

# Effects of estrogenic oral contraceptives on the lipoprotein B particle system defined by apolipoproteins E and C-III content

Christina Khoo,\* Hannia Campos,\* Helena Judge,\* and Frank M. Sacks<sup>1,\*†</sup>

Department of Nutrition,\* Harvard School of Public Health, Boston, MA 02115, and Departments of Medicine,<sup>†</sup> Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

**Abstract** Apolipoproteins E and C-III are modulators of lipoprotein metabolism that could affect development of atherosclerosis. The prevalence in plasma of apoB-containing particles (LpB) that contain either apoE or apoC-III, both or neither, and the effect of estrogen on these lipoproteins are unknown. The LpB particle system, defined by the presence or absence of apoE or C-III, was studied in 13 normolipidemic women, 7 nonusers and 6 users of oral contraceptives. Fasting plasma was separated by anti-apoE and C-III affinity chromatography and ultracentrifugation into four types of VLDL, IDL, and LDL particles: with apoE but not apoC-III (E+C-), apoC-III but not apoE (E-C+), both (E+C+) or neither (E-C-). The predominant VLDL particles were E-C- (42% in nonusers, 56% in users) and E+C+ (39% in nonusers, 24% in users), suggesting that apoE and apoC-III mainly exist together in VLDL. In IDL, E-C- was the major fraction (74% nonusers, 81% users), and in LDL, it was 99% in both groups. The triglycerides in VLDL and IDL were mainly contained in C+ particles (79% and 66% of the total VLDL and IDL triglycerides, respectively). Within VLDL, IDL, and LDL, E-C- particles had the smallest size and E+C+ or E-C+ the largest. Users had higher concentrations of VLDL E-C- (280%) and IDL E-C- (90%) particles than nonusers. They also had higher free cholesterol and cholesteryl ester concentrations associated with these fractions and with VLDL E-C+. The triglyceride contents of VLDL E-C- particles were lower in users of oral contraceptives than in nonusers. This study demonstrates that the elevated VLDL TG concentrations in users of estrogen-dominant oral contraceptives is mainly caused by an increased concentration of small VLDL particles that have reduced TG content, and that do not have apoE and C-III. These particles may have lower atherogenicity than particles enriched with apoE and C-III.—Khoo, C., H. Campos, H. Judge, and F. M. Sacks. **Effects of estrogenic oral contraceptives on the lipoprotein B particle system defined by apolipoproteins E and C-III content.** *J. Lipid Res.* 1999. 40: 202–212.

**Supplementary key words** apoB • apoE • apoC-III • estrogen • oral contraceptives • atherosclerosis • lipoproteins • triglyceride • cholesterol

Very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL) particles are heterogeneous in size and content of triglyceride, cholesterol, and in particular, of specific apolipoproteins. Two major apolipoproteins that are contained in VLDL and IDL, apoC-III and E, play antagonistic roles in the metabolism of VLDL and IDL. ApoC-III inhibits lipoprotein lipase and opposes the apoE-mediated hepatic uptake by cells in culture (1, 2). Transgenic mice, which overexpress the human apoC-III gene, develop hypertriglyceridemia and have a greatly reduced clearance rate of the apoC-III-enriched VLDL particles (3, 4). Hypertriglyceridemic patients have higher synthetic rates of apoC-III and higher concentrations of apoC-III-enriched VLDL (5). Persons deficient in apoC-III and A-I have rapid conversion of VLDL to IDL and low density lipoprotein (LDL) and reduced VLDL triglyceride concentration (6). Responsibility of the apoC-III rather than the apoA-I or high density lipoprotein (HDL) for the low triglycerides in those patients is suggested by the hypotriglyceridemia produced in mice by deleting the apoC-III gene (7). In contrast, apoE acts as a ligand for the LDL receptor and LDL receptor-related protein to mediate clearance of the VLDL, IDL, and LDL particles (8–10). ApoE deficiency in humans and in mice with gene deletion severely impairs VLDL clearance (11–13) and produces hyperlipidemia (11).

