# Plasma lipoprotein distribution of apoC-III in normolipidemic and hypertriglyceridemic subjects: comparison of the apoC-III to apoE ratio in different lipoprotein fractions

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**Abstract** In order to assess the relationship between plasma accumulation of triglyceride-rich lipoproteins (TRL) and lipoprotein levels of apoC-III and apoE, we have measured apoC-III and apoE in lipoproteins separated according to size (by automated gel filtration chromatography) from plasma of normolipidemic subjects (plasma triglyceride (TG): 0.84 ± 0.10 mmol/l; mean ± SE, n = 8), and from type III (n = 8) and type IV (n = 8) hyperlipoproteinemic patients, matched for plasma TG (5.76 ± 0.62 v 5.55 ± 0.45 mmol/l, resp.). Total plasma apoC-III concentration was similar in type III and type IV patients (33.1 ± 3.4 v 37.6 ± 4.4 mg/dl, respectively), but was significantly increased compared to normolipidemic controls (10.0 ± 1.0 mg/dl, $P < 0.001$). TRL apoC-III was lower and high density lipoprotein (HDL) apoC-III was significantly higher in type III versus type IV subjects (14.8 ± 3.2 vs. 22.8 ± 3.0 mg/dl, $P < 0.05$; 8.3 ± 1.0 vs. 5.2 ± 0.5 mg/dl, $P < 0.05$). Plasma concentration of apoC-III in lipoproteins that eluted between TRL and HDL (intermediate-sized lipoproteins, ISL) was similar in the two hypertriglyceridemic groups (10.1 ± 1.3 vs. 9.7 ± 1.6 mg/dl), but was significantly higher ($P < 0.05$) than controls (2.2 ± 0.3 mg/dl). TRL, ISL, and HDL apoE concentrations were significantly higher in type III versus type IV subjects ($P < 0.05$). All lipoprotein fractions in type III patients were characterized by lower apoC-III to apoE ratios. In contrast, the TRL apoC-III to apoE ratio of type IV patients was similar and the ISL apoC-III to apoE ratio was significantly higher, compared to normolipidemic individuals. These results indicate that compared to normolipidemic individuals, remnant-like lipoproteins in the ISL fraction of type IV patients are enriched in apoC-III relative to apoE, whereas those of type III patients are enriched in apoE relative to apoC-III.—**Fredenrich, A., L-M. Giroux, M. Tremblay, L. Krimbou, J. Davignon, and J. S. Cohn.** Plasma lipoprotein distribution of apoC-III in normolipidemic and hypertriglyceridemic subjects: comparison of the apoC-III to apoE ratio in different lipoprotein fractions. *J. Lipid Res.* 1997. **38:** 1421–1432.

**Supplementary key words** atherosclerosis • cholesterol • FPLC • HDL • remnants • triglyceride

Increasing evidence suggests that apolipoprotein (apo)C-III plays an important role in controlling plasma triglyceride metabolism and in determining the plasma concentration of potentially atherogenic triglyceride-rich lipoproteins (TRL) (1). ApoC-III (an 8,800 D glycoprotein) (2) is synthesized by the liver and intestine (3), and is a component of plasma chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (4). Plasma concentration of apoC-III is positively correlated with the level of plasma triglyceride (4,5), and apoC-III production is increased in patients with hypertriglyceridemia (6). ApoC-III gene polymorphisms are associated with increased levels of plasma apoC-III and hypertriglyceridemia (7, 8). Liver perfusion studies have demonstrated that apoC-III inhibits the hepatic uptake of TRL and their remnants (9,10), and in vitro experiments have shown that apoC-III can inhibit the activity of both lipoprotein lipase and hepatic lipase (11–15). ApoC-III therefore modulates the plasma catabolism and clearance of TRL, and this is of pathophysiological significance, as indicated by angiographic studies showing that the plasma lipoprotein distribution of apoC-III is (under certain circumstances) a statistically

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Abbreviations: apo, apolipoprotein; CAD, coronary artery disease; d, density; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediamine-tetraacetate; FPLC, fast protein liquid chromatography; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; ISL, intermediate-sized lipoprotein; LDL, low density lipoprotein; LRP, LDL receptor-related protein; PBS, phosphate-buffered saline; RCT, reverse cholesterol transport; TRL, triglyceride-rich lipoprotein; VLDL, very low density lipoprotein; TG, triglyceride.

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significant independent predictor of the progression or severity of coronary artery disease (CAD) (16–18).

The role of apoC-III in plasma TRL metabolism has been more clearly defined by the results of recent studies in transgenic animals. Mice overexpressing human apoC-III develop marked hypertriglyceridemia, which is proportional to plasma apoC-III levels and liver apoC-III gene expression (19, 20). Overexpression of mouse apoC-III also causes plasma triglyceride concentration to increase significantly (21). Animals deficient in apoC-III on the other hand, are hypotriglyceridemic and have greatly reduced postprandial triglyceridemia (22). Plasma accumulation of TRL in apoC-III transgenic mice has been shown to be associated with reduced plasma VLDL (20) and chylomicron remnant clearance (23), apparently due to reduced binding of TRL to the LDL receptor (21, 23) and/or to heparan sulfate proteoglycans (21). Decreased receptor binding was reversed by addition of exogenous apoE (19, 20). Furthermore, cross-breeding of mice overexpressing human apoC-III with mice overexpressing human apoE resulted in normalization of triglyceride levels in transgenic progeny (21, 23). It has therefore been concluded that apoC-III is able to modulate apoE-mediated clearance of TRL, and that the concentration of apoC-III relative to apoE is a key determinant of triglyceride levels in plasma.

The aforementioned studies have prompted us to reassess, in human subjects, the relationship between plasma accumulation of TRL and plasma lipoprotein levels of apoC-III and apoE. We have thus determined the plasma lipoprotein distribution of apoC-III in lipoproteins separated by size in normolipidemic and hypertriglyceridemic subjects, with the aim of determining the ratio of apoC-III to apoE in different-sized lipoprotein fractions. In order to assess how the presence of remnant lipoproteins in plasma influences relative amounts of apoC-III and apoE, we have compared two groups of hypertriglyceridemic subjects: type III hyperlipoproteinemic individuals, having an apoE 2/2 phenotype and plasma remnant lipoprotein accumulation (as evidenced by the presence of plasma β-VLDL) (24), and type IV hyperlipoproteinemic patients, who (by selection) had a similar level of plasma triglyceride. We have used automated gel filtration chromatography (with an FPLC system) to determine plasma lipoprotein levels of apoC-III and apoE, as this method of lipoprotein separation, unlike ultracentrifugation, does not cause dissociation of apoE from lipoprotein particles (25, 26), and allows for the separation of an apoE-containing intermediate-sized lipoprotein (ISL) fraction, distinct from TRL and HDL, which contains TRL remnant-like lipoproteins (27).

