing 0.1% (w/v) polyethylene glycol 8000, 5 mM of cysteine and 1.5 mM of EDTA]) were added to the wells of a black microplate. The formation of fluorescent degradation products was monitored continuously at 380 ± 5 nm excitation and 460 ± 5 nm emission on a Tecan Safire^{2TM} spectrofluorometer (Tecan Group Ltd, Männedorf, Switzerland). Samples from the same patient (obtained before and after therapy) were processed in the same run.

LDL and HDL Subclass Determination

The plasma LDL and HDL particles were separated using a method previously described by Rainwater *et al.*[22]. A detailed description of the procedure has been published elsewhere[23, 24]. Based on the dominant diameter, we classified the LDL phenotype as phenotype A (>25.5 nm) or phenotype B (≤ 25.5 nm). The relative proportions of the LDL and HDL subclasses were estimated by determining the areas under the peaks of densitometric scans. The relative proportion of small, dense LDL particles was ascertained by computing the area of the densitometric scan at or below 25.5 nm[25]. Likewise, the relative proportion of small HDL particles was assessed by computing the area of the densitometric scan at or below 8.8 nm[25].

Other Measurements in the Plasma

The levels of total cholesterol (TC), triglycerides (TG), LDL-C and HDL-C were determined using reagents obtained from Roche Diagnostics (Roche Diagnostics, Mannheim, Germany), and the C-reactive protein (CRP) level was determined using Beckman Coulter CRP Latex reagent (Beckman Coulter, Inc. Brea, CA, USA), according to the manufacturers' instructions. The serum amyloid A (SAA) level was determined as previously described[18].

Statistical Analysis

All calculations were performed using the SPSS v.20.0 (SPSS Inc., Chicago, IL, USA) software program. Numerical data are shown as the mean ± standard deviation for normally distributed variables and the median and interquartile range for non-normally distributed variables. Categorical variables are presented as relative frequencies. Comparisons of the data obtained before and after 10 weeks of atorvastatin therapy were made using the paired t-test for normally distributed data or Wilcoxon's signed-rank test for non-normally distributed variables. The unpaired Student's *t*-test and Mann-Whitney *U*-test were used to compare data between subgroups with the LDL B and LDL A phenotypes. The analysis of categorical variables was performed using the Chi-square and Fisher-Exact tests (where appropriate). The relationships between the variables were determined using the Spearman rank-order correlation test.

Results

The group of stable angina patients ($n = 43$) comprised 11 men and 32 women (of whom five were smokers), 61.67 ± 9.10 years of age, with a mean body mass index of 27.97 ± 5.13 kg/m^2. The patients had received other therapies prior inclusion into in this study, including beta blockers (44.2%), ACE inhibitors (67.4%), calcium-channel blockers (32.6%), nitrates (39.5%) and acetylsalicylic acid (11.6%). All drugs were administered continuously throughout the study, and the patients were given additional atorvastatin (20 mg/day) therapy daily following their diagnosis of stable angina.

The baseline laboratory parameters and their changes after 10 weeks of atorvastatin treatment are shown in **Fig. 1** and **Table 1**. As to the first part of our hypothesis, atorvastatin treatment did not change the plasma *CTSS* mRNA levels (difference in medians −7.8%, $p = 0.677$), although it lowered the plasma CTSS concentrations (−13.8%, $p = 0.016$) and plasma CTSS activity (−6.5%, $p = 0.043$). As expected, atorvastatin increased the dominant LDL size (+2.1%, $p < 0.001$), although it did not change the percentage of small, dense LDL particles. Simi-

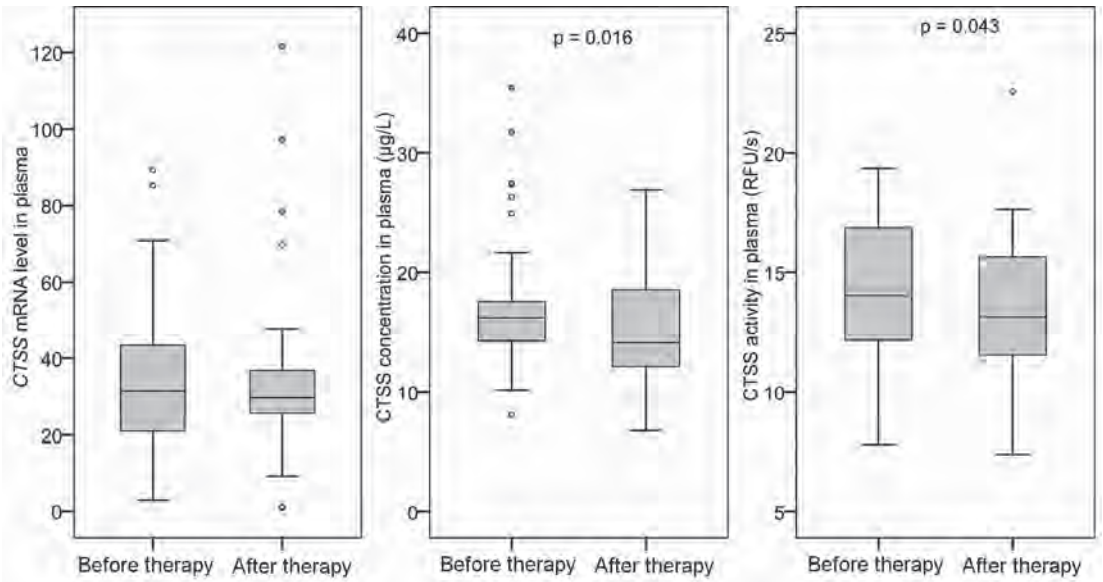

Fig. 1. *CTSS* mRNA levels and CTSS concentrations and activity in the plasma of the 43 patients with stable angina

