Relation between insulin resistance and fast-migrating LDL subfraction as characterized by capillary isotachophoresis

Bo Zhang,[1],* Takuya Kaneshi,[†] Takao Ohta,[†] and Keijiro Saku*

Department of Cardiology,* Fukuoka University School of Medicine, Fukuoka, Japan; and Department of Pediatrics,[†] Faculty of Medicine, University of the Ryukyus, Okinawa, Japan

Abstract The proportion of the electronegative low density lipoprotein [LDL(−)] subfraction, which is atherogenic, is increased in type 2 diabetes but is not reduced by glycemic control. Therefore, we evaluated the ability of a new technique, capillary isotachophoresis (cITP), to quantify charge-based LDL subfractions and examined the relation between insulin resistance and the cITP fast-migrating (f) LDL levels. Seventy-five 10-year-old boys were included. The two cITP LDL subfractions, fLDL and major LDL subfractions, were proportional to the LDL protein content within the range of 0.1–0.8 mg/ml LDL protein. Levels of cITP fLDL were positively correlated with triglyceride (TG) levels and negatively correlated with LDL size. Insulin resistance as assessed by the homeostasis model assessment (HOMA-IR) was positively correlated ($P < 0.01$) with cITP fLDL levels ($r = 0.41$). The relation between HOMA-IR and cITP fLDL levels depended on TG levels but was independent of body mass index and LDL size.▪ cITP lipoprotein analysis is an accurate and sensitive method for quantifying charge-based LDL subfractions in human plasma, and insulin resistance is related to cITP fLDL independent of LDL size.—Zhang, B., T. Kaneshi, T. Ohta, and K. Saku. **Relation between insulin resistance and fast-migrating LDL subfraction as characterized by capillary isotachophoresis.** *J. Lipid Res.* 2005. 46: 2265–2277.

Supplementary key words children • plasma lipoprotein subfraction • low density lipoprotein size • electronegative low density lipoprotein

Electronegative low density lipoprotein [LDL(−)] subfraction in plasma has been shown to have various atherogenic properties [reviewed by Sánchez-Quesada, Benitez, and Ordonez-Llanos (1)]. Although in vitro oxidized LDL can only be taken up by scavenger receptors, LDL(−) can also be taken up by LDL receptors to induce vascular cell adhesion molecule-1 expression through the activation of nuclear factor κB and adaptor protein 1 (2).

Both type 1 and type 2 diabetic patients have been shown to have an increased proportion of LDL(−) (3, 4). However, LDL(−) in type 1 and type 2 diabetes seems to be of different origins. In type 1 diabetes, nonenzymatic glycosylation has been shown to contribute to the increased proportion of LDL(−): glycemic optimization decreased both the glycated LDL and the proportion of LDL(−) (3, 4). However, in type 2 diabetes, glycemic control decreased glycated LDL but had no significant effects on the proportion of LDL(−) (4, 5). It is not clear whether or not insulin resistance contributes to LDL(−) generation in type 2 diabetes.

Insulin resistance is known to be associated with increased levels of triglycerides (TGs) (6). Because LDL(−) separated by anion-exchange chromatography techniques has been shown to contain higher TG content than the major LDL subfraction (7–9), it is possible that there may be a relation between insulin resistance and LDL(−). However, this has not yet been examined. In addition, it would be interesting to know whether or not the relation between insulin resistance and LDL(−) depends on TG levels.

Insulin resistance is also linked to plasma levels of small, dense LDLs (pattern B lipoprotein phenotype), which are associated with an increased risk of coronary heart disease (CHD) (6). In normolipidemic (NL) subjects, LDL(−) is distributed predominantly in small, dense LDL subfractions (10). Therefore, it would also be interesting to determine whether or not the relation between insulin resistance and LDL(−) depends on the size of LDL. Clarifying these points should be important considering that glycemic control failed to decrease the proportion of LDL(−) in type 2 diabetic patients (4).

Chromatography has the advantage that the separated LDL(−) fraction can be collected to characterize the composition of LDL(−) (11–13). However, for routine

[1] To whom correspondence should be addressed.
  e-mail: bozhang@fukuoka-u.ac.jp

analysis, it has the disadvantage that LDL needs to be separated from other plasma proteins (e.g., by ultracentrifugation) for analysis, because protein absorption is monitored by ultraviolet light detection at 280 nm (12, 13). Therefore, it is time-consuming, and although it determines the proportion of LDL(−), it is not able to determine the absolute amount of LDL(−) in plasma.

Capillary isotachophoresis (cITP) is a new technique for separating plasma lipoprotein subfractions based on their electric charges (14–18). The two cITP LDL subfractions, fast-migrating (f) and slow-migrating (s) LDL, represent the LDL(−) and major LDL subfractions, respectively. We and others (14, 15, 19–22) have previously shown that the absolute amount of lipoprotein subfraction can be determined by reference to an internal marker. In cITP analysis, because lipoproteins are stained by the lipophilic dye 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-ceramide and monitored by laser-induced fluorescence detection (excitation, 488 nm; emission, 510 nm), lipoproteins can be analyzed without prior separation from other plasma proteins. However, it is not clear whether or not cITP LDL subfractions in plasma are equivalent to those in LDL separated by ultracentrifugation. Therefore, in the present study, we compared cITP LDL subfractions in plasma with those in LDL separated by ultracentrifugation. It is also not clear whether or not fLDL as determined by the method used in cITP analysis is related to LDL(−) as determined by the detection method used in chromatography. Therefore, we examined the linearity of the relation between levels of cITP LDL subfractions and protein contents of LDL to evaluate the ability of cITP lipoprotein analysis to quantify LDL subfractions. In addition, although cITP separates plasma lipoproteins into subfractions based on their electric charge, there is still no direct evidence that cITP fLDL represents an electronegative fraction of LDL. Therefore, we examined the changes in the distribution of cITP LDL subfractions during the in vitro oxidation of LDL to clarify whether or not cITP fLDL is related to the electronegativity of LDL, because oxidation is known to increase the negative charge of LDL.

Many studies have shown that atherosclerosis may start in childhood and is related to blood lipid levels measured in early life (23, 24), and both insulin resistance and lipid levels are related to age and gender. Therefore, to clarify whether or not insulin resistance contributes to the generation of LDL(−), we examined the relation between insulin resistance and cITP fLDL subfraction and the interaction of TG levels and LDL size in 10 year old nondiabetic boys, who have no CHD and have fewer conventional risk factors such as smoking, hypertension, etc.

Since Sánchez-Quesada et al. (10) reported that LDL(−) as measured by ion-exchange chromatography is distributed predominantly in the dense LDL subclass in NL subjects but is increased in the light LDL subclass in patients with hyperlipidemia, it would be interesting to know the relationship between cITP fLDL and LDL subclasses as measured by density. Therefore, we examined the distribution of cITP fLDL in light and dense LDL subclasses in NL, hypercholesterolemic (HC), and hypertriglyceridemic (HTG) subjects.

METHODS

Subjects

Seventy-five 10-year-old Japanese boys who underwent screening for lifestyle-related diseases in Okinawa, Japan, were included in the study. The study was approved by the Review Board of the University of the Ryukyus and the Ethics Committee of Fukuoka University. Informed consent was obtained from the parents of all of the children. Fasting blood was drawn from the vein. Serum was separated by low-speed centrifugation (1,000 g, 20 min, 4°C). Levels of lipids, lipoproteins, and apolipoproteins were measured in fresh serum. For lipoprotein particle and cITP analysis, aliquots of serum in small volumes were protected with N_2 gas, snap-frozen in liquid nitrogen, and stored at −80°C for <2 months before analysis.

Determination of serum levels of lipids, lipoproteins, and apolipoproteins, LDL size, and insulin resistance

Serum total cholesterol (TC), TG, and high density lipoprotein-cholesterol (HDL-C) levels were measured by enzymatic methods. Low density lipoprotein-cholesterol (LDL-C) was calculated as TC − (HDL-C + TG/5). Serum levels of apolipoprotein A-I (apoA-I), apoA-II, and apoB were determined by the turbidity immunoassay method. LDL size was evaluated by electrophoresis on nondenaturing polyacrylamide gradient gels on precast MULTIGEL-LP (2–15%) according to the procedure specified by the manufacturer (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), as described previously (25). Insulin resistance as assessed by homeostasis model assessment (HOMA-IR) was calculated according to the equation HOMA-IR = fasting glucose (mg/dl) × fasting insulin (μU/ml)/405 (26). A quantitative insulin sensitivity check index (QUICKI), which correlates well with insulin sensitivity from the glucose clamp technique, was determined according to the equation QUICKI = 1/[log(fasting glucose) + log(fasting insulin)] (27).