## METHODS

### Subjects

Healthy male normolipidemic subjects (n = 8), recruited from the staff of our research laboratory, had plasma triglyceride concentrations <2.3 mmol/l, and total plasma cholesterol concentrations <5.2 mmol/l. Hyperlipidemic patients were selected from those attending our Lipid Clinic at the Clinical Research Institute of Montreal. Eight male patients were selected who had type IV hyperlipoproteinemia (plasma triglyceride > 2.3 mmol/l, LDL cholesterol < 3.4 mmol/l), and 8 patients (4 males and 4 females) were selected who had type III hyperlipoproteinemia (plasma triglyceride > 2.3 mmol/l, plasma cholesterol > 6.2 mmol/l, an apoE 2/2 phenotype and a clearly defined β-VLDL band on agarose gel electrophoresis). The normolipidemic and type IV individuals had an apoE 3/3 phenotype, except for one normolipidemic and one type IV subject, who were both apoE 4/3. Neither normolipidemic nor hyperlipidemic subjects were taking medications known to affect plasma lipid levels. Mean plasma lipid and apolipoprotein concentrations for the study subjects are shown in Table 1. Type III and type IV patients were selected so that the mean plasma triglyceride concentrations for the two groups were similar. Type III and type IV patients were significantly older than the normolipidemic control subjects (Table 1).

### Separation of plasma lipoproteins

Blood samples were obtained from subjects who had fasted for at least 12 h overnight. Blood was drawn under vacuum into tubes containing EDTA (final concentration: 1.5 mg/ml). Plasma was obtained by centrifugation at 3,000 rpm (15 min, 4°C) and was separated from red blood cells by aspiration. It was stored at 4°C until lipids or apolipoproteins were assayed, or it was immediately used for plasma lipoprotein separation by automated gel filtration chromatography on a fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology, Uppsala, Sweden). FPLC separation of plasma lipoproteins was carried out as previously described (27). Briefly, plasma samples (1 ml) were manually transferred to a 2-ml sample loop with two washes of 0.5 ml saline solution. They were programmed (Liquid Chromatography Controller LCC-500 Plus) to be loaded and separated on a 50-cm column (16 mm internal diameter) packed with cross-linked agarose gel (Superose 6 prep grade, Pharmacia). The column was eluted with 0.15 mol/l NaCl (0.01% EDTA, 0.02% sodium azide, pH 7.2) at a rate of 1.0 ml/min, and 25 min after addition of sample, 90 1-ml fractions were col-



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TABLE 1. Characteristics of normolipidemic and hyperlipidemic subjects

| | Normolipidemic (n = 8) | Type III (n = 8) | Type IV (n = 8) |
|---|---|---|---|
| Age (years) | 36.5 ± 1.3 | 50.6 ± 3.0[c] | 46.5 ± 3.9[a] |
| Gender (male/female) | 8/0 | 4/4 | 8/0 |
| Total triglyceride (mmol/l) | 0.84 ± 0.10 | 5.76 ± 0.62[c] | 5.55 ± 0.45[c] |
| Total cholesterol (mmol/l) | 4.37 ± 0.21 | 8.68 ± 0.66[c] | 5.76 ± 0.40[b,d] |
| HDL cholesterol (mmol/l) | 1.13 ± 0.10 | 0.80 ± 0.07[a] | 0.67 ± 0.03[b] |
| ApoB (mg/dl) | 104 ± 6 | 130 ± 8[a] | 150 ± 10[b] |
| ApoC-III (mg/dl) | 10.0 ± 1.0 | 33.1 ± 3.4[c] | 37.6 ± 4.4[c] |
| ApoE (mg/dl) | 3.7 ± 0.3 | 27.1 ± 2.5[c] | 7.3 ± 1.0[b,e] |

Values are mean ± SE.
[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$, significantly different from normolipidemic subjects.
[d]$P < 0.01$; [e]$P < 0.001$, significantly different, type III versus type IV subjects.

lected sequentially. Total run time for each sample, including pre- and post-washes was 150 min. Sample elution was monitored spectrophotometrically at 280 nm. Recovery of plasma apoC-III was 82 ± 11%, as assessed by expressing the sum of apoC-III found in individual FPLC elution fractions as a percentage of apoC-III detected in whole plasma. No difference was observed between the recovery of apoC-III from hypertriglyceridemic and normolipidemic samples. Recovery of plasma cholesterol was 90 ± 4%.

### Quantification of apoC-III

ApoC-III was measured by a noncompetitive polyclonal enzyme-linked immunoassay (ELISA), which was developed in our laboratory according to the method of Bury and Rosseneu (28). Immunopurified polyclonal goat anti-human apoC-III antibody (Biodesign, Kennebunk, ME) was used as both the capture and detection antibody. According to the manufacturer's specifications, the antibody was an IgG fraction isolated from goat serum, which was purified by apoC-III affinity column chromatography and repurified by passage through apoC-I and apoE columns. Detection antibody was prepared by conjugating 0.5 mg of horseradish peroxidase to 1.0 mg of anti-apoC-III antibody using the sodium periodate method (29). Conjugated antibody was mixed (1:1, vol/vol) with conjugate stabilizer (Superfreeze®, Pierce, Rockford, IL) and stored in aliquots (100 µl) at −20°C. Ninety-six-well polystyrene plates (Nunc-Immuno Plate Maxisorp, Nunc, Denmark) were coated with immunopurified antibody (1.65 µg per well) dissolved in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.15 M NaCl, 1 mg/ml $NaN_3$, pH 7.4). Outer rows of each plate were not used to avoid outside-well variability (30). Plates were sealed with SealPlate Adhesive film (Elkay, MA), incubated for 3 h at 37°C, and stored at 4°C until use within 6 weeks of preparation. Before each assay, plates were inverted, washed six times (PBS containing 0.5 ml/l Tween 20, Bio-Rad Laboratories, CA) with an automated microplate washer (EL402, Bio-Tek Instruments, VT), and residual binding sites were blocked for 1 h at room temperature with 200 µl PBS containing 0.1% casein (BDH Laboratory Supplies, Poole, UK) and 0.01% merthiolate (Eastman, NY, USA).