The box plots demonstrate the interquartile range (25th and 75th percentiles), with the line inside the box denoting the median. The whiskers indicate the largest and smallest values inside the 1.5 x interquartile range. Outliers (·) are the values between the 1.5 and 3 x interquartile ranges. The plasma mRNA levels were standardized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase as a reference gene. The differences in variables before and after treatment were analyzed using Student's *t*-test for the CTSS activity. Wilcoxon's matched pairs test was used to assess the *CTSS* mRNA level and CTSS concentration. Abbreviations: CTSS, cathepsin S; *CTSS*, gene encoding CTSS; p, statistically significant.

Table 1. Laboratory findings of the 43 patients with stable angina

Parameter	Baseline	After 10 weeks of atorvastatin therapy	Mean difference		
			Absolute	Relative (%)	*p*
Dominant LDL diameter (nm)	25.92 (24.84-27.05)	26.47 (25.53-27.64)	0.55	2.1	< 0.001
Small, dense LDL particles (%)	44.7 (36.9-57.0)	45.1 (38.8-54.3)	0.40	0.9	0.708
Dominant HDL diameter (nm)	9.27 (9.03-10.52)	9.72 (9.33-10.71)	0.45	4.9	0.002
Small-sized HDL particles (%)	32.6 (29.2-40.3)	32.1 (28.2-37.9)	− 0.50	− 1.5	0.003
TC (mmol/L)	7.15 (6.60-7.77)	4.80 (4.20-5.07)	− 2.35	− 33.3	< 0.001
LDL-C (mmol/L)	4.75 (4.32-5.20)	2.65 (2.40-3.00)	− 2.10	− 43.8	< 0.001
HDL-C (mmol/L)[§]	1.33 ± 0.37	1.40 ± 0.34	0.07	5.3	0.074
TG (mmol/L)	1.60 (1.30-2.35)	1.40 (1.20-1.80)	− 0.20	− 12.5	< 0.001
CRP (mg/L)	2.05 (1.10-4.72)	1.90 (1.00-3.50)	− 0.15	− 7.3	0.010
SAA (mg/L)	23.00 (13.27-35.19)	14.44 (9.99-25.27)	− 8.56	− 37.2	< 0.001

The values are presented as the median (Q1-Q3) or mean ± standard deviation.
Wilcoxon's matched pairs test was used for the comparisons (see the Statistical analysis section), except for variables marked with[§], for which Student's *t*-test was used.
Abbreviations: p, level of significance; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; CRP, C-reactive protein; SAA, serum amyloid A.

larly, atorvastatin increased the dominant HDL size (+4.9%, $p=0.002$) and simultaneously lowered the percentage of small HDL particles (−1.5%, $p=0.003$). Finally, atorvastatin treatment lowered the serum concentrations of CRP, SAA, TC, LDL-C and TG, but not HDL-C. Concomitant therapies (administered in addition to atorvastatin) generally had no effect on the statin-induced reductions, with the exception that cal-

Table 2. Plasma *CTSS* mRNA and protein concentrations and activity, lipid profiles and LDL and HDL size and subclasses at baseline and after 10 weeks of atorvastatin therapy in the phenotype B (18 patients) and phenotype A (25 patients) groups

