

# A proteomic study of the apolipoproteins in LDL subclasses in patients with the metabolic syndrome and type 2 diabetes

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**Abstract** The exchangeable apolipoproteins present in small, dense LDL (sdLDL) and large, buoyant LDL subclasses were evaluated with a quantitative proteomic approach in patients with the metabolic syndrome and with type 2 diabetes, both with subclinical atherosclerosis and the B LDL phenotype. The analyses included surface-enhanced laser adsorption/ionization, time-of-flight mass spectrometry, and subsequent identification by mass spectrometry or immunoblotting and were carried out in LDL subclasses isolated by ultracentrifugation in deuterium oxide gradients with near physiological salt concentrations. The sdLDLs of both types of patients were enriched in apolipoprotein C-III (apoC-III) and were depleted of apoC-I, apoA-I, and apoE compared with matched healthy controls with the A phenotype. The LDL complexes formed in serum from patients with diabetes with the arterial proteoglycan (PG) versican were also enriched in apoC-III. In addition, there was a significant correlation between the apoC-III content in sdLDL in patients and the apparent affinity of their LDLs for arterial versican. **■** The unique distribution of exchangeable apolipoproteins in the sdLDLs of the patients studied, especially high apoC-III, coupled with the augmented affinity with arterial PGs, may contribute to the strong association of the dyslipidemia of insulin resistance with increased risk for cardiovascular disease.—Davidsson, P., J. Hulthe, B. Fagerberg, B.-M. Olsson, C. Hallberg, B. Dahllöf, and G. Camejo. **A proteomic study of the apolipoproteins in LDL subclasses in patients with the metabolic syndrome and type 2 diabetes.** *J. Lipid Res.* 2005. 46: 1999–2006.

**Supplementary key words** mass spectrometry • apolipoprotein C-III • apolipoprotein C-I • apolipoprotein E • apolipoprotein A-I • low density lipoproteins • B phenotype

Most of the interactions of LDL with other macromolecules and cells, including those that contribute to its atherogenicity, are controlled by segments of the nonexchangeable apolipoprotein B-100 (apoB-100) exposed at

the particle surface (1–4). However, exchangeable apolipoproteins can also modulate some of these interactions when adsorbed at the LDL surface, as suggested in the pioneering work of Alaupovic (5) and Campos and colleagues (6). The lipoprotein class with densities of 1.019–1.063 is a collection of particles that differ in size, lipid, and apolipoprotein composition (7). Elevated levels of the small, dense LDL (sdLDL) subclass are strongly associated with coronary disease progression, and this phenotype is a marker of the atherogenic dyslipidemia of insulin resistance and type 2 diabetes (8–10). Furthermore, the prevalence of sdLDL is high in subjects with preclinical femoral and carotid atherosclerosis (11). These particles have increased affinity for arterial proteoglycans (PGs) and once associated with them become more easily modifiable by enzymatic and oxidative processes than larger, more buoyant LDLs, causing increased uptake by macrophages (12). Such alterations may lead to preferential entrapment and modifications of sdLDL in the intima PG layer, a critical initial step in atherogenesis (2, 3, 13–15).

High apoC-III content in LDL is receiving much attention because subjects with this phenotype are at much higher risk of cardiovascular events than those with a low content, independent of LDL-cholesterol values (16, 17). ApoC-III, by inhibiting the hydrolysis of triglycerides in VLDL, causes hypertriglyceridemia, but it is not clear why it should increase the atherogenicity of LDL. However, in a detailed study, Olin-Lewis et al. (18) showed that the affinity of LDL subclasses for the PG biglycan increased with decreasing particle diameter, and this trend was associated with increasing apoC-III content and greater positive charge. Furthermore, these investigators found that depletion of apoC-III reduced the affinity for the PG biglycan and that enrichment with apoC-III increased the association. The low content of phospholipids and free

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cholesterol in sdLDL is associated with changes in the exposure of segments of the apoB-100 that modify its affinity for the apoB/E receptor (1, 3, 15, 19, 20). Such changes can also contribute to the increased affinity of sdLDL for PGs, because sequences 3359–3369 (B site) and 3145–3157 (A site), which participate in PG binding, also participate in receptor binding (1, 20, 21). Recent data using LDL from mouse expressing human apoB-100 and human secretory phospholipase A<sub>2</sub> clearly show that the decrease in surface phospholipids increases the affinity for PGs, probably caused by increasing the exposure of the A site (3).

In the present report, in a first hypothesis-seeking study, we explored whether subclasses of LDL of similar density from men with the metabolic syndrome, small LDL particles (pattern B phenotype), and subclinical atherosclerosis differed in the complement of exchangeable apolipoproteins compared with those in healthy controls. In a second study of men with type 2 diabetes and subclinical atherosclerosis and healthy controls, we tested the hypothesis that was raised in the first study. In addition, we evaluated the complement of exchangeable apolipoproteins of plasma LDL with differential affinity for aortic chondroitin sulfate-rich PGs.