A standard curve (ranging from 0.25 to 3.0 ng) was prepared for each assay by making appropriate dilutions of a plasma standard (stored at −70°C), which had been calibrated using purified apoC-III as a primary standard (>98% pure, as assessed by SDS polyacrylamide gel electrophoresis; Chemicon, Temecula, CA). Standards (100 µl) were applied to microtiter plates in duplicate, together with two control plasmas (low and high, stored at −70°C) diluted appropriately. Normolipidemic plasma samples were routinely diluted 1 in 15,000 with sample buffer (PBS, 0.1% casein, 0.01% merthiolate, 0.5 ml/l Tween 20) and hypertriglyceridemic samples were diluted 1 in 15,000 to 1 in 40,000, depending on their triglyceride concentration. All samples were assayed in duplicate.

Plates containing diluted samples were covered and incubated overnight at 37°C on an orbital shaker (Bellco Biotechnology). After vacuum aspiration of samples and 6 automated washes with PBS-Tween, 100 µl of horseradish peroxidase-conjugated antibody, diluted 1:1000 (determined to be the optimal dilution from a titration assay), was added to each well. Plates were covered and incubated for 3 h at 37°C with shaking. After 6 washes, color was developed by addition of 100 µl of freshly prepared substrate solution (sodium phosphate/citrate buffer, pH 5.6, containing 2.4 g/l *o*-phenylenediamine hydrochloride and 0.021% $H_2O_2$) to each well. After 10 to 15 min in the dark, color development was stopped with 100 µl/well of 2.5 M sulfuric acid. Absorbance was measured at 490 nm with a microplate reader (EL310, Bio-Tek Instruments). A standard curve was obtained for each plate by plotting absorbance at 490 nm as a function of apoC-III concen-



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tration. A second-order polynomial curve was fitted to the data with Sigmaplot software (Jandel Scientific, CA) and absorbance values were converted to concentration measurements by regression analysis.

The precision of the apoC-III assay was reflected by a within-assay coefficient of variation of 3.5% (determined by measuring the same sample, with an apoC-III concentration of 15.3 ± 0.5 mg/dl, 20 times on the same plate) and a between-assay coefficient of variation (determined in 10 consecutive assays during a 3-week period) of 9.2% for a low plasma control (9.5 ± 0.9 mg/dl) and 7.8% for a high plasma control (20.8 ± 1.6 mg/dl). The accuracy of the apoC-III assay was assessed by measuring the concentration of apoC-III in 23 frozen plasma samples, duplicate samples of which were sent to the laboratory of Dr. Petar Alaupovic in Oklahoma City for measurement of apoC-III by electroimmunoassay (31). A correlation coefficient of 0.88 was found between values obtained with the two assays. In absolute terms, values obtained by our ELISA assay were 15% higher than those determined by the reference assay: an in-house control plasma from Oklahoma (apoC-III: 13.3 mg/dl) was found to have a mean apoC-III concentration of 15.3 ± 0.5 mg/dl (n = 6). When increasing amounts of ultracentrifugally isolated lipoproteins (VLDL, LDL, and HDL) from one normolipidemic and two hypertriglyceridemic subjects were assayed for apoC-III, parallel concentration curves were obtained, demonstrating that an equal increment in color development was achieved whether apoC-III was contained within large TRL or smaller cholesterol-rich lipoproteins.

### Determination of apoC-III and apoE in different-sized lipoprotein fractions

Plasma apoC-III and apoE characteristically eluted as three overlapping chromatographic peaks, which corresponded to triglyceride-rich lipoprotein (TRL), intermediate-sized lipoprotein (ISL), and high density lipoprotein (HDL)-sized lipoprotein fractions. In order to reproducibly define these fractions, the absorbance profile (at 280 nm) was determined for each plasma sample. The fraction with the lowest absorbance after elution of TRL was taken as the last fraction containing TRL. This nadir in absorbance generally occurred in fraction 16, 17, or 18. The FPLC fraction with the lowest concentration of apoC-III or apoE between ISL and HDL was taken to be the last fraction of ISL. This was generally fraction 31, 32, or 33 for apoE and fraction 34, 35, or 36 for apoC-III in hypertriglyceridemic subjects, reflecting the fact that apoE was associated with larger HDL particles. Plasma concentration of apoC-III or apoE in pooled fractions was determined by adding the amount of apolipoprotein in FPLC fractions corresponding to TRL, ISL, or HDL-sized lipoproteins. As the FPLC recovery of apoC-III and apoE varied somewhat from one subject to another (70–95%), data were corrected to 100% recovery by expressing apolipoprotein in each pooled fraction as a percentage of total recovered apolipoprotein, and this percentage was then multiplied by total apoC-III or apoE concentration determined in whole plasma. The reproducibility of this methodology was very acceptable, as reflected by mean (±SD) TRL, ISL, and HDL apoC-III levels determined on two different occasions 21 months apart (one sample in duplicate, i.e., n = 3) for a normolipidemic subject, TRL apoC-III (mg/dl): 1.7 ± 0.4 (±26%), ISL apoC-III: 1.8 ± 0.2 (±11%), HDL apoC-III: 9.2 ± 1.4 (±15%). ApoE lipoprotein concentrations were determined for the same individual on five different occasions spanning a 13-month period: TRL apoE (mg/dl): 0.36 ± 0.05 (±15%), ISL apoC-III: 1.05 ± 0.14 (±13%), HDL apoC-III: 2.80 ± 0.21 (±7.3%).

### Apolipoprotein and lipid assays

Plasma and lipoprotein cholesterol and triglyceride concentrations were determined enzymatically on an autoanalyzer (Cobas Mira, Roche). HDL cholesterol was determined by assaying cholesterol in the supernatant of the d > 1.006 g/ml fraction, after precipitation of apoB-containing lipoproteins with heparin/manganese. ApoE was assayed by ELISA (27). Plasma apoA-I and apoB concentrations were measured by nephelometry (Behring Nephelometer 100 Analyzer). Lipoproteins were isolated by sequential ultracentrifugation (VLDL: d < 1.006 g/ml; LDL: 1.019 < d < 1.063 g/ml and HDL: 1.063 < d < 1.21 g/ml), using a 50.4 Ti rotor (Beckman Instruments, Inc.), to establish the FPLC elution of ultracentrifugal lipoprotein fractions, to establish the parallelism of lipoprotein apoC-III concentration curves, and to verify the presence of β-VLDL in type III individuals. β-VLDL was determined by agarose gel electrophoretic separation of the d < 1.006 g/ml fraction on a Beckman Paragon Electrophoresis System, followed by staining with Sudan Black. ApoE phenotype was determined by immunoblotting of plasma apoE separated by minigel electrophoresis (32).