Parameter	Patients with LDL B phenotype			Patients with LDL A phenotype		
	Baseline	After 10 weeks	*p*	Baseline	After 10 weeks	*p*
CTSS mRNA†	32.11 (25.90-45.89)	32.56 (27.06-44.17)	0.955	32.37 (19.48-43.69)	29.08 (22.20-34.62)	0.331
CTSS (μg/L)	20.82 ± 6.48§, ***	16.37 ± 4.49	< 0.01	14.69 ± 3.77	14.68 ± 5.17	0.985
CTSS (RFU/s)	15.36 ± 2.97§, **	13.57 ± 2.26	< 0.05	13.39 ± 3.07	13.28 ± 3.24	0.801
Dominant LDL diameter (nm)	24.44 ± 0.64§, ***	25.46 ± 0.93§, ***	< 0.001	27.00 ± 0.87	27.32 ± 0.93	0.066
Small, dense LDL particles (%)	56.9 ± 5.1§, ***	52.0 ± 7.4§, ***	< 0.05	37.2 ± 8.1	41.6 ± 8.1	< 0.01
Dominant HDL diameter (nm)	9.22 ± 0.70§, *	9.72 ± 0.66§, ***	< 0.01	9.74 ± 0.84	10.03 ± 0.92	0.145
Small-sized HDL particles (%)	35.8 ± 9.3	34.1 ± 8.1	0.097	34.4 ± 7.8	32.8 ± 7.6	< 0.05
TC (mmol/L)	7.63 ± 0.99§, *	4.59 ± 0.57	< 0.001	6.96 ± 0.79	4.96 ± 0.79	< 0.001
LDL-C (mmol/L)	5.20 ± 0.98§, *	2.66 ± 0.55	< 0.001	4.73 ± 0.52	2.93 ± 0.72	< 0.001
HDL-C (mmol/L)	1.25 ± 0.36	1.33 ± 0.35	0.245	1.40 ± 0.37	1.46 ± 0.33	0.241
TG (mmol/L)†	2.05 (1.56-3.45)‡, **	1.6 (1.30-2.10)‡, *	< 0.01	1.5 (1.05-1.75)	1.2 (1.05-1.65)	0.152
CRP, mg/L†	1.85 (1.25-4.28)	1.45 (1.00-3.30)	< 0.05	2.80 (1.10-4.95)	2.10 (1.00-3.75)	0.090
SAA, mg/L†	21.60 (12.63-33.09)	14.988 (10.05-24.48)	< 0.01	23.74 (12.52-35.41)	13.59 (9.88-28.02)	< 0.01

The values are presented as the mean ± standard deviation or median (Q1-Q3).
The *CTSS* mRNA levels were standardized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase as a reference gene.
†Data compared according to Wilcoxon's test. §Significantly different from that observed in the patients with the LDL A phenotype at the same point of time, according to the unpaired Student's *t*-test. ‡Significantly different from that observed in the patients with the LDL A phenotype at the same point of time, according to the Mann-Whitney *U*-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
Abbreviations: CTSS, cathepsin S; CTSS, gene encoding CTSS; p, level of significance; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density cholesterol; TG, triglycerides; CRP, C-reactive protein; SAA, serum amyloid A.

cium-channel blockers appeared to be associated with a minor decrease in the TG levels (difference in means −20.3% in patients without vs. −3.8% in patients prescribed calcium-channel blockers, $p = 0.048$).

Regarding the second part of our hypothesis, the CTSS protein concentration and LDL subclass profile were found to be interrelated. For instance, a higher plasma CTSS concentration at baseline was associated with a lower dominant LDL diameter ($r = -0.476$, $p = 0.001$) and a higher proportion of small, dense LDL particles ($r = 0.527$, $p < 0.001$). Furthermore, the relative lowering of the plasma CTSS protein concentration induced by atorvastatin treatment correlated with the relative increment in the dominant LDL diameter ($r = -0.380$, $p = 0.013$) and the relative decrement in the proportion of small, dense LDL particles ($r = 0.384$, $p = 0.012$). A higher plasma CTSS activity was also found to be associated with a higher proportion of small, dense LDL particles (results not shown). Conversely, the changes in the CTSS and HDL subclass profiles were not interrelated. None of the other observed parameters were found to be associated with the above mentioned interrelations, with the exception of CRP plasma concentration-lowering, which correlated with the lowering of the CTSS protein concentration ($r = 0.368$, $p = 0.015$), although not concomitantly with the LDL subclass profile improvements.

In order to further study the CTSS and LDL subclass profile interrelationships, the patients were classified in two groups according to the LDL phenotype. The LDL B phenotype group comprised 18 stable angina patients (five men and 13 women), 63.1 ± 7.6 years of age. The LDL A phenotype group ($n = 25$ patients) was equivalent in terms of age (60.1 ± 10.1 years, $p = 0.405$) and gender (six men and 19 women, $p = 0.779$) to the LDL B phenotype group. Examinees with the LDL B and A phenotypes did not differ in terms of body mass index (28.57 ± 3.99 kg/m2 vs. 27.54 ± 5.86 kg/m2, $p = 0.532$) or the proportion of smokers (11.1% vs. 12%, $p = 0.929$). In addition, there were no differences between the two analyzed groups concerning the administration of beta blockers (55.5% vs. 36%, $p = 0.230$), ACE inhibitors (66.7% vs. 68%, $p = 0.927$), calcium channel blockers (27.8% vs. 36%, $p = 0.744$), nitrates (50% vs. 36%, $p = 0.532$) or acetylsalicylic acid (5.6% vs. 12%, $p = 0.628$). The CTSS plasma profiles and lipid and inflammatory profiles at baseline and after 10 weeks of therapy in each group are given in **Table 2**.