Determination of lipoprotein subfractions by cITP

cITP of lipoproteins in serum, to which was added EDTA-Na_2 at a final concentration of 1 mM before analysis, was performed on a Beckman P/ACE MDQ system (Beckman-Coulter, Inc., Tokyo, Japan) according to the method of Bottcher et al. (14) with some modifications, as described previously (19–22). All of the reagents used for cITP analysis were purchased from Sigma-Aldrich (Tokyo, Japan) unless indicated otherwise. For routine analysis, 6 μl of serum was diluted with 14 μl of leading buffer (LB) consisting of 10 mM HCl (product number 84428; Fluka, Tokyo, Japan) and 18 mM ammediol (2-amino-2-methyl-1,3-propanediol; product number A9074) (LB1, pH 8.8), prestained with 10 μl of 0.1 mg/ml NBD C6-ceramide (product number N1154; Molecular Probes, Inc., Eugene OR), which was prepared by dissolving 1 mg of NBD C6-ceramide in 1 ml of anhydrous methanol (product number 322415) and diluting 10-fold with anhydrous ethylene glycol (product number 324558) for 1 min at room temperature, and mixed with 50 μl of a mixture containing LB with 0.35% hydroxypropylmethylcellulose (product No. H4649) (LB2), spacers, and 5-carboxy-fluorescein (product number C0537) as an internal marker. For experiments to examine the ability of cITP to quantify LDL subfractions, 10 μl of LDL isolated by ultracentrifugation containing 1–8 μg of LDL protein was added to 6 μl of apoB-depleted EDTA plasma (plasma proteins and HDL) and 4 μl of LB1 and used for prestaining. The

spacers included were *N*-(2-acetamido)-2-aminoethanesulfonic acid (product number A7949), D-glucuronic acid (product number 271632), 1-octanesulfonic acid sodium salt (product number O0133), 3-(*N*-tris[hydroxymethyl]methylamino)-2-hydroxypropanesulfonic acid (product number T0432), *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (product number T9659), L-serine (product number S260-0), L-glutamine (product number G320-2), L-methionine (product number M6039), and glycine (product number G7403). The terminating buffer (TB) contained 24 mM β-alanine (product number 239720) and 13 mM ammediol (TB1) and was adjusted to pH 10.5 (TB2) by adding 47 μl of saturated barium hydroxide (product number 11783) solution to 2 ml of TB1. Saturated barium hydroxide solution was prepared under an atmosphere of N_2, kept at 30°C in a water bath, and used within 1 week of preparation. A dimethylpolysiloxane-modified fused silica capillary (AT-1™; part number 14130) was purchased from Alltech Japan, Inc. (Tokyo, Japan). The sample was injected for 18 s at 20 pounds per square inch into a 30 cm long capillary (inner diameter, 180 μm), and separation was performed at a constant 30 μA for 1 min and 10 kV for 7 min with pressure on both sides (10 pounds per square inch). The separated zones were monitored with argon-laser-induced fluorescence detection (excitation, 488 nm; emission, 520 nm). Each peak was identified, and the peak area in relative fluorescence units was analyzed using 32 Karat Software version 5.0 (Beckman-Coulter, Inc., Tokyo, Japan). The peak area for each cITP lipoprotein subfraction relative to that of the internal marker was presented as the level of cITP lipoprotein subfraction (19–22), unless indicated otherwise.

Preparation of apoB-depleted plasma and isolation of LDL

Plasma containing 1 mM EDTA from a healthy volunteer (female, 32 years old) was used for experiments to examine the ability of cITP to quantify LDL subfractions. Whole plasma was used for cITP analysis and LDL isolation by ultracentrifugation immediately after separation, and some was divided into aliquots in small volumes (150 μl), covered with N_2 gas, and stored at −80°C for 1 day. Plasma depleted of apoB-containing lipoproteins was obtained from frozen whole plasma and used within the day of separation without storage.

ApoB-containing lipoproteins in EDTA plasma were precipitated by the phosphotungstate-Mg^{2+} method, as described previously (19). LDL was isolated from fresh EDTA plasma from the same volunteer by sequential ultracentrifugation. The plasma was first adjusted to a density of 1.019 g/ml with solid KBr and subjected to ultracentrifugation in a TLA-100.3 rotor in a Beckman TL-100 Tabletop Ultracentrifuge for 3 h at 100,000 rpm (541,000 *g*) and 10°C. The bottom fraction was collected by cutting the tubes, overlaid with liquid KBr (d = 1.019 g/ml), and ultracentrifuged again to remove any contaminating lipoproteins. The density of the bottom fraction was then increased to 1.063 g/ml with solid KBr, and the sample was subjected to ultracentrifugation for 3 h at 100,000 rpm (541,000 *g*) and 10°C. The top fraction was collected by cutting the tubes and dialyzed in a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) at 4°C against 0.85% NaCl and 0.01% EDTA overnight to remove KBr. LDL was used the day after separation without storage.

Oxidative modification of LDL by incubation with copper

Ultracentrifugally isolated LDL was dialyzed against PBS (pH 7.4) to remove EDTA before oxidation. Oxidation of LDL (0.5 mg/ml) was started by incubation with 10 μM freshly prepared $CuSO_4$ (with water) at 37°C in a shaking water bath. Aliquots were withdrawn at 0, 0.5, 1, 2, and 3 h of oxidation, and oxidation was stopped by cooling the aliquots on ice and adding

EDTA-Na_2 (1 mM) and butylated hydroxytoluene (1 mM). Ten microliters of oxidized LDL was immediately used for cITP separation without removing $CuSO_4$, because cITP patterns were not affected by $CuSO_4$ (data not shown).

Agarose gel electrophoresis of LDL was performed as described by Noble (28) using commercial kits (Gel Universal/8; Corning, Chiba, Japan). One microliter of plasma control and 0.5 mg/ml oxidized LDL were applied and subjected to 1% agarose gel electrophoresis for 40 min at 90 V. Lipoproteins were stained with fat red 7B.

Conjugated dienes were determined by measuring absorbance at 234 nm using an ultraviolet-visible recording spectrophotometer (UV-160A; Shimadzu, Kyoto, Japan). Thiobarbituric acid-reactive substances (TBARS) were assayed by the fluorometric method of Yagi (29). ApoB fluorescence was measured at 430 nm (excitation, 360 nm) using a spectrofluorophotometer (RF-5000; Shimadzu).

Separation of light and dense LDL subfractions by heparin-Mg^{2+} precipitation

To examine the distribution of cITP fLDL in light and dense LDL subclasses, blood was drawn from 8 NL, 8 HC (LDL-C ≥ 140 mg/dl), and 8 HTG (TG ≥ 150 mg/dl) subjects. Serum was frozen with liquid nitrogen immediately after separation and preserved at −80°C under N_2 gas. Serum levels of TC, TG, HDL-C, and LDL-C were measured by enzymatic methods. Six microliters of serum was used to measure lipoprotein subfractions by cITP as described above. The light LDL subclass (d = 1.019–1.044 g/ml) was separated from the dense LDL subclass (d = 1.044–1.063 g/ml) using the heparin-Mg^{2+} precipitation method as described by Hirano, Saegusa, and Yoshino (30), with modifications. Briefly, one part of 300 U/ml heparin sodium (Novo-Heparin Injection 1000; Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) was mixed with one part of 180 mM $MgCl_2$. One part of the heparin-Mg^{2+} solution was then mixed with one part of serum. After the mixture was put on ice for 25 min, it was centrifuged at 4°C and 15,000 rpm for 15 min in a high-speed microrefrigerated centrifuge (MTX-150; Tomy Seiko Co., Ltd., Fukuoka, Japan). Twelve microliters of the supernatant was subjected to cITP analysis to determine the levels of cITP fLDL and sLDL in the small, dense LDL subclass contained in serum depleted of the large, light LDL subclass. Levels of cITP fLDL and sLDL in the large, light LDL subclass were calculated from those determined in whole serum and serum containing the light LDL subclass. The proportion of cITP fLDL was calculated from levels of cITP fLDL and sLDL.

Statistical analysis

All statistical analyses were performed using the SAS (Statistical Analysis System) software package (version 8.2; SAS Institute) at the Fukuoka University. Correlations between variables were examined by Spearman correlations and regression analysis. Variables among tertiles of HOMA-IR and among NL, HC, HTG subjects were compared by ANOVA and Scheffe's multiple comparison test (31). Differences in variables between large and dense LDL subclasses were examined by ANOVA. All *P* values are two-tailed. The significance level was considered to be 5% unless indicated otherwise.

RESULTS

Table 1 shows the anthropometric and biochemical characteristics of the 10 year old boys studied. To show the distribution of continuous variables, mean values, median

TABLE 1. Anthropometric and biochemical characteristics of the 10-year-old boys studied

| Characteristic	Mean ± SD	Median	(Lower Quartile, Upper Quartile)
Height (cm)	140 ± 6	140	137, 144
Body weight (kg)	47 ± 8	48	42, 52
BMI (kg/m²)	23.9 ± 2.6	23.6	21.9, 25.8
SBP (mmHg)	108 ± 12	108	100, 120
DBP (mmHg)	62 ± 12	60	54, 70
Glucose (mg/dl)	93 ± 6	92	89, 96
Insulin (μU/ml)	13.6 ± 8.0	12.1	7.8, 17.6
HOMA-IR	3.1 ± 1.9	2.9	1.7, 4.2
QUICKI	0.14 ± 0.01	0.14	0.14, 0.15
TC (mg/dl)	186 ± 24	185	166, 204
TG (mg/dl)	67 ± 36	58	39, 86
HDL-C (mg/dl)	60 ± 9	60	53, 65
LDL-C (mg/dl)	113 ± 22	109	96, 129
ApoA-I (mg/dl)	135 ± 15	134	125, 145
ApoA-II (mg/dl)	31.2 ± 3.8	30.9	28.9, 34.0
ApoB (mg/dl)	78 ± 15	78	67, 88
LDL size (nm)	26.8 ± 0.7	26.8	26.3, 27.2

apoA-I, apolipoprotein A-I; BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high density lipoprotein-cholesterol; HOMA-IR, insulin resistance as assessed by homeostasis model assessment; LDL-C, low density lipoprotein-cholesterol; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.

values, and lower and upper quartiles are given. As shown, the mean serum TG level was much higher than the median value, indicating that serum TG levels were not normally distributed.