The plasma concentration of apoC-III has been implicated as a risk factor for coronary heart disease (14, 15). The ratio of apoC-III in (VLDL + LDL)/HDL correlated with worsening coronary atherosclerosis, suggesting that apoC-III-containing VLDL is atherogenic in humans (16,

Abbreviations: TG, triglyceride; apo, apolipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein; hyperTG, hypertriglyceridemia; OC, oral contraceptives; CHD, coronary heart disease; CVD, cardiovascular disease; NaSCN, sodium thiocyanate; LpB, apoB-containing lipoprotein.

<sup>1</sup>To whom correspondence should be addressed.

17). Counterintuitively, in view of the properties of apoE in mediating clearance of lipoproteins by the liver, total plasma and VLDL apoE are also associated with coronary heart disease (15, 18–20). In one study, coronary patients had significantly higher mean plasma concentration of apoB-containing lipoprotein particles with apoE (LpB:E) compared to controls, but there was no significant difference in the concentration of apoC-III-containing particles (LpB:C-III) between the two groups (19). An inverse association between apoE concentration and cardiovascular disease is expected as apoE promotes clearance of triglyceride-rich lipoprotein particles and apoE gene deletion in mice promotes atherosclerosis (12). Interpretation of these epidemiological findings (14–20) is complicated by the presence of both apoE and apoC-III on VLDL and HDL particles. Protein–protein interactions between apoE and C-III may regulate the binding of apoE to receptors by decreasing the accessibility of apoE (8). It is also possible that the presence of apoE on a VLDL or IDL particle is a marker for particles that also have apoC-III, and that apoE does not have a direct atherogenic action. Alternatively, apoE on a VLDL particle could directly influence atherosclerosis by causing macrophage foam cell formation (21, 22).

A shortcoming of previous studies (14–20) is that VLDL or IDL particles that contained apoE but not apoC-III, or vice versa, were not isolated separately. Thus, it is not known whether VLDL or IDL particles that contain apoE are distinct from those that contain apoC-III, or whether apoE and C-III mainly coexist on a VLDL or IDL particle as markers for each other. Previous findings (14–20) suggest the need for methods of assessing the presence of remnant lipoproteins in plasma, specifically those enriched in apoE and/or apoC-III. To evaluate the potential for atherogenesis of apoE and C-III in apoB-containing particles, one needs to know the extent of coexistence of apoE and C-III in VLDL and IDL.

The primary purpose of the present study is to examine the enrichment and distribution of apoE and apoC-III in apoB-containing lipoprotein particles using a new affinity chromatography method with anti-apoE and anti-apoC-III immunoaffinity resins in series. VLDL, IDL, and LDL particles were isolated that were enriched in apoE but poor in apoC-III (E+C–), rich in apoC-III but poor in apoE (E–C+), rich in both apoE and apoC-III (E+C+), or neither (E–C–). We chose healthy young women for our first investigation of this topic to characterize the system in normal subjects. In addition, women who were using oral contraceptives, which have well-known hypertriglyceridemic effects due to the estrogen component, were compared to the nonusers to provide insight about estrogenic hypertriglyceridemia. This could have clinical implications as certain particles in VLDL and IDL, those with apoE or C-III, have been linked to cardiovascular disease. We hypothesized that these would not be the particles increased by use of estrogen-rich oral contraceptives as women who use them do not have a higher risk of cardiovascular disease, with the exception of heavy smokers.