## MATERIALS AND METHODS

### Subjects

All subjects described in this report were obtained from a population-based cohort of 61 year old men (22). In the first study, 10 subjects with sdLDL (pattern B phenotype), subclinical atherosclerosis in the carotid arteries as assessed by ultrasound, but without concurrent medication, were compared with 10 age- and sex-matched healthy controls with no risk factors associated with the metabolic syndrome according to the National Cholesterol Education Program (NCEP) definition with the addition of hyperinsulinemia, an indicator of insulin resistance (23). These subjects had no clinical evidence of cardiovascular disease and no subclinical atherosclerosis, as indicated by ultrasound evaluation in the carotid or femoral artery (11). Plaque size was graded as follows: grade 1, one or more small plaques (less than ~10 mm<sup>2</sup>); grade 2, moderate to large plaques. The differentiation between grades 1 and 2 was made subjectively in most cases, and quantitative measurements were made in the computerized system only when the correct classification was not obvious to the observer (24). The characteristics of these subjects are shown in **Table 1**. All of the subjects with pattern B fulfilled the criteria for the metabolic syndrome.

In the second study, 21 patients with type 2 diabetes randomly selected from the 74 available patients in the Atherosclerosis and Insulin Resistance (AIR) Study were compared with 23 age- and sex-matched healthy controls with no risk factors of the metabolic syndrome, as defined by the NCEP guidelines with the addition of hyperinsulinemia, as indicator of insulin resistance (**Table 2**). All subjects were recruited from the AIR Study at 3 year follow-up examination (11, 22). Four of the patients were treated with insulin, 5 subjects were on oral glucose-lowering drugs, and 13 subjects were on antihypertensive drugs. Of the 21 included diabetic patients, 4 had experienced previous myocardial infarction.

The LDL phenotype of all participants was established by gradient gel electrophoresis as described previously (11, 25, 26).

TABLE 1. Characteristics of subjects in study 1: 10 healthy controls and 10 patients with phenotype B and peripheral atherosclerosis

Characteristic	Controls	Phenotype B	P
Waist (cm)	87	97	0.029
LDL (mmol/l)	3.81	3.55	>0.30
HDL (mmol/l)	1.44	0.96	<0.001
Triglyceride (mmol/l)	0.85	2.35	<0.001
Fasting blood glucose	4.7	5.2	0.029
Plasma insulin	29.0	74.5	0.007
Human serum C-reactive protein	0.78	1.88	0.043
Carotid artery plaque			
No	10	0	
Small	0	6	
Moderate/large	0	4	
Femoral artery plaque			
No	10	7	
Small	0	1	
Moderate/large	0	2	

The Ethics Committee at Sahlgrenska University Hospital approved the studies, and the patients gave informed consent to participate.

### LDL fractions

The LDL density subclasses were isolated from serum samples (1.0–1.35 ml) by ultracentrifugation in preformed gradients of buffers containing 140 mM NaCl, 10 mM Na<sub>2</sub>-EDTA, and 10 mM HEPES, pH 7.2, prepared with different amounts of deuterium oxide (D<sub>2</sub>O) as described previously (27). Fractions were collected by upward displacement of the gradient. This method allowed direct measurements of the LDL subclasses without the need for dialysis. Total protein content of LDL fractions was determined using the DC Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions with BSA as the standard. ApoB was determined in LDL fractions by a turbidimetric method using an anti-human apoB antibody (Dakopatts). Total cholesterol was measured photometrically (Roche Diagnostics, Mannheim, Germany). The densities of the solutions used and of the gradients after centrifugation were established by gravimetry (27). Diameters of the LDL subclasses were evaluated by gradient gel electrophoresis essentially as described by Krauss and Burke (26).

TABLE 2. Characteristics of subjects in study 2: 23 healthy controls and 21 patients with type 2 diabetes

Characteristic	Controls	Phenotype B	P
Waist (cm)	89	103	<0.001
LDL (mmol/l)	3.57	3.40	>0.30
HDL (mmol/l)	1.55	1.10	<0.001
Triglyceride (mmol/l)	0.87	2.08	<0.001
Fasting blood glucose	4.64	9.2	<0.001
Plasma insulin	65.1	95.6	0.004
HsCRP	0.78	1.88	0.043
Carotid artery plaque <sup>a</sup>			
No	16	5	
Small	4	3	
Moderate/large	3	9	
Femoral artery plaque <sup>a</sup>			
No	18	11	
Small	1	0	
Moderate/large	3	9	

<sup>a</sup> Because of technical difficulties, five patients had missing data on plaque size in the carotid arteries, and 1 patient and 1 control had missing data on plaque size in the femoral artery.