Figure 1 shows typical electropherograms of plasma lipoprotein subfractions as characterized by cITP for a boy with low HOMA-IR (Fig. 1A) and a boy with high HOMA-IR (Fig. 1B). As shown, plasma lipoproteins were separated into eight fractions by cITP: three HDL fractions [peaks 1–3: fast (f)-, intermediate (i)-, and slow (s)-migrating HDL], a chylomicron/remnant fraction (peak 4), a VLDL/intermediate density lipoprotein (IDL) fraction (peak 5), two LDL fractions (peaks 6 and 7: fLDL and sLDL), and a minor LDL fraction (peak 8).

In **Fig. 2**, cITP LDL subfractions analyzed directly in plasma are compared with those in LDL separated by ultracentrifugation. Figure 2A shows the cITP lipoprotein profile of a female NL volunteer. The cITP HDL fraction was identified by precipitation of apoB-containing lipoproteins (19). As shown in Fig. 2B, peaks 4–7 in Fig. 2A are not seen in apoB-depleted plasma, indicating that peaks 1–3 in Fig. 2A represent HDL subfractions. We identified LDL subfractions by adding ultracentrifugally isolated LDL to apoB-depleted plasma. As shown in Fig. 2C–G, the added LDL subfractions had the same migration time and a similar cITP pattern as those separated from the whole plasma. However, the proportion of fLDL(−) (peak 6) to the major LDL (peak 7, sLDL) seemed to be lower in ultracentrifugally isolated LDL than in plasma LDL, suggesting that more of the cITP fLDL subfraction was lost than cITP sLDL during ultracentrifugation.

One difference between the cITP and chromatographic methods for measuring LDL subfractions is that the cITP method monitors the lipid content of LDL subfractions and the chromatographic method monitors the protein
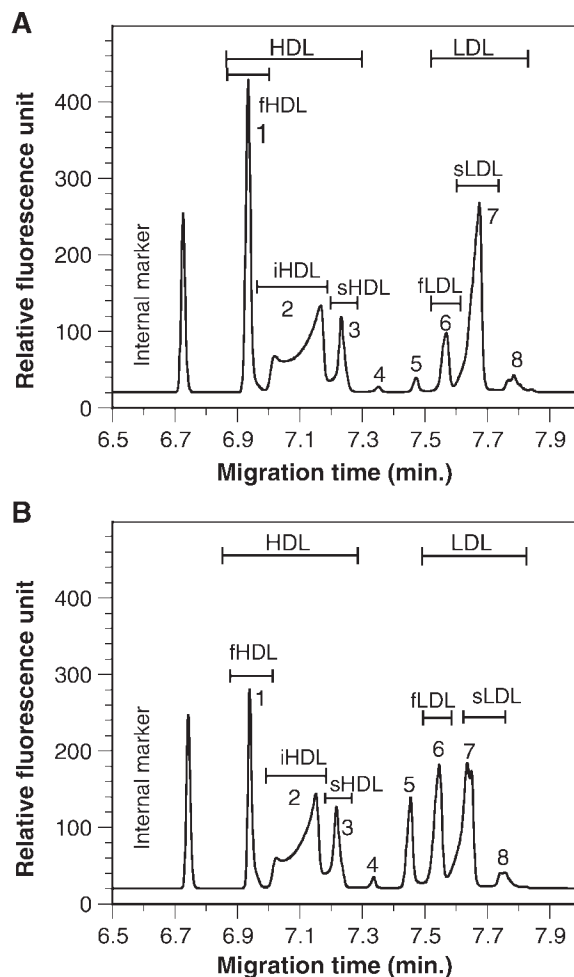
Fig. 1. Lipoprotein profiles as determined by capillary isotachophoresis (cITP) in plasma from a boy with low insulin resistance as assessed by homeostasis model assessment (HOMA-IR; 1.5) (A) and a boy with high HOMA-IR (6.5) (B). Peaks 1–3, fast (f)-, intermediate (i)-, and slow (s)-migrating HDL; peak 4; chylomicron/remnant fraction; peak 5, VLDL/intermediate density lipoprotein (IDL); peaks 6 and 7, fLDL and sLDL; peak 8, a minor LDL fraction.

content of LDL subfractions. In Fig. 2C–G, different amounts of LDL protein were added to apoB-depleted plasma to examine the linearity of the relation between cITP LDL subfractions and the protein content of LDL. As shown, both the cITP fLDL (peak 6) and sLDL (peak 7) subfractions increased with an increasing amount of LDL protein. The relative peak areas of cITP fLDL ($r = 0.998$) and sLDL ($r = 0.997$) subfractions were directly proportional to the amount of LDL protein between 0.1 and 0.8 mg/ml LDL protein content. This result indicates that cITP is an accurate and sensitive method for quantifying charge-based LDL subfractions.

To prove that cITP fLDL is related to the electronegativity of LDL, we tested the hypothesis that the changes in cITP fLDL paralleled the changes in the negative charge of LDL, as indicated by the electrophoretic mobility of LDL in agarose electrophoresis during the in vitro oxidation of LDL. Subjecting LDL to lipid peroxidation by incubation at 37°C in PBS containing 10 μM $CuSO_4$ resulted
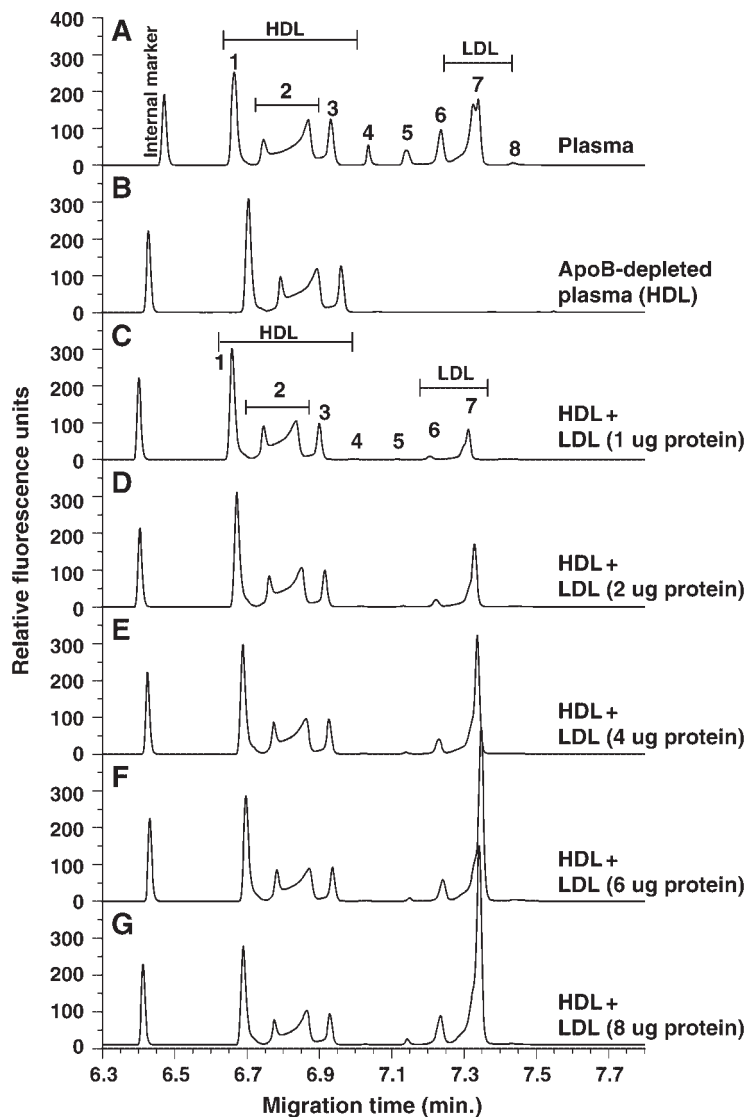
Fig. 2. Electropherograms of plasma lipoproteins (A), HDL fraction obtained by precipitation of apolipoprotein B (apoB)-containing lipoproteins from plasma (B), and ultracentrifugally isolated LDL containing 1 mg (C), 2 mg (D), 4 mg (E), 6 mg (F), and 8 mg (G) of protein in 10 ml of solution that was added to the HDL fraction for cITP separation.

in a rapid increase in conjugated dienes and TBARS (data not shown) and a progressive increase in the electrophoretic mobility on agarose gels (**Fig. 3A**) and apoB fluorescence (data not shown). As shown in Fig. 3A, the increase in the negative charge of oxidized LDL was visible on agarose gels after an oxidation period of 1 h. Changes in cITP LDL subfractions during oxidative modification were monitored by taking aliquots of oxidized LDL at different times and adding them to apoB-depleted EDTA plasma for cITP separation. The electropherograms of oxidized LDLs are shown in Fig. 3B. cITP sLDL (peak 7) gradually decreased from an oxidation time of 0.5 h (Fig. 3Bb–e), whereas cITP fLDL (peak 6) gradually increased from 0.5 to 1.5 h (Fig. 3Bb–d). This result indicates that at the initial stage of oxidation, cITP sLDL subfraction (peak 7) was converted to cITP fLDL subfraction (peak 6), and the increase in cITP fLDL paralleled that in the electrophoretic mobility on agarose gels. From 1.5 to 3 h of oxidation, cITP fLDL (peak 6) decreased, whereas a new cITP LDL subfraction (peak 5), which appeared after 1 h of oxidation (Fig. 3Bc), increased progressively (Fig. 3Bd–f). This result indicates that during the oxidative modifica-

tion of LDL, although sLDL (peak 7) was converted to fLDL (peak 6), fLDL (peak 6) was further converted to a new, more negatively charged LDL subfraction (peak 5). Therefore, the distribution of cITP LDL subfractions changed gradually during the oxidative modification of LDL, with LDL subfractions shifting toward more negative charges. These results indicate that cITP fLDL represents an in vivo LDL($-$) subfraction.