### Statistical analysis

The statistical significance of the difference between the mean values of two groups was assessed by unpaired *t*-test using SigmaStat software (Jandel Scientific, CA). For certain parameters, values were not normally distributed (as assessed by Kolmogorov-Smirnov test), and in these cases mean differences were assessed by Mann-Whitney rank sum test. Pearson correlation coefficients (*r*) were calculated to describe the correlation between different apolipoprotein and lipid parameters.



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## RESULTS

Plasma triglyceride concentrations of type III and type IV hyperlipoproteinemic subjects were (according to patient selection) not significantly different ($P = 0.79$), but were 6 to 7 times greater than that of normolipidemic controls (**Table 1**). Mean plasma apoC-III concentrations were also not significantly different ($P = 0.43$), though they were 3 to 4 times higher than controls. Plasma apoE concentrations were on average two times higher in type IV patients and 7 times higher in type III patients. Total plasma cholesterol and apoB levels were significantly higher and HDL cholesterol levels were significantly lower in patients versus controls (Table 1).

The separation of different-sized plasma lipoprotein fractions by FPLC is shown for two normolipidemic subjects in **Fig. 1**. Elution of lipoprotein cholesterol and triglyceride is indicated and peaks corresponding to TRL, LDL, and HDL are identified in the upper panel. The elution profile of apoC-III is depicted by the closed circles. Peaks of TRL and HDL apoC-III were clearly identifiable in all subjects. A significant proportion of total plasma apoC-III eluted in fractions containing LDL. As the majority of LDL particles do not contain apoC-III (or apoE) (33, 34), we have refered to these fractions (27) as intermediate-sized lipoprotein fractions or ISL. For normolipidemic subjects, 13–27% of total plasma apoC-III was found in these fractions. This is exemplified in Fig. 1, where the subject in the top panel had a plasma triglyceride conc. of 0.86 mmol/l, and a total apoC-III conc. of 11.4 mg/dl, with 20%, 13%, and 67% of total plasma apoC-III in TRL, ISL, and HDL, respectively. The subject in the bottom panel had a triglyceride conc. of 1.15 mmol/l and a total apoC-III conc. of 13.3 mg/dl, and compared to the subject in the top panel had relatively more apoC-III in TRL and ISL (32% and 27%, respectively) and less in HDL (41%).

Plasma apoC-III profiles for one type III and one type IV patient, which were representative of the groups as a whole, are shown in **Fig. 2**. Characteristically, these patients had very pronounced peaks of TRL triglyceride, cholesterol and apoC-III and reduced levels of HDL lipid and apoC-III compared to normolipidemic subjects (N.B. the scales of the y axes in Fig. 1 and Fig. 2 are not the same). In a number of type III patients, the TRL fraction contained chylomicrons of intestinal origin (as evidenced by the presence of lipid-stained material at the origin of agarose gels after electrophoresis), in addition to pre-β-migrating very low density lipoproteins (VLDL), and for this reason we have referred to this fraction in all subjects as TRL rather than VLDL.

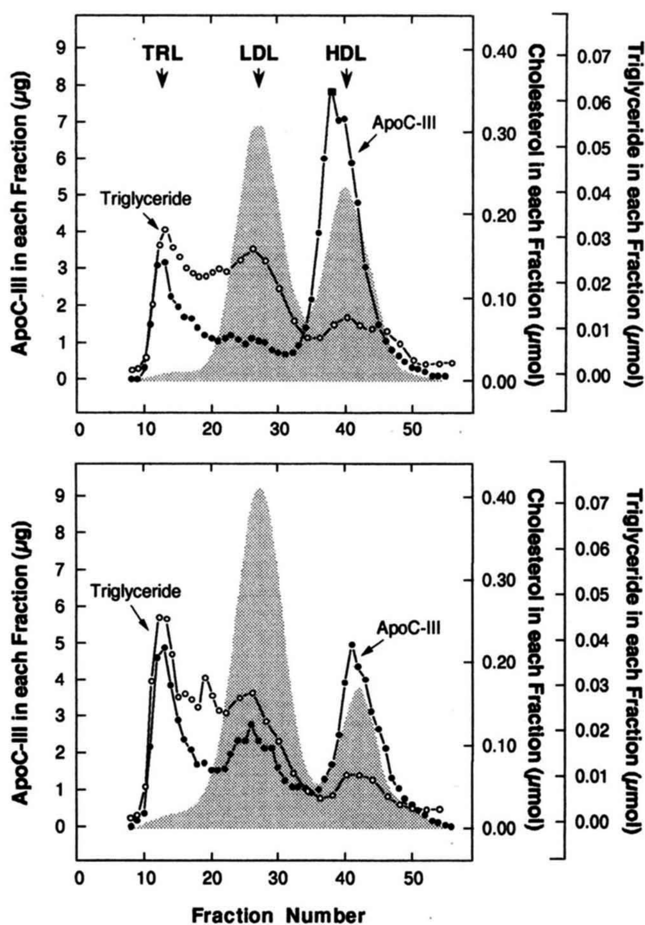


**Fig. 1.** ApoC-III in lipoproteins separated according to size from the plasma of two normolipidemic subjects. Plasma was separated by automated gel filtration chromatography (on an FPLC system) and apoC-III (●–●) was measured by ELISA assay. Triglyceride in each fraction is indicated by the open circles (○–○). The elution of lipoprotein cholesterol is indicated by the shaded area and peaks corresponding to TRL, LDL and HDL are identified in the upper panel. The subject in the top panel had a plasma triglyceride conc. of 0.86 mmol/l, and an apoC-III conc. of 11.4 mg/dl; the subject in the bottom panel had a triglyceride conc. of 1.15 mmol/l and an apoC-III conc. of 13.3 mg/dl.