## Surface-enhanced laser adsorption/ionization analysis of LDL-bound proteins

In the two studies, LDL fraction 4 ( $d = 1.020\text{--}1.040$  g/ml) and fraction 5 ( $d = 1.040\text{--}1.060$  g/ml) from all subjects were analyzed by the surface-enhanced laser adsorption/ionization (SELDI) technique, which is a more simple and fast approach for unbiased protein analysis in small sample volumes compared with the traditional two-dimensional gel electrophoresis method. Two types of protein chip surfaces, cationic (CM10) and anionic (Q10) protein chips, were used with the combination of 50 mM ammonium acetate, pH 6.0, and 50 mM Tris-HCl, pH 9.0, respectively. All samples were processed using a Biomek Laboratory workstation (Beckman-Coulter) modified to make use of a protein chip array bioprocessor (CIPHERGEN Biosystems). Twenty microliters of each LDL fraction was mixed with 80  $\mu$ l of binding buffer, and the mixture was added to the chip surfaces and incubated for 30 min. The spots were then washed three times with 100  $\mu$ l of binding buffer for 5 min to reduce nonspecific binding and finally twice with 100  $\mu$ l of deionized water. Two different types of matrices were used: sinapinic acid (SPA; Aldrich Chemical Co., Milwaukee, WI) and  $\alpha$ -cyano-4-OH-cinnamic acid (CHCA; Bruker Daltonics). A saturated solution of SPA diluted 1:2 (v/v) or CHCA diluted 1:5 (v/v) with 50% acetonitrile containing 0.5% trifluoroacetic acid was applied twice to each dried sample spot to form crystals.

The arrays were subsequently read in a protein chip reader system (PBS II; CIPHERGEN Biosystems). The reader was calibrated externally using the all-in-protein/peptide standards diluted in the SPA/CHCA matrix and applied directly onto a spot of the normal-phase protein chip (NP-20 protein chip array). Protein profile comparisons were performed after normalization for total ion current of all spectra collected in one experiment. The significance threshold was set at  $P < 0.05$ .

### Purification of differentially expressed protein peaks

Aliquots of LDL fractions were pooled and concentrated by vacuum centrifugation, dissolved in 200  $\mu$ l of NuPAGE sample buffer (0.14 M Tris, 0.10 M Tris-HCl, 0.4 mM EDTA, pH 8.5, containing 10% glycerol, 2% LDL, and 3% DTT), boiled for 3 min, and then separated by the NuPAGE system [Novex (precast gels), San Diego, CA] using 4–12% Bis-Tris gels (one well). The NuPAGE-MES buffer system (1 M MES, 1 M Tris, 69 mM SDS, and 20 mM EDTA) was used as running buffer. The mini whole gel eluter (Bio-Rad) was used for electroelution according to the manufacturer's instructions. An elution buffer (25 mM histidine and 30 mM MOPS, pH 6.5) was used, and the elution was performed at 100 mA for 30 min. Fourteen fractions of  $\sim 0.5$  ml were harvested, and aliquots of 250  $\mu$ l/fraction were concentrated and analyzed by the NuPAGE system followed by SYPRO Ruby staining for subsequent identification of protein bands with MS. The remaining part of the gel eluter fractions was mixed with ice-cold ethanol in 1:4 (v/v) ratios, precipitated at  $-20^\circ\text{C}$  for 2 h, centrifuged at 10,000  $g$  for 10 min at  $4^\circ\text{C}$ , dissolved in 10  $\mu$ l of 25 mM  $\text{NH}_4\text{HCO}_3$ , and then analyzed on NP-20 protein chip arrays to follow the purification strategy by SELDI analysis.

### MS analysis of LDL-bound proteins (apoC-III, apoA-I, and apoE)

For subsequent MS analysis, the bands detected on the one-dimensional gels were trypsinized and analyzed by MS as described previously (28). Briefly, the gel pieces were digested by sequencing-grade modified trypsin (Promega, Madison, WI), and the peptides were extracted with formic acid and acetonitrile. To increase peptide ion signals in MS mode and enable MS/MS analysis, desalting and concentration were carried out using POROS

20-resin (PerSeptive Biosystems, Framingham, MA) deposited in a GELoader tip (Eppendorf). The peptides were eluted with 2  $\mu$ l of 70% ACN and 0.1% trifluoroacetic acid directly onto a matrix-assisted laser-desorption/ionization 100 position target plate. The spots were allowed to dry before the application of 1.25  $\mu$ l of matrix solution (CHCA; Agilent Technologies, Waldbronn, Germany) diluted 1:2 (v/v) with 50% acetonitrile, 0.1% trifluoroacetic acid, and a final concentration of 0.2 mg/ml diammoniumcitrate. Analysis was then performed on an Applied Biosystems 4700 proteomics analyzer (matrix-assisted laser-desorption/ionization-time-of-flight/time-of-flight) mass spectrometer (Applied Biosystems, Framingham, MA) in reflector mode. MS and MS/MS data analysis was performed using GPS Explorer™ software (Applied Biosystems), which uses Mascot peptide mass fingerprinting and MS/MS ion search software (Matrix Sciences, London, UK). Identification was considered positive at a confidence level of 95%.

### Immunoblot analysis of apoC-I

After one-dimensional gel analysis of fraction 5 with the NuPAGE system, the proteins were transferred from the gel onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) using the semi-dry blotting technique. The membrane was incubated with an antibody against apoC-I (Biosciences) diluted 1:2,000 (0.02  $\mu$ g/ml). For the immunoblotting procedure, the Western Breeze kit (Invitrogen) was used.