## METHODS

### Subjects

Fourteen healthy premenopausal women, ages 23–36 years, were recruited. Inclusion criteria were cholesterol and triglyceride less than the 95th percentile in oral contraceptive nonusers and users (23), nonsmoking, nondiabetic, not taking medications that affect lipids, and ethanol consumption less than 15 g/day. One subject taking oral contraceptives was studied but excluded from the main analysis because her fasting triglyceride was found to be 209 mg/dl (more than the 95th percentile for oral contraceptive users) (23). Six subjects had been taking estrogen-dominant oral contraceptives (Ortho Novum 1-35, Ortho Novum 777, Desogen, or Modicon) containing 0.035 mg ethinyl estradiol for at least 10 months up to 9 years. The other 7 subjects were nonusers for at least 1 year prior to the study. The two groups were closely matched in terms of age (nonusers  $30 \pm 5$  yrs; users  $29 \pm 4$  yrs), and body mass index (BMI, kg/m<sup>2</sup>) (mean BMI =  $23 \pm 1$  for nonusers;  $24 \pm 2$  for users). Subjects gave informed consent, and the study was approved by the Human Subjects Committee, Harvard School of Public Health.

### Immunoaffinity resin preparation

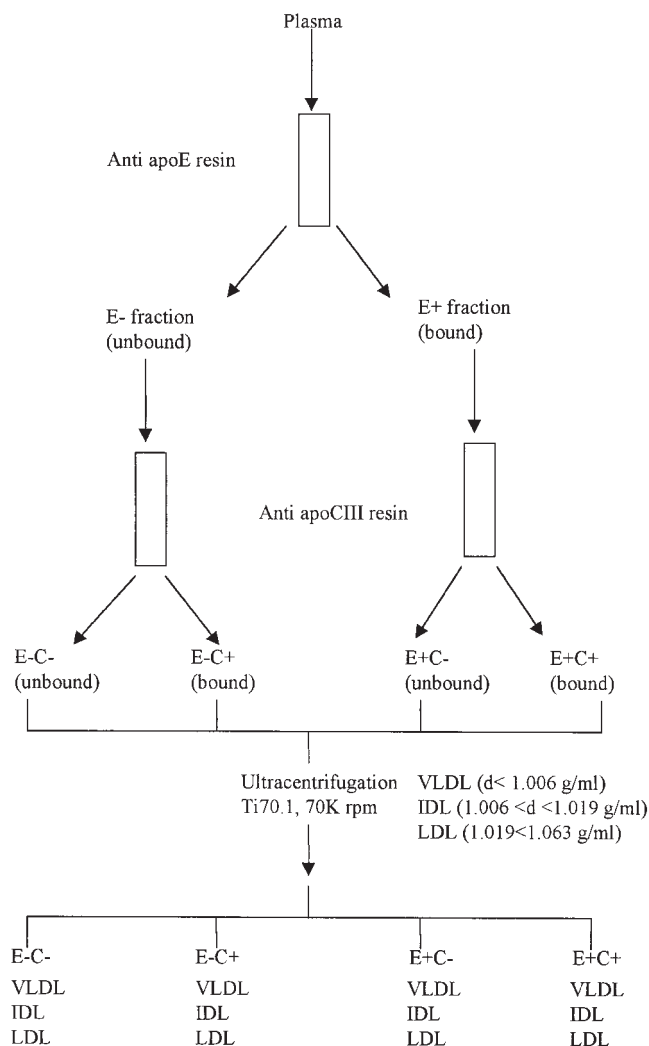
Human apoC-III (10 mg), (Perimmune, Inc., Rockville, MD) was coupled to 3 ml Affigel (Bio-Rad, Hercules, CA). Goat anti-human apoC-III serum (8 ml) (DMA, Inc., Arlington, TX) was loaded onto the column with PBS as the loading buffer, and incubated overnight. The column was washed with PBS until the loading peak reached baseline. The antibody was eluted with 3 M NaSCN and immediately desalted with a Sephadex G25 column. The affinity-purified antibody was coupled to cyanogen-bromide-activated Sephacryl S1000 resin at a concentration of 5 mg antibody/ml resin. Affinity-purified polyclonal anti-apoE was kindly provided by Genzyme Corp. (Cambridge, MA) and coupled at a concentration of 5 mg antibody/ml resin. Plasma from normal donors was used to test the resins.