Levels of cITP fLDL were positively correlated with TG levels ($r = 0.65$, $P < 0.01$) and negatively correlated with LDL size ($r = -0.43$, $P < 0.01$) in all of the boys, suggesting that enrichment of LDL with TG increases the electronegative fraction of LDL and that LDL($-$) is associated with small, dense LDLs.

As shown in **Table 2**, body mass index (BMI), fasting glucose concentration, fasting insulin concentration, and HOMA-IR were significantly higher and QUICKI was significantly lower in the middle and high HOMA-IR tertiles than in the low HOMA-IR tertile, as assessed by ANOVA and Scheffe's multiple comparison test. Also, levels of TG and cITP VLDL/IDL and cITP fLDL fractions were significantly higher, and HDL-C levels, cITP fHDL levels, and
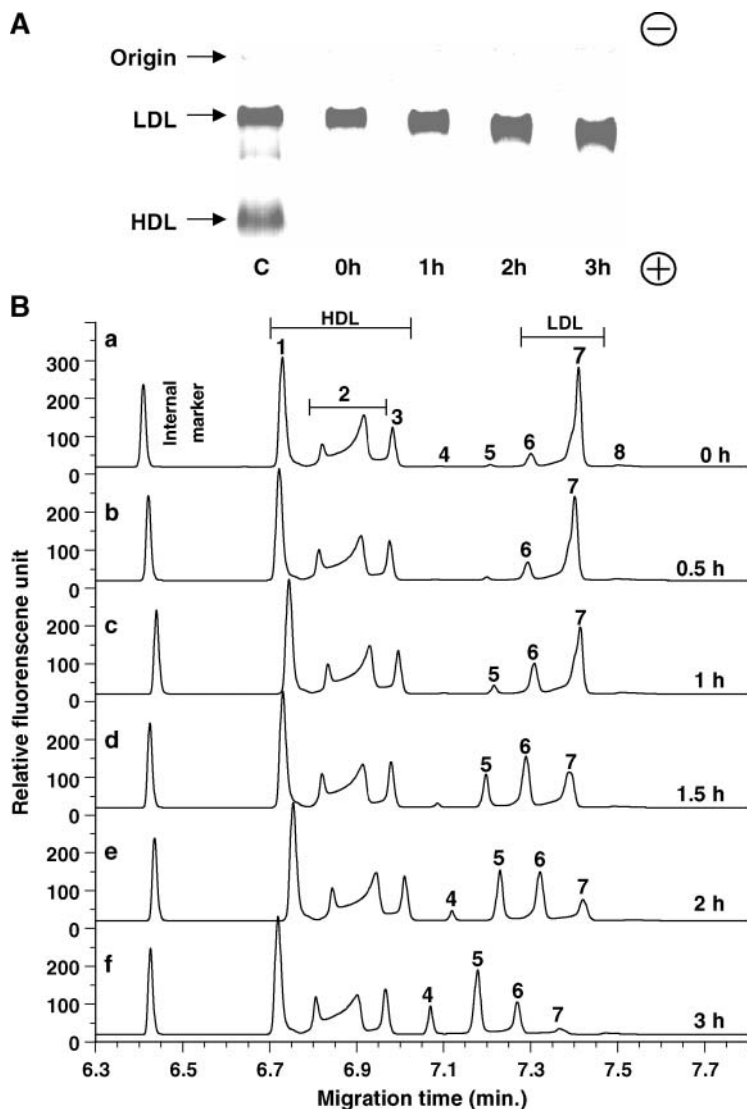
Fig. 3. A: Agarose electrophoresis of the time course to the oxidation of LDL (0.5 mg/ml) catalyzed by Cu^{2+} (10 μM). Lane C represents plasma of the female volunteer who donated blood for the isolation of LDL. B: Electropherograms of ultracentrifugally isolated LDL that was subjected to Cu^{2+}-catalyzed oxidation for 0 h (a), 0.5 h (b), 1 h (c), 1.5 h (d), 2 h (e), and 3 h (f) and added to apoB-depleted plasma (HDL fraction) for cITP separation.

LDL size were significantly lower, in the high HOMA-IR tertile than in low HOMA-IR tertile (Table 2). These results indicate that HOMA-IR is related to the altered distribution of not only cITP HDL subfractions but also LDL subfractions.

Figure 4A, **B** shows the plots of cITP fLDL versus TG levels and cITP fLDL versus LDL size, respectively, in the low and high HOMA-IR groups. As shown in Fig. 4A, cITP fLDL levels did not differ between the low and high HOMA-IR groups after adjusting for TG levels, suggesting that the relation between HOMA-IR and cITP fLDL levels depended on TG levels. As shown in Fig. 4B, the regression line of cITP fLDL levels versus LDL size in the high HOMA-IR group was shifted to higher values compared with that in the low HOMA-IR group (i.e., cITP fLDL levels were higher in the high HOMA-IR group than in the low HOMA-IR group after adjusting for LDL size). These results indicate that the relation between HOMA-IR and cITP fLDL levels was independent of LDL size.

To clarify the relationship between LDL subclasses as measured by density and cITP, the distributions of cITP fLDL were examined in NL, HC, and HTG subjects. **Table 3** shows the anthropometric characteristics and lipoprotein profiles in NL, HC, and HTG subjects. HC and HTG subjects were similar to NL subjects with respect to age but had higher BMI and lower HDL-C levels (Table 3). HC subjects had significantly higher levels of TC and LDL-C than both NL and HTG subjects, and HTG subjects had significantly higher TG levels than both NL and HC subjects (Table 3). HC and HTG subjects also had higher levels of TG and levels of TC and LDL-C than NL subjects, respectively (Table 3).

Hirano, Saegusa, and Yoshino (30) established a method for quantifying small, dense LDL-C levels by depleting large, light LDLs (d < 1.044 g/ml) from serum using heparin-Mg^{2+} precipitation and subsequently measuring the LDL-C levels in the supernatant by a direct homogenous assay. Large, light LDL levels were calculated from LDL-C levels in whole serum and serum depleted of large, light LDLs (30). In the present study, we used cITP analysis to quantify LDL subfractions in whole serum and serum depleted of large, light LDLs. cITP analysis has the advantage that LDL subfractions can be measured in the presence of lipoprotein particles other than LDL because

TABLE 2. BMI, serum levels of lipids, lipoproteins, and apolipoproteins, and lipoprotein subfractions as determined by cITP according to tertiles of HOMA-IR

Variable	Low (n = 25)	Middle (n = 25)	High (n = 25)	ANOVA P Value
		Tertiles of HOMA-IR		
BMI (kg/m^2)	22.3 ± 2.2	24.3 ± 2.7^a	25.0 ± 2.3^a	<0.01
Glucose (mg/dl)	90 ± 4	95 ± 4^a	95 ± 6^a	<0.01
Insulin (μU/ml)	6.4 ± 1.7	12.1 ± 1.5^a	$22.0 \pm 7.9^{a,b}$	<0.01
HOMA-IR	1.4 ± 0.4	2.8 ± 0.3^a	$5.2 \pm 1.9^{a,b}$	<0.01
QUICKI	0.16 ± 0.01	0.14 ± 0.00^a	$0.13 \pm 0.00^{a,b}$	<0.01
TC (mg/dl)	182 ± 8	186 ± 21	188 ± 24	NS
Log (TG)	3.8 ± 0.5	4.0 ± 0.4	$4.4 \pm 0.4^{a,b}$	<0.01
HDL-C (mg/dl)	63 ± 9	61 ± 10	56 ± 7^a	<0.01
LDL-C (mg/dl)	109 ± 3	114 ± 19	114 ± 23	NS
ApoA-I (mg/dl)	138 ± 4	134 ± 18	134 ± 13	NS
ApoA-II (mg/dl)	30.1 ± 3.8	31.1 ± 3.7	32.2 ± 3.3	NS
ApoB (mg/dl)	74 ± 6	77 ± 11	82 ± 16	NS
LDL size (nm)	27.0 ± 0.7	26.9 ± 0.5	26.5 ± 0.6^a	<0.05
cITP lipoprotein subfractions (peak area relative to an internal marker)				
fHDL	1.86 ± 0.30	1.64 ± 0.33	1.62 ± 0.34^a	<0.05
iHDL	2.44 ± 0.31	2.44 ± 0.34	2.48 ± 0.30	NS
sHDL	0.50 ± 0.11	0.51 ± 0.09	0.53 ± 0.11	NS
VLDL/IDL	0.20 ± 0.20	0.22 ± 0.20	$0.36 \pm 0.17^{a,b}$	<0.01
fLDL	0.66 ± 0.23	0.65 ± 0.24	$0.89 \pm 0.20^{a,b}$	<0.01
sLDL	1.99 ± 0.54	2.08 ± 0.49	1.98 ± 0.51	NS

cITP, capillary isotachophoresis; fHDL, fast-migrating HDL; IDL, intermediate density lipoprotein; iHDL, intermediate-migrating HDL; sHDL, slow-migrating HDL. Data are presented as means $\pm$ SD.