### Binding of LDL to arterial PGs

Binding of serum LDL to PGs was performed as described with minor modifications using purified versican isolated from swine aortic intima media (29–31). In brief, 50  $\mu$ l of serum was added to 1.0 ml of buffer A, containing 10  $\mu$ g/ml hexuronate versican, 20 mM NaCl, 10 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , and 5 mM HEPES buffer, pH 7.0. Serum was also added to a blank tube containing buffer A without versican. The tubes were incubated at  $4^\circ\text{C}$  for 1 h and centrifuged for 10 min at 12,000  $g$  at  $4^\circ\text{C}$ , the pellet was washed with 1.0 ml of buffer A, and the supernatant was discarded. The final pellet was dissolved in 100  $\mu$ l of buffer B containing 140 mM NaCl, 5 mM  $\text{Na}_2\text{-EDTA}$ , and 10 mM Tris base, pH 10.5. Aliquots of the solution were used for cholesterol determination and for SELDI analysis of the exchangeable apolipoproteins associated with the LDL precipitated from serum by the PG. For the experiments in which the content of apoC-III in selected sera was increased, the apolipoprotein (Nordic Biosite, Taby, Sweden) was dissolved in buffer A, added to the sera, and incubated at  $4^\circ\text{C}$  overnight. Binding of serum LDL to PGs of the apoC-III-enriched sera was performed as described above.

### Statistical analyses

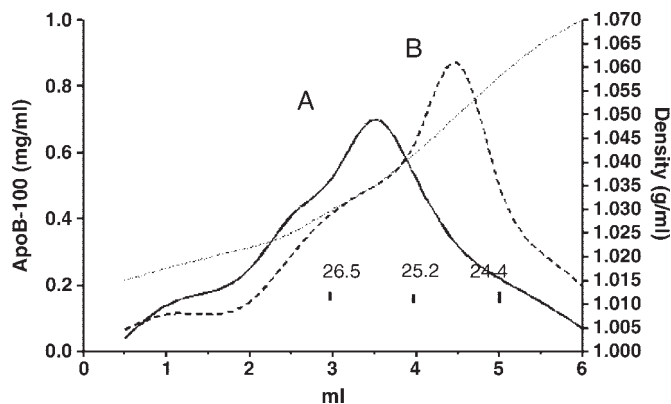
Evaluation of the differences between controls and patients was made with two-tailed Student's  $t$ -test, and  $P < 0.05$  was considered significant. Correlations were evaluated with the GraphPad PRISM 4 program (GraphPad, San Diego, CA).

## RESULTS

### Isolation of LDL subclasses

Density gradient fractionation profiles in  $\text{D}_2\text{O}$  buffers of serum from a subject with the B phenotype and a healthy control are presented in Fig. 1. LDLs isolated with this gradient are indistinguishable in terms of protein content, lipid composition, and electrophoretic properties from lipoproteins from the same plasma obtained with KBr gradients (27). Furthermore, they are more resistant to free radical-mediated oxidation. During frac-



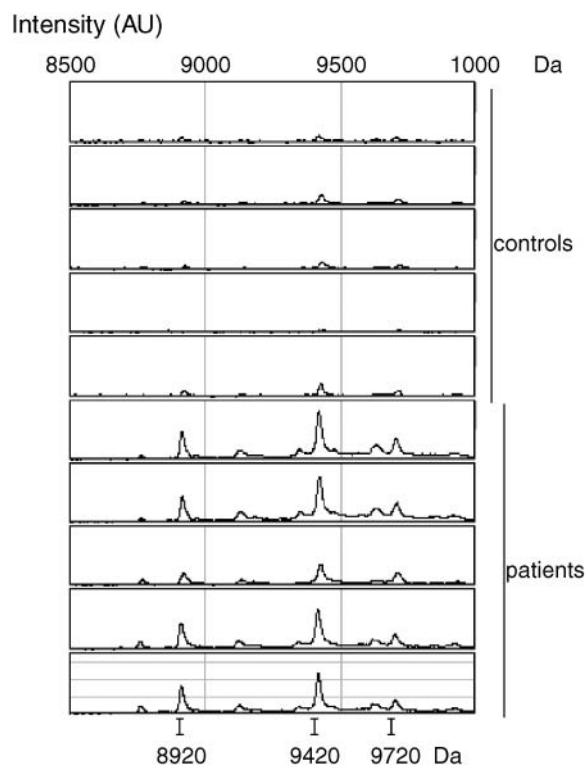


**Fig. 1.** Apolipoprotein B-100 (apoB-100) profiles of serum LDL separated in a preformed gradient of buffers containing 140 mM NaCl, in mixtures of deuterium oxide and water. Profile A is from a healthy control subject. Profile B is from a patient with type 2 diabetes and the LDL B phenotype. The vertical short lines indicate the boundaries of the buoyant fraction 4 and the dense LDL fraction 5 used in subsequent analyses. The numbers indicate the approximate size range in nanometers of these particles. The dotted line indicates the density gradient obtained after 24 h of ultracentrifugation at 147,000 *g*.