### Validation of immunochemical procedures

The bound and unbound fractions from the resins were analyzed for binding efficiency by testing various amounts of resins and plasma in varying ratios based on apoC-III and apoE concentrations. Unbound lipoproteins were incubated back on the resin to test for completeness of extraction of apoE- and C-III-containing lipoproteins. No additional binding occurred. Bound and unbound fractions were ultracentrifuged to obtain the lipoprotein fractions (VLDL, IDL, and LDL). Each fraction was analyzed with SDS-PAGE and immunoblot analysis. Silver staining of duplicate gels was carried out to determine the presence of protein. The gels were transferred to nitrocellulose membranes followed by immunoblotting with apoE (1:1000) and apoC-III (1:3000) polyclonal antibodies to confirm that there was no apoE or apoC-III in the unbound fractions. The immunoblots were able to detect protein levels of about 25–50 ng. In addition, apoC-III (turbidimetric assay) and apoE (ELISA) measurements (detection limit 0.1 mg/dl) were also carried out on bound and unbound fractions to further confirm completeness of binding. As preliminary studies in this laboratory showed that apoB-48 comprised only 5.5% of VLDL and IDL apoB in the fasting state for both oral contraceptive nonusers and users, the hepatic and intestinal lipoproteins were not differentiated in this study.

### Immunoaffinity separation

A flow chart of the method is shown in Fig. 1. Blood was drawn from the subjects after a 12-h fast and immediately centrifuged at 4°C to isolate plasma. A mixture of protease inhibitors (2  $\mu$ M benzamidine, 0.01 mg/ml aprotinin, 17.5  $\mu$ g/ml PMSF)



**Fig. 1.** A flow diagram of the methodology used in this study as described in Methods. Briefly, 3 ml plasma was incubated with anti-apoE resin to yield an E- fraction (unbound) and an E+ fraction (bound). After desalting of the bound fractions, each fraction was individually incubated with the anti-apoC-III resin to yield four different immunofractions; E-C-, E-C+, E+C-, and E+C+. These fractions were individually separated into three lipoprotein density fractions by ultracentrifugation to obtain VLDL ( $d < 1.006$  g/ml), IDL ( $1.006 < d < 1.019$  g/ml), and LDL ( $1.019 < d < 1.063$  g/ml).

and 0.05 mg/ml gentamicin was added to the plasma and the samples were stored at 4°C. The samples were analyzed within 3 days after collection. Three ml plasma from each subject was incubated with 1.0 ml anti-apoE resin in a  $1 \times 10$ -in disposable Econopac column (Bio-Rad, Hercules, CA). All incubations and rinses were carried out in these columns. Samples were incubated for 1 h at room temperature with constant mixing by rotating all the columns on the Rotatorque™ rotator (Cole Palmer, Vernon Hills, IL). The nonbinding fractions (E-) were collected by gravity flow from the Econopac columns and the resin was washed with  $3 \times 1$  ml PBS, each time allowing the washes to flow by gravity through the resin bed. These washes were added to the nonbinding fractions as E- fractions. The resin was further rinsed with 1 ml PBS, and then left to incubate with 0.75 ml 3 m NaSCN at room temperature in the same columns for 3 min with constant rotation. The bound fraction was collected into a tube containing 0.5 ml PBS and designated E+.

The resin was again incubated with 0.5 ml 3 m NaSCN for 1.5 min. This fraction and the final wash with 0.5 ml PBS were added to the E+ fraction. The total E+ fraction was immediately dialyzed by exchange in microconcentrators (Amicon, Beverly, MA) using PBS. The ultrafiltrate from E+ fractions concentrated with 100 kDa cutoff Ultrafree™ concentrators (Millipore, Bedford, MA) was analyzed by ELISA. Free apoE was not detected in the ultrafiltrates showing that apoE was not lost from the particles after elution with NaSCN.

The E- fractions and the dialyzed E+ fractions were further incubated with 1.0 ml anti-apoC-III resin for 4 h at 4°C. The same elution protocol used for the anti-apoE resin was carried out to yield four immunofractions, E-C-, E-C+, E+C-, E+C+, which were dialyzed. The recoveries for C+ fractions were similar whether the anti-E or anti-C-III resin was used first in the protocol, demonstrating that the elution protocol for the anti-apoE resin did not cause loss of apoC-III from particles.