As shown in **Table 2** and **Fig. 2**, the relative changes in the CTSS concentrations and activity were

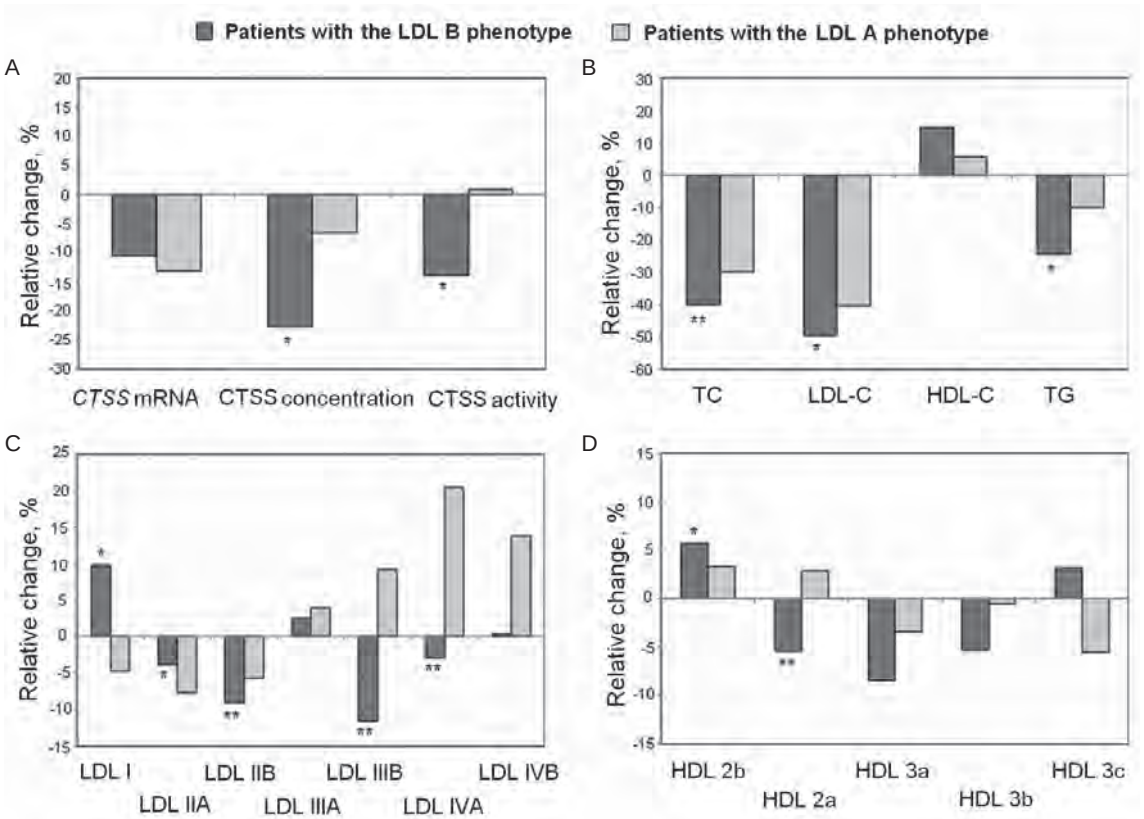

Fig. 2. Relative changes in the examined parameters after 10 weeks of atorvastatin therapy in the patients with the LDL B (18 patients) and LDL A (25 patients) phenotypes

A, plasma *CTSS* mRNA levels and CTSS concentrations and activity; B, lipid parameters; C, LDL subclasses; D, HDL subclasses. The data were compared according to the Mann-Whitney *U*-test and are presented as the median: $^{*}p<0.05$; $^{**}p<0.01$. Abbreviations: CTSS, cathepsin S; *CTSS*, gene encoding CTSS; TC, total cholesterol; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density cholesterol; TG, triglycerides; p, statistically significant.

significantly higher in the patients with the LDL B phenotype. In addition, this group experienced significantly greater changes in the LDL and HDL subclass distribution after treatment (**Fig. 2**). Furthermore, the CTSS and LDL subclass profiles were found to be interrelated in the patients with the LDL B phenotype only. For instance, the relative lowering of the plasma CTSS activity induced by atorvastatin correlated with the relative decrement in the proportion of small, dense LDL particles ($r=0.553$, $p<0.05$). The relative lowering of the plasma *CTSS* mRNA level also correlated with the relative decrement in the proportion of small, dense LDL particles ($r=0.625$, $p<0.05$). Interestingly, we found an association between the changes in the plasma CTSS concentrations and the changes in the CTSS plasma activity in the patients with the LDL B phenotype only ($r=0.617$, $p<0.01$).

Discussion

In the present study of patients with stable angina, treatment with atorvastatin (20 mg/day, for 10 weeks) did not change the plasma *CTSS* mRNA levels, although it lowered the plasma CTSS concentrations and activity. An increased plasma CTSS concentration and activity were both found to be associated with a more atherogenic LDL subclass profile (a decreased dominant LDL size and increased percentage of small, dense LDL particles), while atorvastatin-induced lowering of the plasma CTSS concentration correlated with favourable changes in the LDL subclass profile. The beneficial effects of atorvastatin on the CTSS level and its association with the atherogenic LDL subclass profile were observed in the LDL phenotype B patients only. In this subgroup of patients, the atorvastatin-induced lowering of the

plasma *CTSS* mRNA level also correlated with the lowering of the proportion of small, dense LDL particles. Conversely, the atorvastatin-induced changes in the CTSS levels and HDL subclass profiles were not found to be interrelated.