A direct comparison of apoC-III lipoprotein profiles for normolipidemic, type III, and type IV subjects is presented in **Fig. 3**. ApoE was assayed in the same samples and these data are presented on the right-hand side of Fig. 3. Data points represent mean values for 8 subjects in each group. In qualitative terms, more apoC-III eluted with TRL and less with HDL in type IV compared to type III individuals. Mean plasma apoC-III profiles for the plasma of type III and type IV subjects were virtually superimposable for those fractions containing ISL. Peaks of TRL and ISL apoC-III, but not HDL, were considerably smaller in normolipidemic subjects. In contrast, apoE was present in higher amounts in all FPLC fractions of plasma from type III subjects. More apoE



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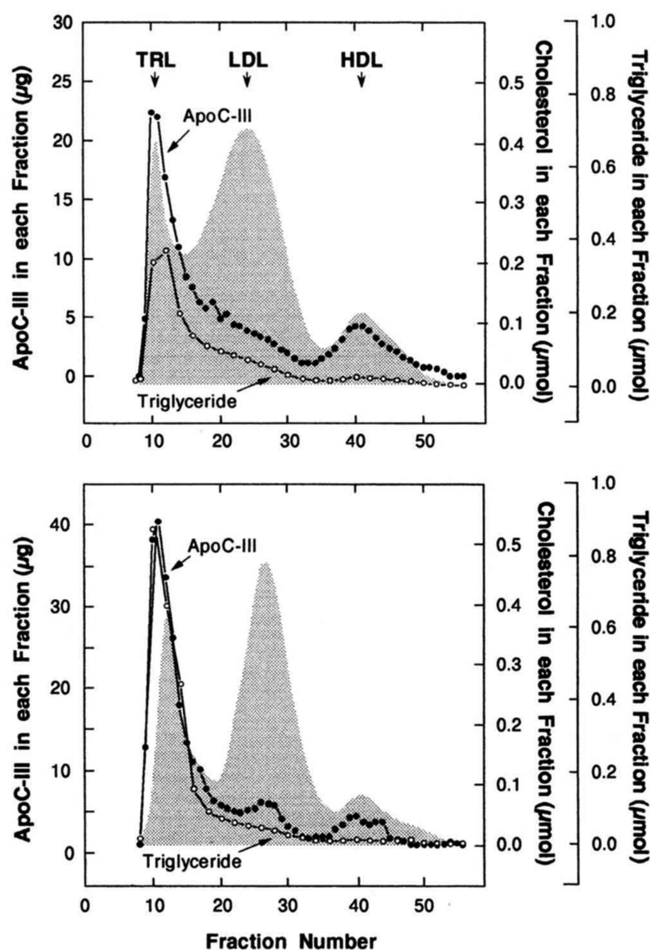


**Fig. 2.** Plasma lipoprotein distribution of apoC-III in a type III hyperlipoproteinemic subject (top panel) and a type IV subject (bottom panel). Triglyceride in each fraction is indicated by the open circles (○–○) and apoC-III by the closed circles (●–●). The elution of lipoprotein cholesterol is indicated by the shaded areas and peaks corresponding to TRL, LDL, and HDL are identified in the upper panel. The subject in the top panel had a plasma triglyceride conc. of 4.86 mmol/l, and an apoC-III conc. of 29.7 mg/dl, with 56%, 23%, and 21% of total plasma apoC-III in TRL, ISL, and HDL, respectively. The subject in the bottom panel had a triglyceride conc. of 5.75 mmol/l and an apoC-III conc. of 43.3 mg/dl, with 62%, 26%, and 12% of total plasma apoC-III in TRL, ISL,and HDL, respectively.

was also detected in the TRL and ISL fractions (but not HDL) of type IV subjects compared to controls.

Individual FPLC fractions were grouped together, so that relative (%) and absolute (mg/dl) amounts of apoC-III and apoE in three different-sized lipoprotein fractions (TRL, ISL, HDL) could be determined (as described in Methods). Relative and absolute triglyceride and cholesterol concentrations were also determined for these same fractions (**Table 2**). Plasma TRL, ISL, and HDL triglyceride concentrations were significantly higher in hypertriglyceridemic patients compared to controls, but were not significantly different between type III and type IV subjects. The similar distribution of triglyceride between lipoproteins of type III and type IV subjects was associated with dissimilar patterns of apoC-III distribution. In relative terms, more plasma apoC-III in type IV individuals was associated with TRL (60% vs. 43%) and less with HDL (15% vs. 26%), and subsequently TRL apoC-III concentration was significantly higher and HDL apoC-III concentration was significantly lower in type IV subjects. Relative amounts of plasma apoC-III in ISL, and ISL apoC-III concentrations, were not significantly different. The molar ratio of TRL triglyceride to TRL apoC-III (representing the number of molecules of triglyceride for each molecule of apoC-III in TRL) was thus 115 ± 12, 219 ± 21, and 145 ± 10 for the normolipidemic, type III, and type IV groups, respectively (type III vs. type IV: $P < 0.01$; type IV vs. normolipidemic: $P = 0.08$, n.s). The molar ratio of triglyceride to apoC-III in ISL was not, however, significantly different among the three groups: 227 ± 39, 162 ± 11, and 149 ± 20, respectively. Total plasma, TRL, and ISL apoC-III concentrations (for the three groups combined, n = 24) were significantly correlated with total plasma, TRL, and ISL triglyceride concentrations, respectively ($r = 0.92, 0.92, 0.86, P < 0.001$). HDL apoC-III was not, however, correlated with HDL triglyceride ($r = 0.18$, $P = 0.41$, n.s.). Mean TRL and ISL (though not HDL) cholesterol levels were higher in type III compared to type IV patients, and mean apoE concentrations in all three fractions in this group were also significantly elevated (Table 2).

In order to compare the amount of apoC-III in each FPLC elution fraction with that of apoE, profiles in Fig. 3 were regrouped for each patient type and were redrawn to the same scale (**Fig. 4**). The amount of apoC-III in each FPLC elution fraction was expressed as a ratio relative to apoE, and these ratios were then converted to a molar ratio (taking into account the difference in molecular weight between apoC-III and apoE, 8,746 vs. 34,200 daltons, respectively). FPLC fractions were grouped and averaged for TRL, ISL (which was divided evenly into three different-sized fractions: ISL1, ISL2, and ISL3, large to small, respectively), and HDL (which was divided into large and small HDL: HDL1 and HDL2). In those cases where apoE and apoC-III cut-points did not coincide (this difference was not greater than four fractions), a division was made midway between the two. As shown in **Fig. 5**, the type III patients had a decreased apoC-III to apoE ratio in all fractions, compared to the other subjects. TRL from type III patients contained on average 6 ± 1 molecules of apoC-III for every molecule of apoE; the average apoC-III to apoE ratio for the three ISL fractions combined was 5 ± 1, which was not significantly different from TRL. In the normolipidemic subjects, ISL3 and ISL2 had significantly lower apoC-III to apoE ratios