The four immunofractions (E-C-, E-C+, E+C-, E+C+) isolated from plasma were ultracentrifuged separately using a Ti 70.1 rotor at 70,000 rpm in an L8-70M instrument (Beckman Instruments, Palo Alto, CA) (24). VLDL (density  $< 1.006$  g/ml) was prepared by overlaying 4 ml of the fractions with 2 ml PBS and ultracentrifugation for 2.5 h. The infranatant density was raised to 1.019 g/ml, overlaid with 1 ml KBr ( $d < 1.019$  g/ml) before ultracentrifugation for 3 h to collect IDL ( $d < 1.019$  g/ml). LDL ( $d < 1.063$  g/ml) was prepared in the same way except that ultracentrifugation was carried out for 4 h. All samples were collected in a total volume of 0.75 ml. IDL and LDL fractions were dialyzed against PBS for further analysis.

Recovery after separation by immunoaffinity chromatography and ultracentrifugation, expressed as % of total plasma apoB or cholesterol concentrations, was 70% for both users and nonusers and 65% for VLDL, IDL, and LDL. Equivalent losses were found for all the immunofractions. The lipoprotein-poor fraction ( $d > 1.21$  g/ml) contained 25% of the total apoE, but only 3% of the apoC-III. Preliminary tests with this methodology using plasma from normal donors showed that distributions of apoE in total control plasma samples were 9%, 2%, and 5% in VLDL, IDL, and LDL, respectively, very similar to the distributions of the sum of the apoE in the immunofractions separated with the resins which were 11%, 4%, and 9%, respectively. The distributions for apoC-III for total plasma samples were 11%, 4%, and 12%, compared to 14%, 3%, and 14% in VLDL, IDL, and LDL, respectively. The quality of the separation was confirmed by the lack of apoA-I in the VLDL and IDL fractions and less than 1% of the total plasma apoA-I in the LDL fraction (detection limit 0.5 mg/dl). No detectable apoA-II was found in the VLDL, IDL, or LDL fractions. More than 99% of all apoA-I and apoA-II were in the HDL fraction ( $d > 1.063$  g/ml).

Cholesterol, triglyceride (Roche Diagnostics Systems, Inc. NJ), and free cholesterol concentrations (Wako Chemicals USA, Inc., Richmond, VA) were determined by enzymatic methods using the COBAS MIRA Plus analyzer. Cholesteryl ester concentration was calculated by subtracting free cholesterol from total cholesterol and expressed in molar concentrations. ApoB (Alerchek, Inc., Portland, ME), and apoE (PerImmune, Inc., Rockville, MD) were measured with ELISA (detection limit 0.1 mg/dl). ApoC-III was measured by turbidimetric immunoassay (detection limit 0.1 mg/dl) (Wako Chemicals USA, Inc., Richmond, VA) using the COBAS MIRA Plus analyzer. Intra-assay coefficients of variation (CV) for cholesterol, triglyceride, free cholesterol, apoB, apoC-III, and apoE were 2%, 3%, 5%, 4%, 5%, and 5%, and inter-assay CVs were 4%, 9%, 10%, 21, 10%, and 7%, respectively, for both nonusers and users.

### Electron microscopy

VLDL, IDL, and LDL immunofractions were prepared from a pool of plasma from 7 normolipidemic subjects, 4 female and 3



TABLE 1. Plasma lipid and apolipoprotein content of oral contraceptive (OC) users and nonusers