The present results indicate that atorvastatin treatment exerts a 'beneficial' effect on the CTSS concentration; however, not by lowering the gene expression, but rather by lowering the extracellular protein concentration and enzyme activity. The absence of atorvastatin-induced lowering of the *CTSS* expression, as assessed non-invasively according to a plasma mRNA analysis, is contradictory to the decreased gene expression observed in the myocardium of apoE$^{-/-}$ mice after 32 weeks of simvastatin treatment[12] but in line with the findings of our previous study in which atorvastatin-treated patients with coronary atherosclerosis had a higher *CTSS* expression than healthy subjects[13]. In the present study, despite the lack of changes in the gene expression, atorvastatin lowered the extracellular CTSS protein concentrations and enzyme activity. This finding is notable and requires explanation. Current studies suggest that statins increase the CTSS concentration in the plasma via the statin-induced apoptosis of vascular cells[26] and/or statin-enhanced lysosomal enzyme activity in vascular cells[27], which in turn can again induce apoptosis and CTSS extracellular leakage. However, this suggestion has limitations, as statin treatment did not change the CTSS activity in the wall of abdominal aortic aneurysms in one small case-control study[11] and CTSS deficiency by itself reduces the apoptosis of vascular macrophages[7]. An alternative explanation is that atorvastatin downregulated inflammation, matrix remodelling and oxidative stress in our patients[28], which further reduced the *CTSS* expression and amount of CTSS extracellular leakage. Indeed, the plasma CRP lowering observed in our study correlated with lowering of the CTSS protein concentration in the plasma; however, again, this suggestion has limitations, as we found no concomitant associations with the changes in the plasma *CTSS* mRNA levels. Another reasonable explanation is that, in our study, atorvastatin improved the balance between the activity of CTSS and the level of cystatin C (a potent endogenous CTSS inhibitor), as documented in a previous study[11], thus reducing the level of CTSS-mediated vascular cell apoptosis, which further decreased the extracellular CTSS concentration and enzyme activity. Unfortunately, we did not measure the cystatin C levels, which considerably limits this interpretation. Whatever the background mechanism, atorvastatin treatment exhibits a beneficial effect on the CTSS concentration. This effect is independent of any concomitantly prescribed drugs, although all conclusions should be drawn cautiously due to the use of polyvalent therapy.

In this study, we demonstrated an interrelationship between an increased CTSS level and an atherogenic LDL subclass profile in the plasma of stable angina patients, which can be improved with atorvastatin treatment. That atorvastatin therapy induces improvements in an atherogenic LDL subclass profile has been documented previously[29]; however, the association with CTSS has not yet been studied. It appears that the impact of CTSS on lipid metabolism has been elucidated only in part. *In vitro* cathepsins degrade apolipoprotein B in LDL, and modified LDL exhibits increased binding to proteoglycans as well as enhanced aggregation and fusion and augmented foam cell formation[15, 30]. However, in our study, an increased CTSS concentration and activity at baseline correlated with a higher proportion of small, dense LDL particles in the plasma. It appears that CTSS may also degrade apolipoprotein B and LDL in the plasma in the way that small, dense LDL particles accumulate intravascularly. This observation is further supported by the findings obtained after atorvastatin therapy. Namely, the atorvastatin-induced lowering of the CTSS concentration was in line with the favourable changes noted in the dominant LDL size and proportion of small, dense LDL particles in the plasma. The close interrelationship between an increased CTSS level and atherogenic LDL subclass profile in the plasma requires further clarification, as such a relationship may suggest a new mechanism underlying CTSS-mediated atherogenesis. Small, dense LDL particles easily penetrate the vascular intima, reside longer in the subendothelium and are more prone to oxidation, as compared to their larger counterparts[31], and, once formed, oxidized LDL displays potent proinflammatory, immunogenic, apoptotic and cytotoxic effects[31]. The spectrum of proinflammatory activities also involves the differentiation of monocytes into macrophages. Therefore, our findings offer an additional explanation for the occurrence of CTSS-mediated lipid accumulation in the vascular wall[5] and the observation that CTSS-mediated atherogenesis is associated with CD40-mediated inflammatory and immune responses[10]. However, the alternative explanation that atorvastatin first improved the atherogenic LDL subclass profile, which then lowered the plasma CTSS concentration (*via* decreased atherosclerotic stress that reduced apoptosis and/or the *CTSS* expression in vascular cells) is also possible, although it has some limitations, as discussed in the previous paragraph. The actions of cystatin C, which was

unfortunately not measured in this study, may explain the mechanism underlying the close interrelationship observed between an increased CTSS level and an atherogenic LDL subclass profile in the plasma. The finding of an association between cystatin C and a "beneficial" LDL subclass profile in the plasma in future studies would convincingly support the suggested mechanism of CTSS-mediated atherogenesis.