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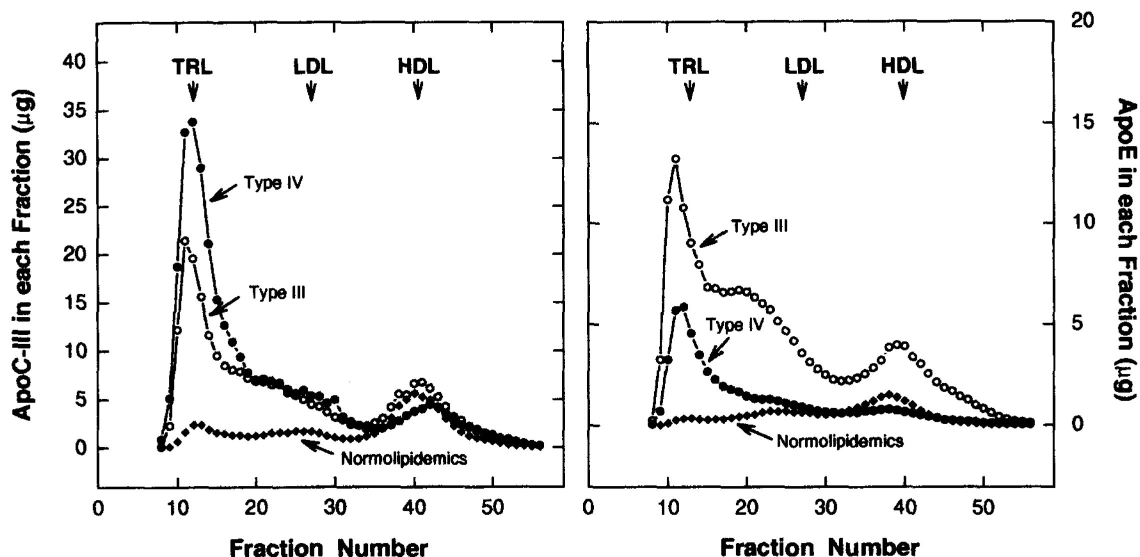


**Fig. 3.** Plasma lipoprotein distribution of apoC-III (left-hand panel) and apoE (right-hand panel) in normolipidemic (♦–♦), type III (○–○), and type IV subjects (●–●). Data points represent mean values for 8 subjects in each group. The elution positions of TRL, LDL, and HDL are indicated.

TABLE 2. Plasma apoC-III and apoE concentrations in lipoprotein fractions isolated from normolipidemic and hyperlipidemic subjects

| | Normolipidemic (n = 8) | Type III (n = 8) | Type IV (n = 8) |
|---|---|---|---|
| ApoC-III | | | |
| TRL mg/dl | 1.9 ± 0.5 | 14.8 ± 3.2[a] | 22.8 ± 3.0[a,b] |
| (%) | (16.9 ± 3.4) | (42.9 ± 5.6)[a] | (60.2 ± 2.2)[a,b] |
| ISL mg/dl | 2.2 ± 0.3 | 10.1 ± 1.3[a] | 9.7 ± 1.6[a] |
| (%) | (22.0 ± 1.5) | (31.1 ± 3.1)[a] | (25.4 ± 1.7) |
| HDL mg/dl | 5.9 ± 0.5 | 8.3 ± 1.0[a] | 5.2 ± 0.5[b] |
| (%) | (61.1 ± 4.2) | (26.1 ± 3.6)[a] | (14.6 ± 1.6)[a,b] |
| ApoE | | | |
| TRL mg/dl | 0.3 ± 0.1 | 10.7 ± 1.6[a] | 4.3 ± 0.7[a,b] |
| (%) | (7.6 ± 1.5) | (39.2 ± 4.2)[a] | (58.2 ± 2.2)[a,b] |
| ISL mg/dl | 1.2 ± 0.1 | 10.0 ± 1.6[a] | 1.9 ± 0.3[a,b] |
| (%) | (34.1 ± 2.4) | (36.4 ± 3.8) | (26.4 ± 2.0)[a,b] |
| HDL mg/dl | 2.2 ± 0.2 | 6.4 ± 0.7[a] | 1.2 ± 0.3[a,b] |
| (%) | (58.4 ± 2.7) | (24.4 ± 2.5)[a] | (15.5 ± 1.7)[a,b] |
| Triglyceride | | | |
| TRL mmol/l | 0.2 ± 0.1 | 3.5 ± 0.6[a] | 3.7 ± 0.4[a] |
| (%) | (24.9 ± 3.8) | (58.3 ± 4.1)[a] | (65.0 ± 2.8)[a] |
| ISL mmol/l | 0.5 ± 0.1 | 1.9 ± 0.3[a] | 1.5 ± 0.1[a] |
| (%) | (55.3 ± 2.8) | (34.4 ± 3.8)[a] | (27.2 ± 2.1)[a] |
| HDL mmol/l | 0.2 ± 0.1 | 0.4 ± 0.1[a] | 0.4 ± 0.1[a] |
| (%) | (19.8 ± 1.3) | (7.7 ± 0.6)[a] | (7.8 ± 1.0)[a] |
| Cholesterol | | | |
| TRL mmol/l | 0.1 ± 0.1 | 2.4 ± 0.3[a] | 1.3 ± 0.2[a,b] |
| (%) | (1.3 ± 0.3) | (28.6 ± 3.8)[a] | (21.6 ± 2.1)[a] |
| ISL mmol/l | 2.9 ± 0.2 | 5.0 ± 0.7[a] | 3.3 ± 0.3[b] |
| (%) | (65.4 ± 2.5) | (59.2 ± 4.5) | (56.3 ± 2.1)[a] |
| HDL mmol/l | 1.4 ± 0.1 | 1.0 ± 0.1[a] | 1.1 ± 0.1[a] |
| (%) | (33.3 ± 2.6) | (12.2 ± 1.0)[a] | (22.1 ± 2.3)[a,b] |

Values are mean ± SE.
[a]Significantly different ($P < 0.05$) from normolipidemics.
[b]Significantly different ($P < 0.05$), type III versus type IV.