[a] $P < 0.05$, vs. low tertile, assessed by ANOVA and Scheffe's multiple comparison test.

[b] $P < 0.05$, high tertile vs. middle tertile, assessed by ANOVA and Scheffe's multiple comparison test.

HDL, TG-rich lipoprotein (TRL), and LDL are separated into discrete fractions (14, 15, 19–22).

Figure 5 shows typical cITP lipoprotein profiles in whole serum and serum depleted of large, light LDLs by heparin-Mg^{2+} (30) in NL, HC, and HTG subjects. As shown, cITP sLDL (peak 7) in serum depleted of large, light LDLs (Fig. 5B, D, F) was greatly reduced compared with that in whole serum LDL (Fig. 5A, C, E) in NL (Fig. 5A, B), HC (Fig. 5C, D), and HTG (Fig. 5E, F) subjects, whereas cITP fLDL (peak 6) in serum depleted of large, light LDLs was only slightly reduced (Fig. 5). These results indicate that the distribution of cITP LDL subfractions was different between whole serum and light LDL-depleted serum. cITP HDL subfractions (peaks 1–3) were similar in whole serum and serum depleted of large, light LDLs, as expected (Fig. 5). As shown in Fig. 5E, F, the VLDL/IDL subfraction (peak 5) in the HTG subject was also reduced in serum depleted of large, light LDLs compared with that in whole serum, indicating that heparin-Mg^{2+} precipitation also affects TRL. Hirano, Saegusa, and Yoshino (30) did not address the effects of heparin-Mg^{2+} precipitation on TRL because changes in TRL did not affect their measurement of LDL-C levels in whole serum or serum depleted of large, light LDLs. Similarly, because cITP completely separates HDL, TRL, and LDL subfractions, the effects of heparin-Mg^{2+} precipitation on TRL also do not interfere with the measurement of cITP LDL subfractions. Therefore, distributions of cITP fLDL subfractions in different LDL density subclasses can be compared among
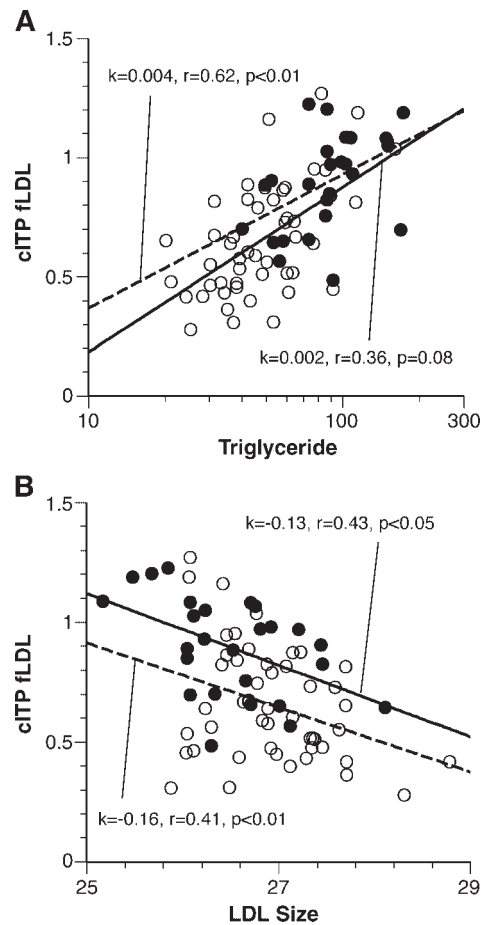
Fig. 4. Correlations between cITP fLDL and triglyceride (A) or LDL size (B) in the low (open circles) and high (closed circles) HOMA-IR groups.

NL, HC, and HTG groups while ignoring the effects of heparin-Mg^{2+} precipitation on lipoprotein particles other than LDL.

Figure 6A shows the cITP fLDL levels in NL, HC, and HTG groups according to LDL density subclasses separated by precipitation method (30). In whole serum (Fig. 6A, left panel), both the HC and HTG groups had significantly higher cITP fLDL levels than the NL group (indicated by asterisks). Levels of cITP fLDL in the small,

TABLE 3. Anthropometric characteristics and lipoprotein profiles of NL, HC, and HTG subjects

Variable	NL (n = 8)	HC (n = 8)	HTG (n = 8)
Age (years)	76 ± 5	72 ± 9	76 ± 4
BMI (kg/cm^2)	17.9 ± 1.6	22.7 ± 3.4^a	21.7 ± 2.2^a
TC (mg/dl)	193 ± 21	243 ± 11^a	$209 \pm 28^{a,b}$
TG (mg/dl)	54 ± 13	117 ± 20^a	$198 \pm 44^{a,b}$
HDL-C (mg/dl)	85 ± 13	47 ± 5^a	48 ± 7^a
LDL-C (mg/dl)	86 ± 14	160 ± 10^a	$119 \pm 28^{a,b}$

HC, hypercholesterolemic; HTG, hypertriglyceridemic; NL, normolipidemic.

[a] $P < 0.05$ vs. NL, assessed by ANOVA and Scheffe's multiple comparison test.

[b] $P < 0.05$, HTG vs. HC, assessed by ANOVA and Scheffe's multiple comparison test.
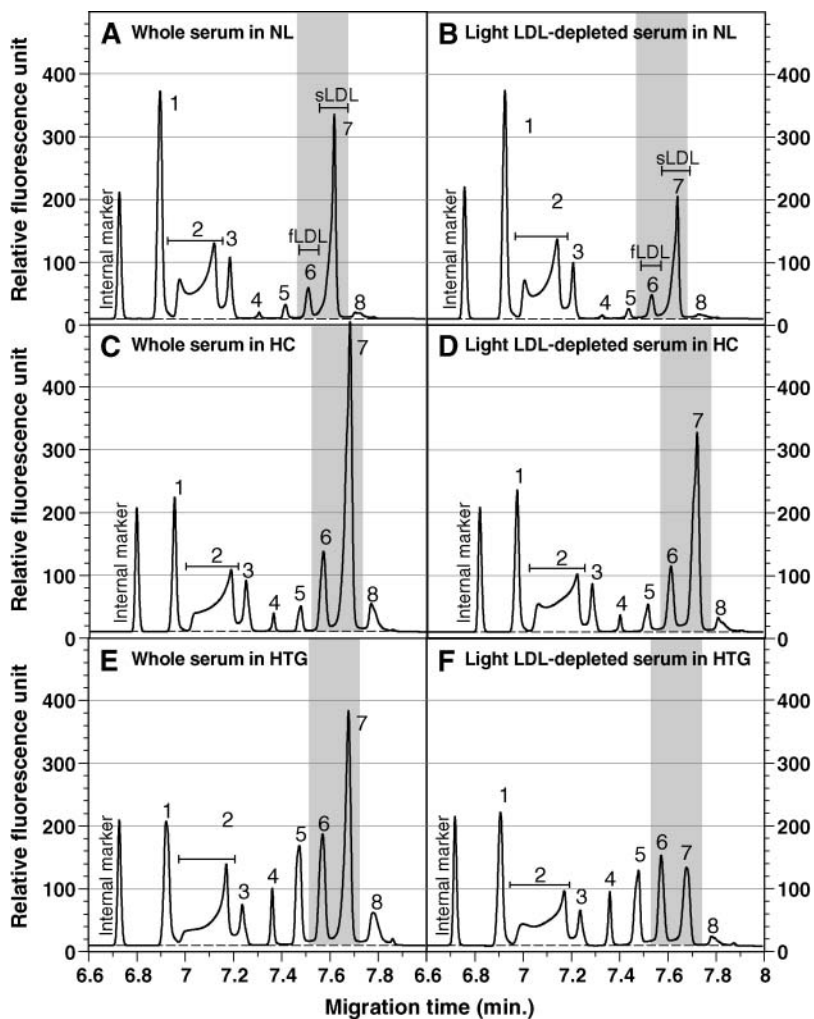
Fig. 5. Typical lipoprotein profiles as determined by cITP in whole serum (A, C, E) and serum-depleted of large, light LDLs (d = 1.019–1.044 g/ml) by heparin-Mg^{2+} precipitation (30) (B, D, F) from a normolipidemic (NL) subject (A, B), a hypercholesterolemic (HC) subject (C, D), and a hypertriglyceridemic (HTG) subject (E, F). Peaks 1–3, HDL subfractions; peak 4; chylomicron/remnant fraction; peak 5, VLDL/IDL; peaks 6 and 7, fLDL and sLDL; peak 8, a minor LDL fraction.

dense LDL subclass were higher than those in the large, light LDL subclass (indicated by daggers) in all three groups (Fig. 6A, right panel), indicating that cITP fLDL is distributed mainly in the small, dense LDL subclass. However, cITP fLDL levels in both the dense and light LDL subclasses (Fig. 6A, right panel) were significantly higher (indicated by asterisks) in the HC and HTG groups than in the NL group. Similar results were obtained when cITP fLDL was expressed as a proportion of total cITP LDL (Fig. 6B). These results indicate that cITP fLDL, expressed as either a peak area relative to that of an internal marker or as a proportion of total LDL, in HC and HTG subjects was increased in both light and dense LDL subfractions, but more of the cITP fLDL was distributed in small, dense LDL subclasses.