	Nonusers (n = 7)	OC Users (n = 6)	P Value
<b>Lipid</b>			
Triglyceride (mg/dl)	71 ± 13 (808 ± 149 μmol/L)	119 ± 40 (1360 ± 458 μmol/L) <sup>a</sup>	0.01
Total cholesterol (mg/dl)	139 ± 16 (3580 ± 421 μmol/L)	165 ± 29 (4250 ± 743 μmol/L)	0.07
Free cholesterol (mg/dl)	51 ± 6 (1320 ± 144 μmol/L)	59 ± 7 (1530 ± 173 μmol/L) <sup>a</sup>	0.03
Cholesteryl ester (mg/dl)	88 ± 11 (2260 ± 290 μmol/L)	106 ± 23 (2720 ± 579 μmol/L)	0.09
LDL cholesterol (mg/dl)	71 ± 9 (1820 ± 240 μmol/L)	86 ± 29 (2200 ± 757 μmol/L)	0.2
HDL cholesterol (mg/dl)	54 ± 16 (1400 ± 402 μmol/L)	58 ± 10 (1490 ± 246 μmol/L)	0.6
<b>Apolipoproteins</b>			
ApoB (mg/dl)	68 ± 7 (1220 ± 126 nmol/L)	87 ± 17 (1570 ± 306 nmol/L) <sup>a</sup>	0.02
ApoE (mg/dl)	14 ± 3 (4100 ± 880 nmol/L)	17 ± 6 (5160 ± 1760 nmol/L)	0.3
ApoC-III (mg/dl)	7.4 ± 1.7 (8140 ± 1870 nmol/L)	11 ± 2 (12100 ± 2200 nmol/L) <sup>a</sup>	0.002

Values are given as mean ± SD.

<sup>a</sup>*P* ≤ 0.05.

male, ages 23 to 38 years. They were analyzed for particle sizes by transmission electron microscopy, Model JEOL 1200EX (JEOL, Inc., Tokyo, Japan), at the Electron Microscopy Core (Harvard Medical School, Boston, MA) with a magnification of 30000×. Grids were drawn for the electron micrographs to determine particle sizes and 5 particles were measured per 1.5 × 1.5 in square for a total of 60 particles. Two independent observers who were blinded to the identity of lipoprotein fraction shown on the micrographs carried out size measurements.

### Statistical analysis

The lipoprotein measurements in oral contraceptive users were compared to the nonusers by the *t* test for two independent samples with two-tailed *P* values. Associations among the lipoprotein particle types were tested by Pearson's correlation analysis.

## RESULTS

The premenopausal women in the study were normolipidemic. Plasma total triglycerides, free cholesterol, apoB, and apoC-III were significantly higher in the users compared to the nonusers of oral contraceptives (Table 1). There were trends towards increases in total plasma cholesterol and cholesteryl ester in the oral contraceptive users as well.

The predominant type of VLDL particle in these healthy, premenopausal women was unretained by either the anti-apoE or anti-apoC-III resin, denoted E-C-; it comprised 42% of total VLDL particles in nonusers and 56% in users (Fig. 2, Table 2), based on the assumption that each VLDL particle has one molecule of apoB. Oral contraceptive users had significantly higher concentrations of VLDL E-C- particles than nonusers (*P* = 0.007). The second most prevalent VLDL particle in these women was retained by both anti-apoE and anti-apoC-III resins, denoted E+C+; it comprised 39% of the total VLDL particles in nonusers and 24% in users (*P* = 0.2 between the groups). VLDL that had either apoE or apoC-III (but not both) were minor fractions, comprising 6% of the total VLDL in both groups for E+C-, and 12–13% for E-C+. The concentrations of these types of VLDL particles were significantly higher in oral contraceptive users than nonusers (E+C-, *P* = 0.05; E-C+, *P* = 0.02).

IDL E-C- particles represented 74% of all IDL particles in nonusers and 81% in users (*P* = ns) (Fig. 2, Table