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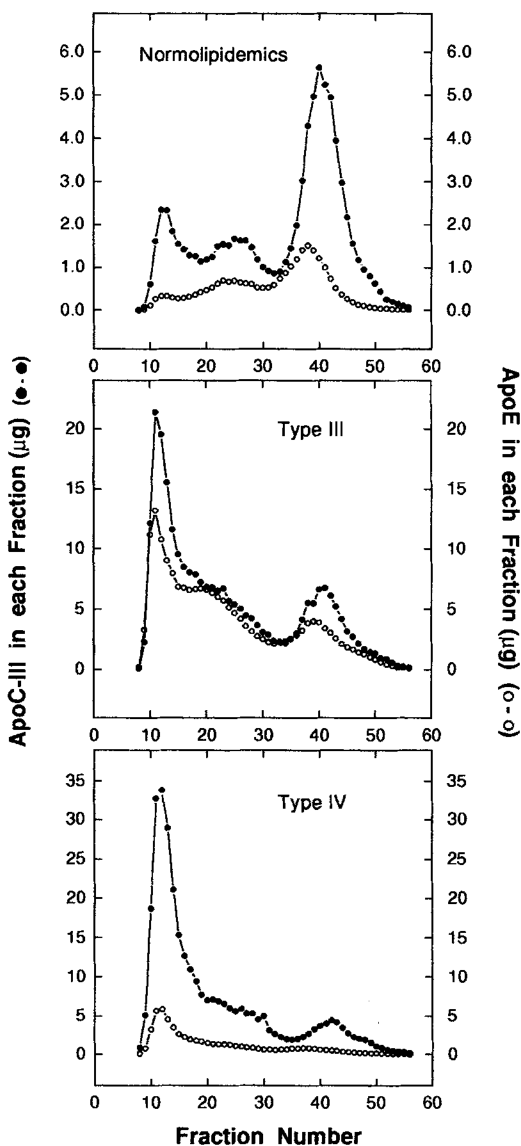


**Fig. 4.** Comparison between the plasma lipoprotein distribution of apoC-III and apoE in normolipidemic (top panel), type III (middle panel), and type IV subjects (bottom panel). Data points represent mean values for 8 subjects in each group. The scale of the y-axis is the same for both apolipoproteins but is different for each patient group.

compared to ISL1 ($P < 0.05$), and the ratio of ISL1 was in turn significantly lower than that of TRL ($P < 0.01$). TRL from normolipidemic patients contained on average 24 ± 3 molecules of apoC-III for every molecule of apoE. The apoC-III to apoE ratio of type IV TRL (24 ± 3 molecules of apoC-III for each molecule of apoE) was not significantly different from that of normolipidemic TRL, and no significant decrease in this ratio was evident in the ISL fractions of type IV subjects. The ratio of apoC-III to apoE was significantly lower in HDL1 compared to HDL2 for all three subject groups ($P < 0.05$).

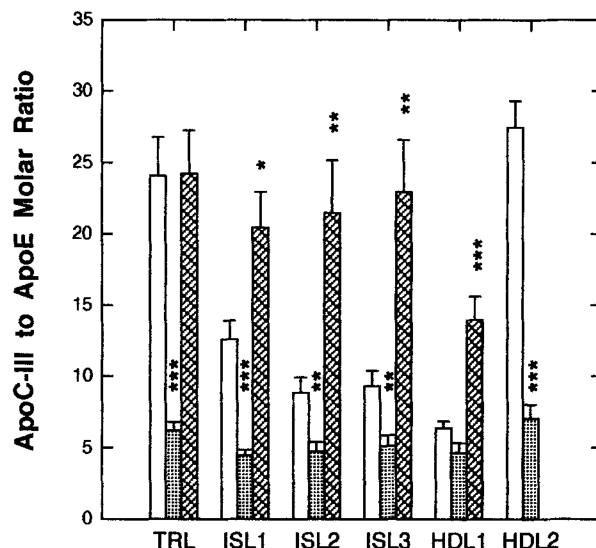


**Fig. 5.** ApoC-III to apoE molar ratio of different-sized lipoprotein fractions isolated from normolipidemic (empty bar), type III (dotted bar), and type IV subjects (hatched bar). The height of each bar represents the mean (± SE) for 8 subjects in each group. Data for each lipoprotein fraction from type III and type IV patients were compared to that of normolipidemics: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data for HDL2 in type IV patients were not included in the figure because they were out of scale (40 ± 5, $P < 0.01$ vs. normolipidemics).

## DISCUSSION

The measurement of apoC-III in plasma lipoproteins isolated by automated gel filtration chromatography (FPLC) has allowed us in the present study to compare the amount of apoC-III with that of apoE in different-sized lipoproteins of normolipidemic and hypertriglyceridemic subjects. The results have shown that the TRL fraction of normolipidemic subjects contained 24 ± 3 molecules of apoC-III for every molecule of apoE, whereas smaller remnant-like lipoproteins isolated in the ISL fraction had significantly less (10 ± 1 molecules). This is consistent with the concept that plasma remnant lipoproteins are enriched in apoE and depleted in apoC-III compared to their TRL precursors (35–37). However, this was not evident for the hypertriglyceridemic patients. In type III patients, all lipoprotein fractions contained increased amounts of apoE (Fig. 3), resulting in greatly reduced molar apoC-III to apoE ratios in both TRL and ISL (6 ± 1 and 5 ± 1, respectively). In contrast, type IV patients with an elevation in total and TRL triglyceride similar to the type III patients had an ISL molar ratio of 22 ± 3, which was not significantly different from the apoC-III to apoE ratio (24 ± 3) of their TRL, nor from the ratio of TRL from normolipidemic subjects. These results indicate that compared to normolipidemic individuals, remnant-like lipoproteins in the ISL fraction of type IV pa-



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tients are enriched in apoC-III relative to apoE, whereas those of type III patients are enriched in apoE relative to apoC-III.