Because the storage of samples is inevitable in most clinical studies, we evaluated the effects of short-term (**Fig. 7A**), intermediate-term (Fig. 7B), long-term (**Fig. 8A**), and very-long-term (Fig. 8B) storage of serum samples on cITP lipoprotein analysis. Fig. 7Aa shows the cITP

lipoprotein profile in fresh serum from a volunteer subject (female, 62 years old). Small aliquots of fresh serum from the subject were protected with N_2 gas, snap-frozen with liquid nitrogen, and stored at −80°C for 1 day. Figure 7Ac shows the cITP lipoprotein profile in the frozen serum sample. As shown, cITP lipoprotein profiles in fresh serum (Fig. 7Aa) and the briefly stored serum (Fig. 7Ac) were similar, indicating that the storage of a serum sample under the indicated conditions for a very short time does not significantly affect cITP lipoprotein analysis. Both fresh serum and briefly stored serum were then depleted of large, light LDLs by heparin-Mg^{2+} precipitation and subjected to cITP analysis. As shown in Fig. 7Ab, d, cITP lipoprotein profiles in fresh serum and stored serum that were depleted of large, light LDLs were also similar, indicating that the storage of serum samples under the indicated conditions for a very short time does not significantly affect cITP lipoprotein subfractions in different LDL density subclasses.

The impact of intermediate-term storage of serum was
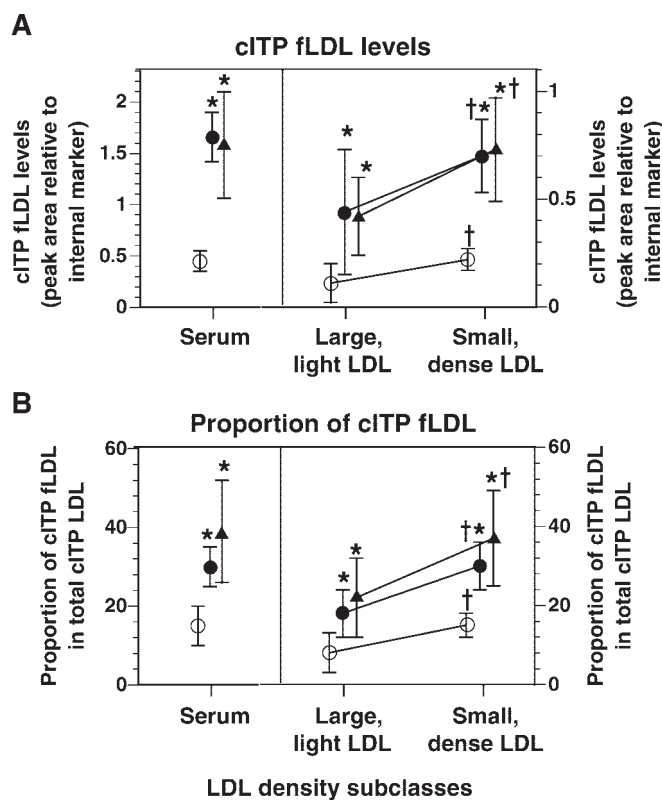
Fig. 6. Distributions of levels of cITP fLDL expressed as the peak area relative to that of an internal marker (A) and the proportion of cITP fLDL in total cITP LDL (B) in whole serum (left panel) and in light and dense LDL subclasses separated by heparin-Mg^{2+} (30) (right panel) in NL (open circles), HC (closed circles), and HTG (closed triangles) subjects. * $P < 0.05$ versus NL subjects, assessed by ANOVA and Scheffe's multiple comparison test; † $P < 0.05$, dense LDLs versus light LDLs, assessed by ANOVA.

assessed using serum samples from another volunteer (male, 36 years old), which had been used as a quality control for routine cITP analysis (Fig. 7B). Fig. 7Ba–d shows cITP lipoprotein profiles in serum of the subject that was not stored (0 Mo.) and stored at −80°C under protection with N_2 gas for 1, 2, and 3 months, respectively. As shown, cITP HDL (peaks 1–3), VLDL/IDL (peak 5), and LDL subfractions (peaks 6–8) were similar in serum samples that were not stored (Fig. 7Ba) and stored for 1 (Fig. 7Bb), 2 (Fig. 7Bc), and 3 (Fig. 7Bd) months. This result indicates that the storage of serum samples under the indicated conditions does not significantly affect these cITP lipoprotein subfractions. However, the cITP chylomicron/remnant subfraction (peak 4) seemed to be reduced in stored serum samples compared with that in serum that was not stored. This cITP subfraction was not included in the data analysis in the present study.

The impact of long-term and very-long-term storage of serum was assessed using serum samples from a 12-year-old boy (Fig. 8A) and a 12 year old girl (Fig. 8B). Fig. 8Aa, b shows cITP lipoprotein profiles in serum from the boy that was not stored and stored at −80°C for 9 months (not protected by N_2 gas), respectively. As shown, the distribution patterns of cITP HDL (peaks 1–3), VLDL/IDL (peak

5), and LDL subfractions (peaks 6–8) were not markedly different in serum samples that were not stored (Fig. 8Aa) and stored for 9 months (Fig. 8Ab). Fig. 8Ba, b shows cITP lipoprotein profiles in serum from the girl that was not stored and stored at −80°C for 18 months (not protected by N_2 gas), respectively. As shown, VLDL/IDL (peak 5) and fLDL subfractions (peak 6) were apparently different in serum samples that were not stored (Fig. 8Ba) and stored for 18 months (Fig. 8Bb). cITP HDL (peaks 1–3) and sLDL subfractions (peak 7) were less affected. These results indicate that 9 months should be the maximum duration of storage of serum samples at −80°C for cITP analysis, if not protected by N_2 gas. Very-long-term storage of serum samples is not recommended for cITP analysis.

DISCUSSION

The LDL(−) subfraction in plasma includes various forms of modified LDL, and the proportion of LDL(−) to major LDL has been used as a marker for LDL modification (1, 4, 7, 11, 12). Both type 1 and type 2 diabetes have been shown to have increased glycated LDL and a greater proportion of the LDL(−) fraction (3, 4). However, glycemic control reduced LDL(−) in type 1 diabetes but not in type 2 diabetes (3–5). This encouraged us to examine the possibility of an association between insulin resistance and LDL(−).

In the present study, we used the cITP method, originally developed by Böttcher et al. (14) and Schmitz, Mollers, and Richter (15), to separate and quantify the LDL(−) subfraction (cITP fLDL). It is not clear whether or not the proportion of cITP fLDL that is measured directly in plasma is equivalent to that in LDL separated by ultracentrifugation. Therefore, we compared cITP LDL subfractions measured directly in plasma and those measured in ultracentrifugally separated LDL. We found that the proportion of cITP fLDL in ultracentrifugally separated LDL was much lower than that in plasma (Fig. 2). Therefore, separation of LDL by ultracentrifugation may reduce the accuracy of the measurement of the proportion of LDL(−) because LDL(−) is not distributed uniformly in fractions of LDL with different densities.

We examined whether or not the two different methods for monitoring LDL subfractions used in the cITP and chromatography methods are related. We found that levels of cITP fLDL and sLDL, prestained by the lipophilic dye NBD-ceramide, are proportional to the protein content of LDL between 0.1 and 0.8 mg/ml LDL protein. This finding indicates that cITP is an accurate and sensitive method for quantifying charge-based LDL subfractions. It has the advantage that LDL subfractions can be measured directly in plasma. We also examined changes in cITP LDL subfractions during the in vitro oxidation of LDL to prove that fLDL is related to the electronegativity of LDL. We found that cITP sLDL was converted to fLDL during the oxidation of LDL and that the progressive increase in fLDL at the initial stage of oxidation paralleled
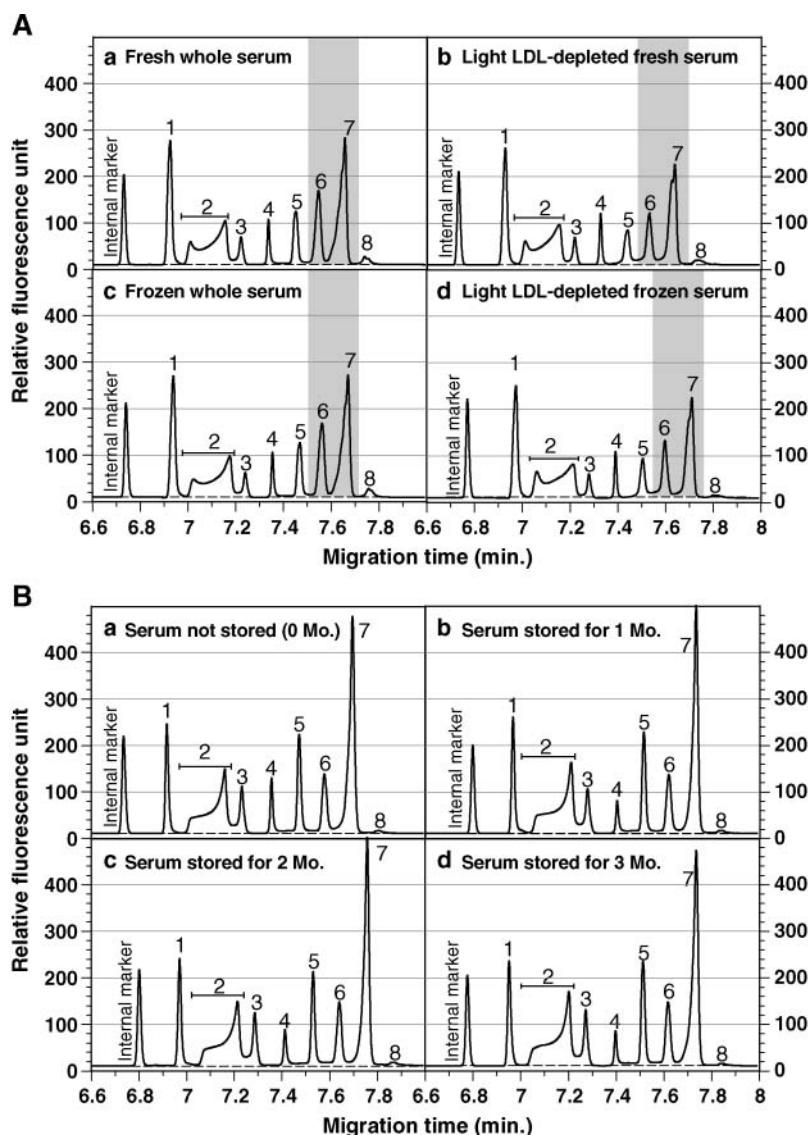
Fig. 7. A: Effects of snap-freezing serum with liquid nitrogen and short-term storage of serum at −80°C on cITP lipoprotein subfractions in serum (a, c) and serum depleted of large, light LDLs by heparin-Mg²⁺ precipitation (b, d). a: Fresh whole serum; b: fresh serum depleted of large, light LDLs; c: frozen, preserved (for 1 day) whole serum; d: frozen serum that was depleted of large, light LDLs after thawing. B: Effects of intermediate-term storage of serum at −80°C on cITP lipoprotein subfractions. a: Fresh serum without being preserved (0 Mo.); b, c, d: serum that had been stored at −80°C under N₂ gas for 1, 2, and 3 months, respectively. Peaks 1–3, HDL subfractions; peak 4; chylomicron/remnant fraction; peak 5, VLDL/IDL; peaks 6 and 7, fLDL and sLDL; peak 8, a minor LDL fraction.