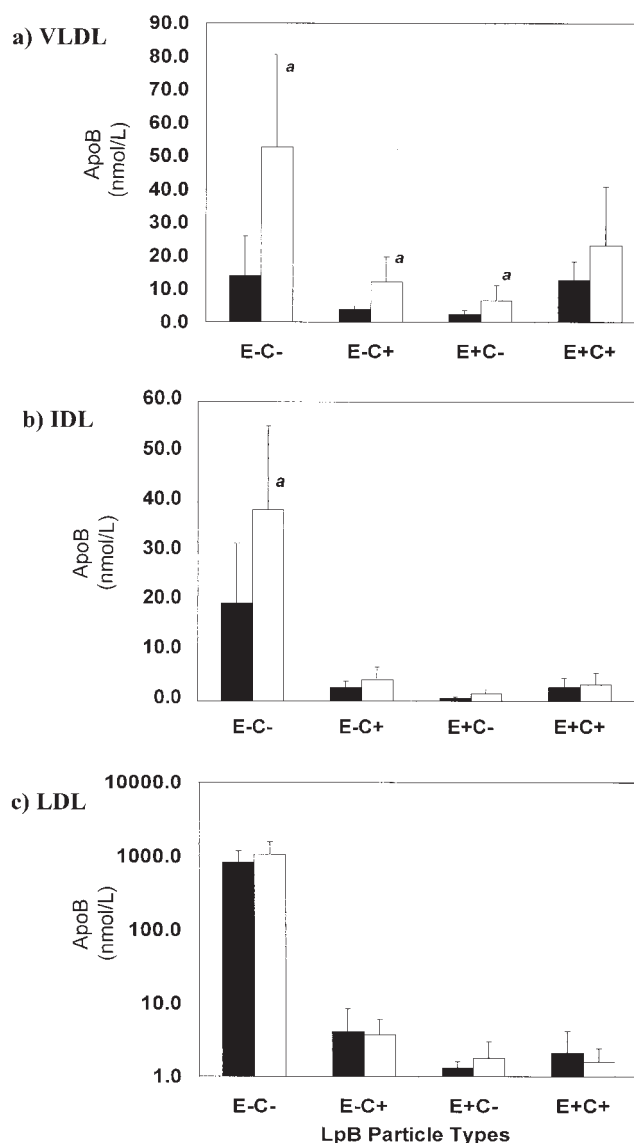


Fig. 2. Concentration of apoB in lipoprotein subfractions in normolipidemic women. Plasma from nonusers (■) and users (□) of oral contraceptives was separated by anti-apoE and apoC-III immunoaffinity resins and then ultracentrifugation to yield a) VLDL, b) IDL, and c) LDL particles enriched with apoE and C-III (E+C+), with apoC-III (E-C+), with apoE (E+C-) or neither (E-C-). ApoB concentration for each fraction was measured by ELISA. <sup>a</sup>denotes *P* < 0.05. To convert nmol/L to mg/dl, divide by 18.