tiation with the D<sub>2</sub>O gradients, the LDL is always maintained at physiological ionic strength and pH, and they can be immediately analyzed by electrophoresis and the proteomic approach used here without the need for prolonged dialysis or buffer exchange. The apoB profiles were obtained by collecting 0.5 ml fractions; however, for the proteomic evaluation, fractions of 1.0 ml were used to maintain the subsequent analyses within manageable numbers. As indicated in Fig. 1, with this procedure, fraction 4 contained LDLs with densities of 1.030–1.040 ± 0.005 g/ml, the most abundant LDL subclass from all control subjects in study 1. This fraction corresponds approximately in size range to that of the LDL2b–LDL3a range of Krauss (9). Fraction 5 contained LDLs with densities of 1.040–1.060 ± 0.005 g/ml, which corresponds approximately to the size range of fractions LDL3b–LDL4b of Krauss (9) (Fig. 1). This fraction was the most abundant class in 8 of the 10 studied subjects with the B phenotype in study 1. In the second study, 20 of the 23 controls showed a maximum at fraction 4, and 19 of the 21 patients with type 2 diabetes showed a maximum at fraction 5. These results indicate that the D<sub>2</sub>O density gradient correlates as expected with the LDL phenotyping using the gel electrophoresis procedure and show the high prevalence of sdLDL in both types of patients (11).

#### SELDI analysis of LDL-bound proteins in patients and controls

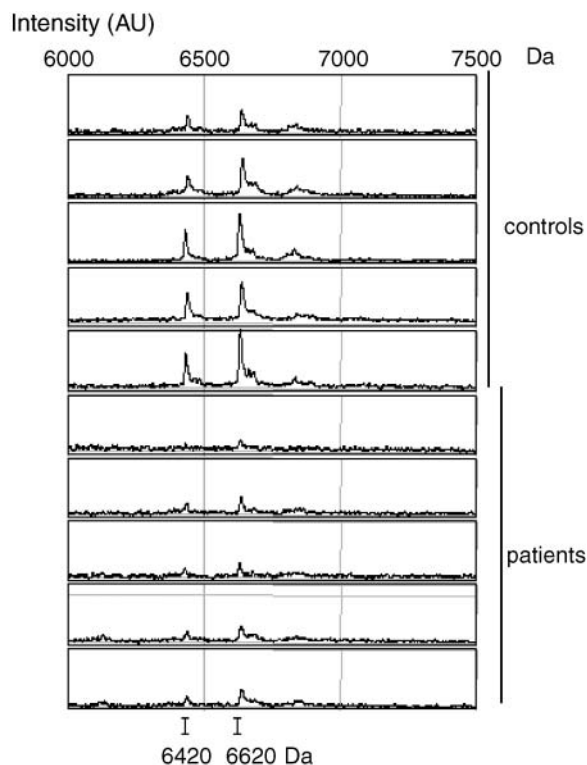
Bound proteins to the LDL subclasses were analyzed on the Q10 (anionic) and CM10 (cationic) protein chip arrays by SELDI. **Figure 2** shows representative SELDI profiles of sdLDLs from patients and controls in study 2 on the Q10 protein chip arrays. Clearly, the sdLDLs from the patients had higher intensity of the bands with masses of 8,920, 9,420, and 9,720 Da compared with those of the



**Fig. 2.** Surface-enhanced laser adsorption/ionization time-of-flight (SELDI-TOF) MS protein spectra of small, dense LDLs (sdLDLs) of 5 representatives from 23 healthy controls and 5 representatives from 21 diabetic patients (study 2) analyzed on Q10 protein chip arrays using 50 mM Tris-HCl, pH 9.0, as incubating buffer. Sinapinic acid (SPA) was used as a matrix. The three bands with molecular masses of 8,920, 9,420, and 9,720 Da were increased in sdLDLs in patients compared with controls ( $P < 0.001$ ). AU, arbitrary units.

controls. The three bands were identified as apoC-III after purification with one-dimensional gels and electroelution and subsequent identification of their matched peptide ions by MS/MS analysis. The purification procedure was followed by SELDI analysis. The differences in molecular mass of the three polymorphs suggest that they represent different states of sialidation (32). **Figure 3** displays SELDI profiles of patients and controls from study 2 on the CM10 protein chip arrays. The two bands at 6,420 and 6,620 Da were clearly more prominent in the sdLDLs of controls than in those of patients. These two bands were identified as apoC-I isoforms after purification with one-dimensional gels and electroelution and subsequent identification by immunoblotting using specific antibodies against apoC-I. The two bands of apoC-I probably represent different states of glycation. Also, SELDI analysis of the mass region between 10,000 and 50,000 Da allowed the identification and evaluation of apoA-I and apoE in the two LDL subclasses. ApoA-I and apoE were most prominent in the profiles of the sdLDLs of controls compared with those of patients (data not shown).