that in the negative charge of LDL, as indicated by the electrophoretic mobility of LDL on agarose gels. These findings indicate that cITP fLDL is related to the electronegativity of LDL and represents an electronegative subfraction of LDL.

We found that cITP fLDL is strongly and positively correlated with TG levels. This finding supports those of other authors that LDL(−) separated by anion-exchange fast protein liquid chromatography has a higher TG content than LDL(+) (7–9). Therefore, an increased TG level in plasma contributes to the qualitative modification of LDL. However, our finding disagrees with that of Cazzolato, Avogaro, and Bittolo-Bon (32), who separated LDL(−) using anion-exchange high-pressure liquid chro-

matography and found no significant correlation between the percentage concentration of LDL(−) in total native LDL and TG levels. Considering that the TG level was not correlated with the major LDL subfraction (cITP sLDL; data not shown) in the present study, this discrepancy suggests that the proportion of LDL(−) determined by anion-exchange chromatography may not be sensitive enough to detect changes in LDL(−) related to TG levels.

Our finding that cITP fLDL is negatively correlated with the size of LDL agrees with the finding of Sánchez-Quesada et al. (10) that most of the LDL(−) in NL subjects was contained in dense LDL subclasses. They also reported that half of the LDL(−) from patients with hypertriglyceridemia was contained in dense LDL subclasses,
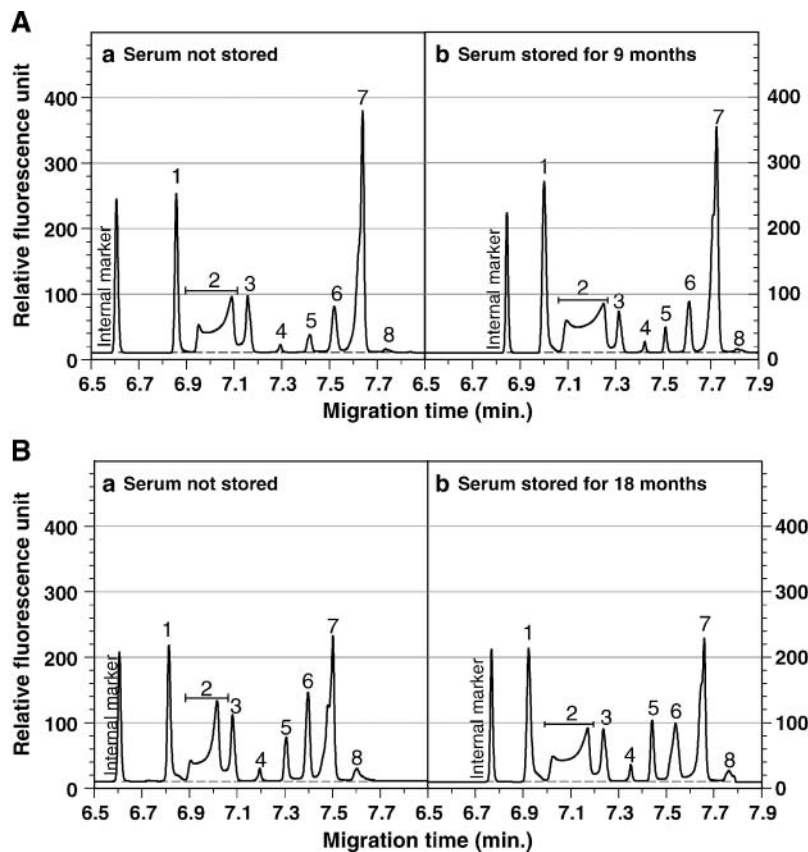
Fig. 8. A: Effects of long-term storage of serum at $-80°C$ (not protected by N_2 gas) on cITP lipoprotein subfractions in a 12-year-old boy. a: Fresh serum without being preserved; b: serum that had been stored at $-80°C$ for 9 months. B: Effects of very-long-term storage of serum at $-80°C$ (not protected by N_2 gas) on cITP lipoprotein subfractions in a 12-year-old girl. a: Fresh serum without being preserved; b: serum that had been stored at $-80°C$ for 18 months. Peaks 1–3, HDL subfractions; peak 4; chylomicron/remnant fraction; peak 5, VLDL/IDL; peaks 6 and 7, fLDL and sLDL; peak 8, a minor LDL fraction.

whereas most of the LDL(−) from patients with familial hypercholesterolemia was contained in light LDL subclasses (10). Because LDL(−) from both NL and familial hypercholesterolemia subjects has been shown to have proinflammatory activity on human endothelial cells (7, 11), it could be important to establish the relationship between LDL subfractions as measured by density and charge. Therefore, we examined the distribution of the cITP fLDL subfraction in light and dense LDL subclasses in NL, HC, and HTG subjects. Hirano, Saegusa, and Yoshino (30) recently reported a novel and simple method for the quantification of small, dense LDLs in serum depleted of large, light LDLs (d > 1.044 g/ml) by heparin-Mg^{2+} precipitation. We used their method to separate large, light LDL subclasses from small, dense LDL subclasses. Our findings that HC and HTG subjects had increased levels and a higher proportion of cITP fLDL agree with those of Sánchez-Quesada et al. (10), who reported that the proportion of LDL(−) determined by ion-exchange chromatography was increased in both patients with familial hypercholesterolemia and those with HTG. Our finding that cITP fLDL was distributed more in small, dense LDL subclasses in NL, HC, and HTG subjects is consistent with that of Sánchez-Quesada et al. (10) in NL

subjects. Our finding that HC and HTG subjects had increased cITP fLDL in large, light LDL subclasses supports the suggestion of Sánchez-Quesada et al. (10) that hyperlipidemia could promote the formation of light LDL(−). We found that both the proportion and the levels of cITP fLDL were also increased in small, dense LDL subclasses in HC and HTG subjects. This finding disagrees with the finding of Sánchez-Quesada et al. (10), who reported that NL subjects had a higher percentage of LDL(−) in dense LDL fractions (LDL4–6 by gradient centrifugation) than patients with familial hypercholesterolemia and HTG. This discrepancy may be attributable to the different methods used to separate LDL density subclasses and quantify charge-based LDL subfractions. However, our findings and those of Sánchez-Quesada et al. (10) consistently indicate that the quantification of the minimally modified negatively charged LDL subfraction in LDL density subclasses could be clinically important. Further investigations are needed to clarify whether or not the combination of charge-based LDL subfractions and LDL density subclasses, which can be routinely measured with the cITP method and the precipitation method, respectively, could be a novel and potentially useful marker for atherosclerosis.

This is the first study to show that HOMA-IR is associated with increased cITP fLDL levels. The origin of the association between cITP fLDL and insulin resistance is not clear. However, our finding that the relation between HOMA-IR and cITP fLDL depended on TG levels indicates that an increased TG level is a contributing factor to cITP fLDL associated with insulin resistance. Because increased TG levels are preceding factors in the development of insulin resistance in type 2 diabetic patients (33, 34), our finding suggests that an increased TG level may be an important contributor to the risk of CHD in type 2 diabetes.

We found that the relation between insulin resistance and cITP fLDL levels was independent of LDL size. This novel finding suggests that insulin resistance may contribute to the risk of CHD independent of small, dense LDLs, considering that LDL with an increased negative charge is atherogenic (1). However, because the present study did not directly compare cITP fLDL with LDL(−) isolated by anion-exchange HPLC, further investigation will be needed to determine whether or not an association between insulin resistance and cITP fLDL may explain the failure of glycemic control to reduce the proportion of LDL(−) in type 2 diabetes (4). It would also be interesting to determine whether or not antidiabetic drugs such as thiazolidinediones, which are high-affinity peroxisomal proliferator-activated receptor γ (PPARγ) ligands, may reduce cITP fLDL or LDL(−) in type 2 diabetes. However, it is possible that these drugs may at least limit the inflammatory response to LDL(−), because pioglitazone, a synthetic PPARγ activator, has been shown to inhibit the expression of vascular cell adhesion molecule-1 on human umbilical vein endothelial cells after interleukin-1β stimulation (2).