The beneficial effects of atorvastatin on the CTSS concentration and LDL subclass were observed in the LDL phenotype B patients only, which further supports the assumption that the CTSS level and LDL subclass in the plasma are causally related. Variability in plasma lipoprotein patterns (LDL phenotypes A and B) has recently been recognised to be important in selecting adequate lipid-lower therapy. In the present study, atorvastatin treatment had a beneficial effect on the LDL particles size distribution in the patients with the LDL B phenotype (**Table 2**), who were initially characterised by an increased proportion of small, dense LDL particles and, consequently, a higher risk of cardiovascular disease. This finding is consistent with those of previous studies[32]. However, the most intriguing finding of our study is that statin-induced CTSS-lowering was demonstrated in the LDL phenotype B patients only. Furthermore, our unique simultaneous measurements of plasma mRNA and its protein revealed that decreases in plasma *CTSS* mRNA and its enzyme activity were associated with lowering of the proportion of small, dense LDL particles. Such beneficial, concomitant and interrelated reductions in the atherogenic lipoprotein levels and CTSS concentration and its enzyme activity are likely to be causally related and, importantly, lead to improvements in a patient's overall condition. Unfortunately, the extent of coronary atherosclerosis was not evaluated in this study. The finding of an association of the interrelationship between the CTSS concentration and LDL subclass profile with the severity of atherosclerosis in future studies would further support the role of CTSS as an important factor to be considered when selecting adequate lipid-lower therapy.

In the present study, the HDL subclass analysis revealed that atorvastatin increased the dominant HDL size, with a concomitant decrease in the proportion of small, dense subclasses. This finding is in line with the results of previous studies[33, 34] showing the beneficial effects of statins via an increase in the level of large HDL particles. Indeed, statin therapy induces a minor increase in the HDL-C level (by 5-10%), consistent with a reduction in the cholesteryl ester transfer protein activity and stimulation of apolipoprotein A-I production[35]. As a result, statins preferentially increase the level of large HDL particles[34, 36]. Data regarding the effects of statins on HDL subfractions are inconsistent and difficult to interpret. More recent findings suggest that small, dense HDL has a significantly higher atheroprotective activity than large HDL[37]. However, certain impairments of the protein/lipid content in small, dense HDL may decrease its anti-atherogenic capacity or even induce pro-atherogenic properties[35, 38]. On the other hand, a higher level of large HDL particles is associated with reduced atherosclerotic progression and a lower risk of cardiovascular events[35]. Likewise, the presence of a large HDL 2b subclass was found to be highly inversely correlated with the risk of coronary disease in the prospective Malmo Diet and Cancer Study[39]. Regardless, the changes in the HDL subclass profiles observed in our study were not associated with statin-induced CTSS-lowering, as we had hypothesized based on previously published *in vitro* evidence[14].

Conclusions

In conclusion, among the patients with stable angina treated in the present study, atorvastatin (20 mg/day, 10 weeks) did not change the plasma *CTSS* mRNA levels, although it lowered the plasma CTSS concentrations and activity. In addition, an increased plasma CTSS concentration and activity were found to be associated with a more atherogenic LDL subclass profile (decreased dominant LDL size and increased percentage of small, dense LDL particles). Furthermore, the atorvastatin-induced CTSS-lowering observed in this study was concomitant with improvements in the LDL subclass profile and the changes were interrelated, which requires further clarification. Concomitant and interrelated changes in the CTSS concentrations and LDL subclasses were observed in the LDL phenotype B patients only.

Conflict of Interest Statement

All authors declare no competing interests.