**Table 3** presents the relative content of the identified apolipoproteins detected by SELDI in the sdLDLs (fraction 5) after correction for apoB-100 content in both stud-



**Fig. 3.** SELDI-TOF MS protein spectra of sdLDLs of 5 representatives from 23 healthy controls and 5 representatives from 21 diabetic patients (study 2) analyzed on CM10 protein chip arrays using 50 mM ammonium acetate, pH 6.0, as incubating buffer. SPA was used as a matrix. The two bands with molecular masses of 6,420 and 6,620 Da were reduced in sdLDLs in patients compared with controls ( $P < 0.05$ ).

ies. The sdLDLs from the patients had significantly higher content of apoC-III compared with the equivalent dense fraction from the matched controls in study 1. On the other hand, the patients showed a significantly lower content of apoC-I, apoA-I, and apoE in its sdLDL class than did the controls. In study 2, the sdLDLs of patients with type 2 diabetes also contained significantly more apoC-III

and lower contents of apoC-I, apoA-I, and apoE than did the dense fraction of the matched controls (Table 3). In the controls, there was a significantly higher content in the sdLDLs than in the buoyant particles of apoC-III [ $3.48 \pm 1.54$  vs.  $2.18 \pm 1.31$  arbitrary units (AU)] and apoC-I ( $7.24 \pm 3.09$  vs.  $5.55 \pm 3.15$  AU). In the patients, there was also a higher content in the sdLDLs than in the buoyant LDLs of apoC-III ( $6.40 \pm 2.43$  vs.  $4.10 \pm 2.49$  AU) but not of apoC-I ( $4.30 \pm 2.99$  vs.  $4.37 \pm 2.40$  AU). The differences found between the exchangeable apolipoproteins in the buoyant LDLs of controls and patients in the two studies after correction for apoB-100 content were not as prominent as those found in sdLDLs.

### Binding of LDL to arterial PGs

Incubation of serum with arterial versican performed as described causes the precipitation of mainly LDL (30, 31, 33, 34). When corrected for the apoB-100 differences, the amount of PG-bound LDL-cholesterol of the patient sera from study 2 was  $\sim 32\%$  higher than from control sera, as expected ( $115 \pm 15$   $\mu$ g PG-bound cholesterol/mg apoB for patients vs.  $80.0 \pm 9$  for controls;  $P < 0.05$ ). Furthermore, SELDI analyses of the PG-LDL complexes indicated that the PG-insolubilized LDL from patients contained 57, 65, and 58% more apoC-III-1, apoC-III-2, and apoC-III-3, respectively, than the complexes from controls. These differences were all statistically significant. The profiles of the LDL-PG complexes from two controls and two patients with type 2 diabetes are shown in Fig. 4.

In agreement with these findings, we found a significant correlation between the total apoC-III content of sdLDLs from patients in study 2 and the amount of LDL-cholesterol precipitated by the PG from serum (Fig. 5). There was no significant correlation between the apoC-III content in sdLDLs of controls and the LDL-cholesterol insolubilized by the arterial versican (Fig. 5). To further explore the effect of apoC-III on the association of LDL in serum with versican, the content of apoC-III in five randomly selected controls and five patients with type 2 diabetes was increased by adding purified human apoC-III.

**TABLE 3.** Exchangeable apolipoproteins in sdLDLs (fraction 5) of patients with LDL phenotype B and matched controls in both studies

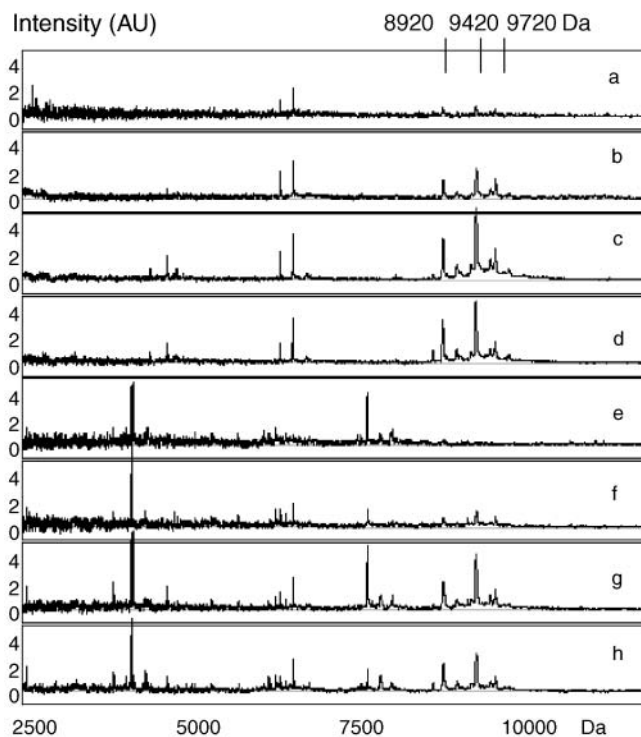
Apolipoprotein	Study 1		Study 2	
	Controls (n = 10)	Patients (n = 10)	Controls (n = 23)	Patients (n = 21)
C-III-1 (8,920)	$4.29 \pm 2.76$	$6.89 \pm 4.71^a$	$3.48 \pm 1.54$	$6.40 \pm 2.43^a$
C-III-2 (9,420)	$7.30 \pm 5.16$	$11.84 \pm 6.14^a$	$6.23 \pm 2.78$	$11.00 \pm 4.19^a$
C-III-3 (9,720)	$3.83 \pm 2.84$	$5.38 \pm 2.09^a$	$3.36 \pm 1.22$	$5.28 \pm 1.87^a$
C-I-1 (6,430)	$7.57 \pm 1.46$	$6.64 \pm 2.76^b$	$7.24 \pm 3.09$	$4.30 \pm 2.79^c$
C-I-2 (6,630)	$6.20 \pm 1.59$	$4.57 \pm 1.65^b$	$11.33 \pm 5.66$	$6.95 \pm 4.64^c$
A-I (28,130)	$0.40 \pm 0.44$	$0.29 \pm 0.26^b$	$1.22 \pm 1.29$	$0.57 \pm 0.58^c$
E (33,570)	$0.85 \pm 0.59$	$0.42 \pm 0.35^b$	$1.49 \pm 0.64$	$1.13 \pm 0.53^b$