In conclusion, cITP lipoprotein analysis is an accurate and sensitive method for quantifying the charge-based LDL subfractions in human plasma, and insulin resistance is associated with an increased cITP fLDL fraction independent of LDL size. Further studies are needed to clarify whether or not cITP fLDL is useful for controlling the risk factors of CHD in type 2 diabetes.

REFERENCES

1. Sánchez-Quesada, J. L., S. Benitez, and J. Ordonez-Llanos. 2004. Electronegative low-density lipoprotein. *Curr. Opin. Lipidol.* **15:** 329–335.

2. Ziouzenkova, O., L. Asatryan, D. Sahady, G. Orasanu, S. Perrey, B. Cutak, T. Hassell, T. E. Akiyama, J. P. Berger, A. Sevanian, et al. 2003. Dual roles for lipolysis and oxidation in peroxisome proliferation-activator receptor responses to electronegative low density lipoprotein. *J. Biol. Chem.* **278:** 39874–39881.

3. Sánchez-Quesada, J. L., A. Perez, A. Caixas, J. Ordonmez-Llanos, G. Carreras, A. Payes, F. Gonzalez-Sastre, and A. de Leiva. 1996. Electronegative low density lipoprotein subform is increased in patients with short-duration IDDM and is closely related to glycaemic control. *Diabetologia.* **39:** 1469–1476.

4. Sánchez-Quesada, J. L., A. Perez, A. Caixas, M. Rigla, A. Payes, S. Benitez, and J. Ordonez-Llanos. 2001. Effect of glycemic optimization on electronegative low-density lipoprotein in diabetes: relation to nonenzymatic glycosylation and oxidative modification. *J. Clin. Endocrinol. Metab.* **86:** 3243–3249.

5. Gambino, R., B. Uberti, N. Alemanno, E. Pisu, G. Pagano, and M. Cassader. 2004. In vivo oxidizability of LDL in type 2 diabetic patients in good and poor glycemic control. *Atherosclerosis.* **173:** 103–107.

6. Reaven, G. M. 2002. Multiple CHD risk factors in type 2 diabetes: beyond hyperglycaemia. *Diabetes Obes. Metab.* **4 (Suppl. 1):** 13–18.

7. De Castellarnau, C., J. L. Sánchez-Quesada, S. Benitez, R. Rosa, L. Caveda, L. Vila, and J. Ordonez-Llanos. 2000. Electronegative LDL from normolipemic subjects induces IL-8 and monocyte chemotactic protein secretion by human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **20:** 2281–2287.

8. Chappey, B., I. Myara, M. O. Benoit, C. Maziere, J. C. Maziere, and N. Moatti. 1995. Characteristics of ten charge-differing subfractions isolated from human native low-density lipoproteins (LDL). No evidence of peroxidative modifications. *Biochim. Biophys. Acta.* **1259:** 261–270.

9. Demuth, K., I. Myara, B. Chappey, B. Vedie, M. A. Pech-Amsellem, M. E. Haberland, and N. Moatti. 1996. A cytotoxic electronegative LDL subfraction is present in human plasma. *Arterioscler. Thromb. Vasc. Biol.* **16:** 773–783.

10. Sánchez-Quesada, J. L., S. Benitez, C. Otal, M. Franco, F. Blanco-Vaca, and J. Ordonez-Llanos. 2002. Density distribution of electronegative LDL in normolipemic and hyperlipemic subjects. *J. Lipid Res.* **43:** 699–705.

11. Sánchez-Quesada, J. L., M. Camacho, R. Anton, S. Benitez, L. Vila, and J. Ordonez-Llanos. 2003. Electronegative LDL of FH subjects: chemical characterization and induction of chemokine release from human endothelial cells. *Atherosclerosis.* **166:** 261–270.

12. Vedie, B., I. Myara, M. A. Pech, J. C. Maziere, C. Maziere, A. Caprani, and N. Moatti. 1991. Fractionation of charge-modified low density lipoproteins by fast protein liquid chromatography. *J. Lipid Res.* **32:** 1359–1369.

13. Hodis, H. N., D. M. Kramsch, P. Avogaro, G. Bittolo-Bon, G. Cazzolato, J. Hwang, H. Peterson, and A. Sevanian. 1994. Biochemical and cytotoxic characteristics of an in vivo circulating oxidized low density lipoprotein (LDL−). *J. Lipid Res.* **35:** 669–677.

14. Böttcher, A., J. Schlosser, F. Kronenberg, H. Dieplinger, G. Knipping, K. J. Lackner, and G. Schmitz. 2000. Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions. *J. Lipid Res.* **41:** 905–915.

15. Schmitz, G., C. Mollers, and V. Richter. 1997. Analytical capillary isotachophoresis of human serum lipoproteins. *Electrophoresis.* **18:** 1807–1813.

16. Schmitz, G., U. Borgmann, and G. Assmann. 1985. Analytical capillary isotachophoresis: a routine technique for the analysis of lipoproteins and lipoprotein subfractions in whole serum. *J. Chromatogr.* **320:** 253–262.

17. Schmitz, G., and C. Mollers. 1994. Analysis of lipoproteins with analytical capillary isotachophoresis. *Electrophoresis.* **15:** 31–39.

18. Böttcher, A., C. Mollers, K. J. Lackner, and G. Schmitz. 1998. Automated free-solution isotachophoresis: instrumentation and fractionation of human serum proteins. *Electrophoresis.* **19:** 1110–1116.

19. Zhang, B., P. Fan, E. Shimoji, H. Xu, K. Takeuchi, C. Bian, and K. Saku. 2004. Inhibition of cholesteryl ester transfer protein activity by JTT-705 increases apolipoprotein E-containing high-density lipoprotein and favorably affects the function and enzyme composition of high-density lipoprotein in rabbits. *Arterioscler. Thromb. Vasc. Biol.* **24:** 1910–1915

20. Zhang, B., A. Matsunaga, K. Saku, S. Nakano, and T. Yamada. 2004. Associations among plasma lipoprotein subfractions as characterized by analytical capillary isotachophoresis, apolipoprotein E phenotype, Alzheimer disease, and mild cognitive impairment. *Arterioscler. Thromb. Vasc. Biol.* **24:** e144–e146.

21. Zhang, B., K. Noda, and K. Saku. 2003. Effect of atorvastatin on total lipid profiles assessed by analytical capillary isotachophoresis. *Cardiology.* **99:** 211–213.

22. Zhang, B., H. Tomura, A. Kuwabara, T. Kimura, S. Miura, K. Noda, F. Okajima, and K. Saku. 2005. Correlation of high density lipoprotein (HDL)-associated sphingosine 1-phosphate with serum levels of HDL-cholesterol and apolipoproteins. *Atherosclerosis.* **178:** 199–205.

23. Klag, M. J., D. E. Ford, L. A. Mead, J. He, P. K. Whelton, K. Y. Liang, and D. M. Levine. 1993. Serum cholesterol in young men and subsequent cardiovascular disease. *N. Engl. J. Med.* **328:** 313–318.

24. Berenson, G. S., W. A. Wattigney, R. E. Tracy, W. P. Newman 3rd, S. R. Srinivasan, L. S. Webber, E. R. Dalferes, Jr., and J. P. Strong. 1992. Atherosclerosis of the aorta and coronary arteries and cardiovascular risk factors in persons aged 6 to 30 years and studied at necropsy. The Bogalusa Heart Study. *Am. J. Cardiol.* **70:** 851–858.

25. Shimabukuro, T., M. Sunagawa, and T. Ohta. 2004. Low-density lipoprotein particle size and its regulatory factors in school children. *J. Clin. Endocrinol. Metab.* **89:** 2923–2927.

26. Matthews, D. R., J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, and R. C. Turner. 1985. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* **28:** 412–419.

27. Katz, A., S. S. Nambi, K. Mather, A. D. Baron, D. A. Follmann, G. Sullivan, and M. J. Quon. 2000. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J. Clin. Endocrinol. Metab.* **85:** 2402–2410.

28. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.

29. Yagi, K. 1976. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15:** 212–216.

30. Hirano, T., Y. Ito, H. Saegusa, and G. Yoshino. 2003. A novel and simple method for quantification of small, dense LDL. *J. Lipid Res.* **44:** 2193–2201.

31. SAS/STAT User's Guide. Version 6, 4th edition, Vol. 2. Cary, NC, SAS Institute, Inc., 1990.

32. Cazzolato, G., P. Avogaro, and G. Bittolo-Bon. 1991. Characterization of a more electronegatively charged LDL subfraction by ion exchange HPLC. *Free Radic. Biol. Med.* **11:** 247–253.

33. Sane, T., and M. R. Taskinen. 1993. Does familial hypertriglyceridemia predispose to NIDDM? *Diabetes Care.* **16:** 1494–1501.

34. Mingrone, G., F. L. Henriksen, A. V. Greco, L. N. Krogh, E. Capristo, A. Gastaldelli, M. Castagneto, E. Ferrannini, G. Gasbarrini, and H. Beck-Nielsen. 1999. Triglyceride-induced diabetes associated with familial lipoprotein lipase deficiency. *Diabetes.* **48:** 1258–1263.