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References

1) Liu J, Sukhova GK, Sun JS, Xu WH, Libby P, Shi GP: Lysosomal cysteine proteases in atherosclerosis. Arterioscler Thromb Vasc Biol, 2004; 24: 359-366
2) Cheng XW, Huang Z, Kuzuya M, Okumura K, Murohara T: Cysteine protease cathepsins in atherosclerosis-based vascular disease and its complications. Hypertension, 2011; 58: 978-986
3) Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P: Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. J Clin Invest, 1998; 102: 576-583
4) Jormsjö S, Wuttge D, Sirsjö A, Whatling C, Hamsten A, Stemme S, Eriksson P: Differential expression of cysteine and aspartic proteases during progression of atherosclerosis in apolipoprotein E- deficient mice. Am J Pathol, 2002; 161: 939-945
5) Lutgens SP, Cleutjens KB, Daemen MJ, Heeneman S: Cathepsin cysteine proteases in cardiovascular disease. FASEB J, 2007; 21: 3029-3041
6) Sukhova GK, Zhang Y, Pan JH, Wada Y, Yamamoto T, Naito M, Kodama T, Tsimikas S, Witztum JL, Lu ML, Sakara Y, Chin MT, Libby P, Shi GP: Deficiency of cathepsin S reduces atherosclerosis in LDL receptor-deficient mice. J Clin Invest, 2003; 111: 897-906
7) de Nooijer R, Bot I, von der Thüsen JH, Leeuwenburgh MA, Overkleeft HS, Kraaijeveld AO, Dorland R, van Santbrink PJ, van Heiningen SH, Westra MM, Kovanen PT, Jukema JW, van der Wall EE, van Berkel TJ, Shi GP, Biessen EA: Leukocyte cathepsin S is a potent regulator of both cell and matrix turnover in advanced atherosclerosis. Arterioscler Thromb Vasc Biol, 2009; 29: 188-194
8) Rodgers KJ, Watkins DJ, Miller AL, Chan PY, Karanam S, Brissette WH, Long CJ, Jackson CL: Destabilizing role of cathepsin S in murine atherosclerotic plaques. Arterioscler Thromb Vasc Biol, 2006; 26: 851-856
9) Liu J, Ma L, Yang J, Ren A, Sun Z, Yan G, Sun J, Fu H, Xu W, Hu C, Shi GP: Increased serum cathepsin S in patients with atherosclerosis and diabetes. Atherosclerosis, 2006; 186: 411-419
10) Cerne D, Stern I, Marc J, Cerne A, Zorman D, Krzisnik-Zorman S, Kranjec I: CTSS activation coexists with CD40 activation in human atheroma: evidence from plasma mRNA analysis. Clin Biochem, 2011; 44: 438-440
11) Abisi S, Burnand KG, Humphries J, Waltham M, Taylor P, Smith A: Effect of statins on proteolytic activity in the wall of abdominal aortic aneurysms. Br J Surg, 2008; 95: 333-337
12) Qin YW, Ye P, He JQ, Sheng L, Wang LY, Du J: Simvastatin inhibited cardiac hypertrofi and fibrosis in apolipoprotein E-deficient mice fed a "Western-style diet" by increasing PPAR α and γ expression and reducing TC, MMP-9, and Cat S levels. Acta Pharmacol Sin, 2010; 31: 1350-1358
13) Stern I, Marc J, Kranjec I, Zorman D, Cerne A, Cerne D: Increased plasma levels of CATS mRNA but not CATB mRNA in patients with coronary atherosclerosis. Clin Biochem, 2010; 43: 1427-1430
14) Lindstedt L, Lee M, Öörni K, Brömme D, Kovanen PT: Cathepsins F and S block HDL3-induced cholesterol efflux from macrophage foam cells. Biochem Biophys Res Commun, 2003; 312: 1019-1024
15) Hakala JK, Oksjoki R, Laine P, Du H, Grabowski GA, Kovanen PT, Pentikäinen MO: Lysosomal enzymes are released from cultured human macrophages, hydrolyze LDL in vitro, and are present extra cellularly in human atherosclerotic lesions. Arterioscler Thromb Vasc Biol, 2003; 23: 1430-1436
16) Lutgens E, Lutgens SP, Faber BC, Heeneman S, Gijbels MM, de Winther MP, Frederik P, van der Made I, Daugherty A, Sijbers AM, Fisher A, Long CJ, Saftig P, Black D, Daemen MJ, Cleutjens KB: Disruption of the cathepsin K gene reduces atherosclerosis progression and induces plaque fibrosis but accelerates macrophage foam cell formation. Circulation, 2006; 113: 98-107
17) Fox K, Garcia MA, Ardissino D, Buszman P, Camici PG, Crea F, Daly C, De Backer G, Hjemdahl P, Lopez-Sendon J, Marko J, Morais J, Pepper J, Sechtem U, Simoons M, Thygesen K, Priori SG, Blanc JJ, Budaj A, Camm J, Dean V, Deckers J, Dickstein K, Lekakis J, McGregor K, Metra M, Morais J, Osterspey A, Tamargo J, Zamorano JL: Guidelines on the management of stable angina pectoris: executive summary: The Task Force on the Management of Stable Angina Pectoris of the European Society of Cardiology. Eur Heart J, 2006; 27: 1341-1381
18) Mirjanic-Azaric B, Rizzo M, Sormaz L, Stojanovic D, Uletilovic S, Sodin-Semrl S, Lakota K, Artenjak A, Marc J, Cerne D: Atorvastatin in stable angina patients lowers CCL2 and ICAM1 expression: Pleiotropic evidence from plasma mRNA analyses. Clin Biochem, 2013; 46: 1526-1531
19) Cerne D, Stern I, Kranjec I, Marc J: Optimisation of methods for quantifying plasma mRNA levels from genes responsible for coronary artery plaque development and destabilization. Med Glas (Zenica), 2011; 8: 90-96
20) Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. Methods, 2001; 25: 402-408
21) Kos J, Sekirnik A, Kopitar G, Cimerman N, Kayser K, Stremmer A, Fiehn W, Werle B: Cathepsin S in tumours, regional lymph nodes and sera of patients with lung cancer: relation to prognosis. Brit J Cancer, 2001; 85: 1193-1200
22) Rainwater DL, Andres DW, Ford AL, Lowe WF, Blanche PJ, Krauss RM: Production of polyacrylamide gradient gels for the electrophoretic resolution of lipoproteins. J Lipid Res, 992; 33: 1876-1881
23) Vekic J, Topic A, Zeljkovic A, Jelic-Ivanovic Z, Spasojevic-Kalimanovska V: LDL and HDL subclasses and their relationship with Framingham risk score in middle-aged Serbian population. Clin Biochem, 2007; 40: 310-316
24) Zeljkovic A, Spasojevic-Kalimanovska V, Vekic J, Jelic-Ivanovic Z, Topic A, Bogavac-Stanojevic N, Spasic S, Vujovic A, Kalimanovska-Ostric D: Does simultaneous determination of LDL and HDL particle size improve prediction of coronary artery disease risk? Clin Exp Med, 2008; 8: 109-116