sdLDL, small, dense LDL. Values are means  $\pm$  SD in intensity arbitrary units analyzed by the surface-enhanced laser adsorption/ionization technique and corrected by apolipoprotein B-100 content of sdLDLs in patients and controls from both studies. Values in parentheses are molecular masses in daltons. Statistical significance of the differences in apolipoprotein content between the dense LDL class fraction (fraction 5) and the same fraction from controls was evaluated with the *t*-test.

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

<sup>b</sup>  $P < 0.05$ .

<sup>c</sup>  $P < 0.01$ .

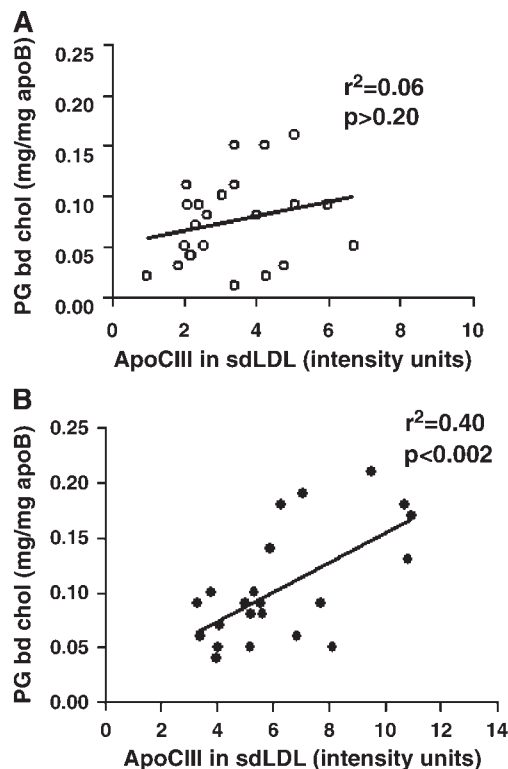


**Fig. 4.** Comparison of SELDI-TOF MS protein profiles on sdLDLs after deuterium fractionation (a–d) and on proteoglycan (PG)-LDL complexes (e–h) of 2 representatives from 23 healthy controls (a–b, e–f) and 2 representatives from 21 diabetic patients (c–d, g–h) (study 2) analyzed on Q10 protein chip arrays. The three bands with molecular masses of 8,920, 9,420, and 9,720 Da were increased in sdLDLs in patients compared with controls ( $P < 0.001$ ) as well as in PG-LDL complexes in patients compared with controls ( $P < 0.001$ ).

Then, these sera were used to evaluate complex formation with the PG. The results shown in **Fig. 6** indicate that upon increasing apoC-III, the amount of LDL-cholesterol precipitated from the patient sera increased significantly, but this was not the case for the controls. These results suggest that in patients, the amount of apoC-III is a more important determinant of the association of LDL with versican than in controls.

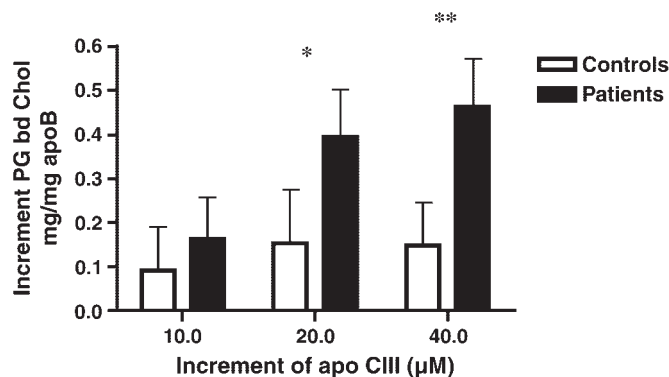
## DISCUSSION

The patients in the first study had the B LDL phenotype, insulin resistance and to a large extent peripheral atherosclerosis, compared with controls (Table 1). None of the matched controls showed the B phenotype. Therefore, this study provided two distinct LDL profiles suitable to raise and test our hypothesis, besides providing information about the resolution and reproducibility of the methods used for lipoprotein fractionation and apolipoprotein evaluation. The  $D_2O$ -based procedure used for lipoprotein subclass isolation minimizes the possibilities of altering the distribution of exchangeable proteins and apolipoproteins bound to the LDL particles in circulation that occurs with ultracentrifugation at high salt concentrations (35, 36). Furthermore, the unbiased proteomic ap-