The apoE enrichment of TRL and their remnants in type III patients has been documented by the early work of Havel et al. (38, 39) and is consistent with the well-accepted concept that incomplete conversion of apoE2-containing remnants to LDL (40) and abnormal binding of apoE2 to hepatic receptors (41) are primary (though not exclusive) reasons for the reduced plasma clearance (42) and subsequent plasma accumulation of remnant lipoproteins in type III hyperlipoproteinemia. The apoC-III enrichment of ISL relative to apoE in type IV patients in the present study was, however, more unexpected. Lipolysis of TRL is generally believed to be associated with a loss of C apolipoproteins (including apoC-III) to HDL (35). TRL particles thus become enriched with surface-active apoE, which is competent to bind to cell receptors (43), and which facilitates the plasma clearance of remnant lipoproteins. Type IV patients in the present study were not, however, found to have a significantly decreased ISL apoC-III to apoE ratio in comparison to TRL, and hence relative to normolipidemic subjects their ISL apoC-III, to apoE ratio was significantly increased (Fig. 5). At the same time, ISL triglyceride concentration was 3-fold higher in type IV patients compared to normolipidemics (Table 2). These results suggest that smaller remnant-like lipoproteins in type IV patients are enriched in apoC-III relative to apoE, which may be a cause or a consequence of triglyceride accumulation in this fraction. Recent evidence would support the theory that apoC-III is the cause of ISL triglyceride accumulation. Overexpression of human or mouse apoC-III in transgenic mice causes marked hypertriglyceridemia, which is proportional to the level of apoC-III expression (19, 20). Hypertriglyceridemia is due to the plasma accumulation of triglyceride-rich ($d < 1.006$ g/ml) lipoproteins, which are enriched in apoC-III and depleted in apoE. Data from two laboratories have shown that these lipoproteins are poorly recognized by cell receptors and their plasma clearance is thus greatly reduced (20, 23). This is consistent with the observation that C apolipoproteins can inhibit the apoE-dependent interaction of TRL with: *a)* the LDL receptor (44, 45), *b)* the LDL receptor-related-protein (LRP) (46), and *c)* lipoprotein lipase–heparin sulfate proteoglycan complexes (47). TRL from apoC-III transgenic mice were not found to be deficient in their ability to act as substrate for lipoprotein lipase in vitro. This absence of lipoprotein lipase inhibition by apoC-III, despite earlier reports to the contrary (11–15), is supported by a recent study showing that VLDL from different hyperlipidemic patient groups act as normal substrate for lipoprotein lipase, in spite of differences in apoC-III content (48). These findings together suggest that apoC-III not only plays an important role in determining the level of plasma triglyceride, but it also regulates the plasma clearance of TRL remnant lipoproteins, which in type IV individuals appear to represent triglyceride- and apoC-III-enriched lipoproteins, in contrast to the apoE- and cholesterol-enriched remnants typical of type III hyperlipoproteinemia. Interestingly, it has recently been shown that apoC-III enrichment of IDL and LDL is also a characteristic feature of dyslipoproteinemia in patients with chronic renal failure (49).

The plasma lipoprotein distribution of apoC-III, as assessed by gel filtration chromatography, was first reported by Alaupovic (50). Gibson et al. (51) subsequently quantitated apoC-III in different-sized lipoproteins of both normolipidemic subjects and patients with homozygous familial hypercholesterolemia. Normolipidemic control subjects with a total plasma apoC-III concentration of 14.6 mg/dl had a majority of plasma apoC-III associated with HDL-sized lipoproteins, and 9.3%, 19.8%, and 69.5% of plasma apoC-III was found in TRL, ISL, and HDL fractions: 1.7, 3.1, and 9.8 mg/dl of apoC-III, respectively. Our data for normolipidemic subjects (Tables 1 and 2) are consistent with these results, providing validation of the current methodology. The data are also in agreement with earlier results obtained with ultracentrifugally isolated lipoproteins (4, 28), where 62% and 74% of plasma apoC-III was on average found associated with HDL in normolipidemic subjects. Ultracentrifugation caused less than 5% of total apoC-III to be isolated in the density $> 1.21$ g/ml fraction (4), compared to 20–40% for plasma apoE (39,52), indicating that apoC-III is less susceptible than apoE to lipoprotein dissociation during ultracentrifugal isolation.

ApoE- and apoC-III-containing lipoproteins in the ISL fraction have, in the present study, been referred to as remnant-like lipoproteins. This interpretation is based on our previous work involving the characterization of ISL apoE (27), which showed that: *a)* ISL apoE is associated with lipoproteins that co-elute with LDL, but have a size-distribution favoring lipoproteins intermediate in size between TRL and LDL; *b)* ISL apoE has slow pre-β or β migration on agarose gel electrophoresis, characteristic of remnant lipoproteins (24, 37); and *c)* type III hyperlipoproteinemics, with greatly increased levels of circulating remnants, have the highest levels of ISL apoE, when compared with other hyperlipidemic patient groups. Agarose and non-denaturing polyacrylamide gel electrophoretic analysis of ISL apoC-III (data not shown) has also demonstrated that ISL apoC-III is associated with a remnant-like population of lipoproteins that co-elutes with LDL. This is consistent with the



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work of Rubinstein et al. (53), where lipoprotein lipase- or hepatic lipase-deficient patients were infused with heparin and ISL and apoE and apoC-III were found to behave in a fashion consistent with their being associated with TRL remnants. Studies that have used ultracentrifugation to isolate LDL have given rise to the concept that apoE- and apoC-III-containing lipoproteins in this density range are of minor importance (33). In contrast, experiments involving immunoaffinity techniques have shown that these lipoproteins are of quantitative significance (54, 55), and the present results demonstrate that 20–35% of plasma apoC-III and 25–40% of plasma apoE can be found in the ISL fraction of normolipidemic and hypertriglyceridemic subjects. These apolipoproteins presumably reside on lipoproteins intermediate in their conversion from TRL to LDL and are metabolically distinct from the majority of apoB-only-containing lipoproteins in the ISL fraction. Whether apoC-III and apoE are both present on the same lipoprotein particles in this fraction or whether they are present on separate lipoproteins remains to be determined.

A number of recent studies have drawn attention to the role of apoC-III in the pathogenesis of atherosclerosis and to the possibility that the plasma lipoprotein distribution of apoC-III could be used to better predict patients at increased risk of CAD. In the Cholesterol Lowering Atherosclerosis Study (CLAS), the amount of apoC-III in HDL was negatively correlated with the progression of CAD in colestipol plus niacin-treated men with previous coronary bypass surgery (16). In the Monitored Atherosclerosis Regression Study (MARS), the amount of apoC-III associated with VLDL and LDL was a statistically significant predictor of CAD progression in patients treated with lovastatin (17). The plasma lipoprotein distribution of apoC-III has also been independently related to the severity of CAD in normotensive, non-diabetic subject (18), and to the presence of CAD in subjects from France and Northern Ireland participating in the ECTIM study (56). In all these studies, apoC-III was measured in two major classes of plasma lipoprotein, i.e., apoB- and non-apoB-containing lipoproteins. ApoB:apoC-III-containing lipoproteins can, however, be divided on the basis of size into different subclasses (e.g., TRL and ISL), and it is likely that this size heterogeneity affects the potential atherogenicity of these lipoproteins. Very large TRL containing apoC-III (diameter > 75 nm) are probably restricted by the vascular endothelial cell layer from entering the intimal layer of the arterial wall, and are therefore less atherogenic. Smaller apoB:apoC-III-containing lipoproteins may, however, penetrate the endothelial layer more readily. The degree to which these lipoproteins contribute to atherosclerotic lesion formation is then dependent on the extent to which they are trapped beneath the endothelium (57). Whether apoC-III and/or apoE are important in determining the selective retention of lipoproteins within the intimal layer remains to be determined.

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