25) Zeljkovic A, Vekic J, Spasojevic-Kalimanovska V, Jelic-Ivanovic Z, Bogavac-Stanojevic N, Gulan B, Spasic S: LDL and HDL subclasses in acute ischemic stroke: prediction of risk and short-term mortality. Atherosclerosis, 2010; 210: 548-554
26) Katsiki N, Tziomalos K, Chatzizisis Y, Elisaf M, Hatzitolios AI: Effect of HMG-CoA reductase inhibitors on vascular cell apoptosis: beneficial or detrimental? Atherosclerosis, 2010; 211: 9-14
27) Isusi E, Aspichueta P, Liza M, Hernández ML, Díaz C, Hernández G, Martínez MJ, Ochoa B: Short- and long-term effects of atorvastatin, lovastatin and simvastatin on the cellular metabolism of cholesteryl esters and VLDL secretion in rat hepatocytes. Atherosclerosis, 2000; 153: 283-294
28) Bonetti PO, Lerman LO, Napoli C, Lerman A: Statin effects beyond lipid lowering - are they clinically relevant? Eur Heart J, 2003; 24: 225-248
29) Pontrelli L, Parris W, Adeli K, Cheung RC: Atorvastatin treatment beneficially alters the lipoprotein profile and increases low-density lipoprotein particle diameter in patients with combined dyslipidemia and impaired fasting glucose/type 2 diabetes. Metabolism, 2002; 51: 334-342
30) Öörni K, Sneck M, Brömme D, Pentikäinen MO, Lindstedt KA, Mäyränpää M, Aitio H, Kovanen PT: Cysteine protease cathepsin F is expressed in human atherosclerotic lesions, is secreted by by cultured macrophages, and modifies low density lipoprotein particles in vitro. J Biol Chem, 2004; 279: 34776-34784
31) Rizzo M, Berneis K, Zeljkovic A, Vekic J: Should we routinely measure low-density and high-density lipoprotein subclasses? Clin Lab, 2009; 55: 421-429
32) Kostapanos MS, Milionis HJ, Lagos KG, Rizos CB, Tselepis AD, Elisaf MS: Baseline triglyceride levels and insulin sensitivity are major determinants of the increase of LDL particle size and buoyancy induced by rosuvastatin treatment in patients with primary hyperlipidemia. Eur J Pharmacol, 2008; 590: 327-332
33) Kostapanos MS, Milionis HJ, Filippatos TD, Christogiannis LG, Bairaktari ET, Tselepis AD, Elisaf MS: Dose-dependent effect of rosuvastatin treatment on HDL-subfraction phenotype in patients with primary hyperlipidemia. J Cardiovasc Pharmacol Ther, 2009; 14: 5-13
34) Schaefer EJ, McNamara JR, Tayler T, Daly JA, Gleason JA, Seman LJ, Ferrari A, Rubenstein JJ: Effects of atorvastatin on fasting and postprandial lipoprotein subclasses in coronary heart disease patients versus control subjects. Am J Cardiol, 2002; 90: 689-696
35) Kontush A, Chapman MJ: Functionally defective high-density-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. Pharmacol Rev, 2006; 58: 342-374
36) Soedamah-Muthu SS, Colhoun HM, Thomason MJ, Betteridge DJ, Durrington PN, Hitman GA, Fuller JH, Julier K, Mackness MI, Neil HA: The effect of atorvastatin on serum lipids, lipoproteins and NMR spectroscopy defined lipoprotein subclasses in type 2 diabetic patients with ischaemic heart disease. Atherosclerosis, 2003; 167: 243-255
37) Kontush A, Chapman MJ: Antiatherogenic small, dense HDL-guardian angel of the artherial wall? Nat Clin Pract Cardiovas Med, 2006; 3: 144-156
38) de Souza JA, Vindis C, Hansel B, Negre-Salvayre A, Therond P, Serrano CV Jr, Chantepie S, Salvayre R, Bruckert E, Chapman MJ, Kontush A: Metabolic syndrome features small, apolipoprotein A-I-poor, triglyceride-rich HDL3 particles with defective anti-apoptotic activity. Atheroslerosis, 2008; 197: 84-94
39) Musunuru K, Orho-Melander M, Caulfield MP, Li S, Salameh WA, Reitz RE, Berglund G, Hedblad B, Engström G, Williams PT, Kathiresan S, Melander O, Krauss RM: Ion mobility analysis of lipoprotein subfractions identifies three independent axes of cardiovascular risk. Arterioscler Thromb Vasc Biol, 2009; 29: 1975-1980