**Fig. 5.** Correlation between the total content of apoC-III in the sdLDLs of controls (A) and patients with type 2 diabetes (B) and the amount of LDL-cholesterol insolubilized by the association with aortic versican in vitro. PG bd chol, proteoglycan-bound cholesterol.

proach allowed quantitative evaluation and identification of several apolipoproteins with an acceptable coefficient of variation. The results show that the sdLDL subclass with densities of  $1.040\text{--}1.060 \pm 0.005$  g/ml in patients with subclinical peripheral atherosclerosis and with the B phenotype (Table 3) is enriched in apoC-III and depleted of apoC-I, apoA-I, and apoE compared with the equivalent fraction in healthy controls. The patients in the larger study 2 (Table 2) with type 2 diabetes and showing the B phenotype and subclinical peripheral atherosclerosis also had a larger content of apoC-III in the sdLDL than the



**Fig. 6.** Increase of PG-bound cholesterol (PG bd Chol) in sera from healthy controls (open bars) and patients with type 2 diabetes (closed bars) after increasing the content of apoC-III in the sera. The values shown are means  $\pm$  SD. \*  $P < 0.05$ , \*\*  $P < 0.01$ .



corresponding fraction of controls. Also, the sdLDL fraction from these patients showed a lower content of apoC-I, apoA-I, and apoE (Table 3).

Total plasma apoC-III and that associated with LDL are strong predictors of coronary risk, especially in women and men affected by the metabolic syndrome (37). Furthermore, in patients with type 2 diabetes and coronary disease, those in the quartile of LDL with the highest apoC-III have a >6-fold higher relative risk of new coronary events than do those in the quartile with the lowest apoC-III content (16). sdLDL particles are the main catabolic product of large, triglyceride-rich VLDLs, and recent evidence indicates that its apoC-III content is correlated to its rate of production and decreased catabolism (38, 39). These results suggest a mechanism by which apoC-III-enriched dense LDLs can be generated in patients with the metabolic syndrome and type 2 diabetes, like those we studied. The mechanism leading to the reduced content of apoC-I, apoA-I, and apoE that we also found characterized the sdLDL subclass of patients with the metabolic syndrome or type 2 diabetes remains to be elucidated. One possibility is that this reflects the reduced availability of these apolipoproteins in the plasma of these patients and a reduced capacity of the sdLDL particle to retain them, as has been shown to occur for apoE in large VLDLs (40). Total plasma apoA-I was determined in study 2 for the 21 diabetic patients and 23 controls, and the level of apoA-I was reduced in patients ( $0.970 \pm 0.303$  g/l) compared with controls ( $1.278 \pm 0.350$  g/l); these results support the theory mentioned above.

It is still not clear why sdLDL is more atherogenic than buoyant particles, but its high affinity for arterial PGs could increase its retention in the intima, an important first step in atherogenesis (2–4, 12, 41). In patients with clinical manifestations of atherosclerosis and with the atherogenic lipoprotein phenotype associated with insulin resistance, a higher affinity of LDL for arterial PGs than in controls has been described with the simple procedure used here (30, 42–44). In the present study, we observed a significant correlation between the content of apoC-III in sdLDL and the affinity of the corresponding LDL in serum for arterial versican (Fig. 5). Furthermore, all patients showed an enrichment of apoC-III in the isolated PG-LDL complexes. Thus, particles with these characteristics may be preferentially retained in the arterial intima, adding to their atherogenicity. Furthermore, the findings by Olin-Lewis et al. (18) clearly documented that there is a significant inverse correlation between LDL size and affinity for the PG biglycan and that the ratio of apoC-III to apoB is an important contributor to this correlation. Our results (Figs. 5, 6) suggest that the apoC-III content is a more important factor for the interaction with versican in the sera and LDL from patients with diabetes than in healthy controls. However, to explore the structural basis of these differences and establish causality, it will be necessary to conduct a detailed comparison of the interaction of isolated LDL subclasses from patients and controls with versican. This could provide some answers about the properties that control the interaction of the sdLDL with

arterial PGs in patients with diabetes that may be behind the link between the B phenotype and arterial disease.

The major limitation of our report is that only middle-aged men were examined in a cross-sectional, case-control study; therefore, it is not possible to generalize our findings. However, the cohort studied, with subjects with high and low risk of cardiovascular disease, was obtained from a general population sample, and the results may be representative of subjects with these risk profiles within that population.

In summary, our results indicate that if the above assumption is valid, men with the metabolic syndrome and patients with type 2 diabetes, the B phenotype, and subclinical atherosclerosis have high circulating levels of sdLDL rich in apoC-III that could increase its interaction with the arterial intima and thus facilitate its in situ oxidative and enzymatic modification. In addition, these sdLDL particles appear to be impoverished on apoA-I, an apolipoprotein that has been postulated to protect LDL in the arterial wall against atherogenic modifications (45). We speculate that this class of sdLDL could explain in part the striking association of increased apoC-III in LDL with the augmented risk of cardiovascular disease in insulin resistance and type 2 diabetes (16, 17). ■

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