TABLE 2. Lipid and apoprotein distribution in VLDL, IDL, and LDL subfractions

Concentrations	VLDL		IDL		LDL	
	Nonusers (n = 7)	Users (n = 6)	Nonusers (n = 7)	Users (n = 6)	Nonusers (n = 7)	Users (n = 6)
<b>E-C-</b>						
Triglyceride <sup>x</sup>	44.7 ± 19.5	124.9 ± 105.1 <sup>a</sup>	48.9 ± 49.6	78.2 ± 53.6	85.8 ± 20.0	163.5 ± 68.5 <sup>c</sup>
Cholesteryl ester	20.0 ± 20.2	74.5 ± 50.0 <sup>b</sup>	21.2 ± 10.2	50.7 ± 25.0 <sup>c</sup>	1790 ± 351	1960 ± 506
Free cholesterol	22.5 ± 15.0	64.1 ± 41.8 <sup>b</sup>	16.5 ± 6.6	33.8 ± 12.8 <sup>c</sup>	629 ± 130	698 ± 156
ApoB <sup>y</sup>	14.0 ± 12.0	52.6 ± 28.0 <sup>c</sup>	19.6 ± 12.1	38.4 ± 16.8 <sup>b</sup>	832 ± 345	1070 ± 527
<b>E+C-</b>						
Triglyceride	16.2 ± 11.8	28.6 ± 21.1	5.6 ± 5.3	7.7 ± 7.8	2.5 ± 3.1	2.6 ± 3.6
Cholesteryl ester	0.4 ± 0.8	7.6 ± 9.2 <sup>a</sup>	nd	0.3 ± 0.8	1.5 ± 1.2	3.0 ± 3.5
Free cholesterol	3.3 ± 2.2	9.0 ± 7.2 <sup>a</sup>	0.4 ± 0.6	1.3 ± 1.2	2.6 ± 1.2	3.6 ± 2.4
ApoB	2.5 ± 1.1	6.5 ± 4.7 <sup>b</sup>	0.6 ± 0.3	1.5 ± 0.8	1.3 ± 0.3	1.8 ± 1.1
ApoE	29.9 ± 25.2	35.5 ± 37.5	13.3 ± 10.5	15.3 ± 7.6	17.3 ± 10.9	11.0 ± 4.2
<b>E+C+</b>						
Triglyceride	125.1 ± 76.8	155.7 ± 104.3	27.7 ± 11.8	41.0 ± 34.2	27.7 ± 18.8	20.0 ± 18.8
Cholesteryl ester	17.9 ± 8.4	32.1 ± 27.2	0.7 ± 1.4	1.7 ± 2.0	3.7 ± 3.6	3.4 ± 3.6
Free cholesterol	24.2 ± 10.6	33.0 ± 21.4	2.6 ± 1.4	2.0 ± 1.6	4.6 ± 2.4	4.9 ± 2.2
ApoB	12.6 ± 5.7	23.1 ± 17.7	2.8 ± 1.8	3.3 ± 2.4	2.1 ± 2.1	1.6 ± 0.8
ApoE	204 ± 110	179 ± 109	15.2 ± 9.3	22.9 ± 22.3	22.3 ± 10.2	24.7 ± 11.1
ApoC-III	238 ± 170	432 ± 351	nd	nd	nd	nd
<b>E-C+</b>						
Triglyceride	108.7 ± 46.4	144.2 ± 50.6	51.5 ± 34.1	62.0 ± 27.9	30.8 ± 12.1	38.5 ± 17.5
Cholesteryl ester	4.4 ± 4.2	18.0 ± 11.2 <sup>c</sup>	0.9 ± 1.2	3.3 ± 2.8 <sup>a</sup>	8.1 ± 6.3	8.6 ± 7.0
Free cholesterol	10.3 ± 4.0	21.3 ± 12.6 <sup>b</sup>	3.1 ± 1.4	4.5 ± 1.8	6.2 ± 2.0	7.2 ± 3.0
ApoB	3.8 ± 1.1	12.1 ± 7.6 <sup>b</sup>	2.8 ± 1.2	4.3 ± 2.5	4.1 ± 4.3	3.7 ± 2.3
ApoC-III	108.5 ± 107.6	503.9 ± 339.0 <sup>b</sup>	18.4 ± 13.7	23.6 ± 24.8	nd	nd

Fasting plasma was separated by anti-apoE and anti-apoC-III affinity resin to obtain the four immunofractions: particles with apoE and C-III (E+C+), particles without either (E-C-), and particles with either apoE alone (E+C-) or apoC-III alone (E-C+). Ultracentrifugation was carried out to obtain VLDL, IDL, and LDL. To convert values for TG and cholesterol from  $\mu\text{m}$  to mg/dl, divide by 11.4 and 25.8, respectively. To convert values for apoB, apoE, and apoC-III from nm to mg/dl, divide by 18, 293, and 1130, respectively.

Between nonusers and users. <sup>a</sup>0.05 >  $P \leq 0.1$ ; <sup>b</sup> $P < 0.05$ ; <sup>c</sup> $P < 0.01$ ; nd, not detectable.

<sup>x</sup>Values for lipids are in  $\mu\text{m}$ .

<sup>y</sup>Values for apolipoproteins are in nm.

2). IDL E-C+ and E+C+ particles each comprised approximately 11% of total IDL particles. The level of IDL E+C- particles was very low, 2-3% of total IDL. Oral contraceptive users had nearly double the concentration of IDL E-C- particles of the nonusers ( $P = 0.04$ ), while the concentrations of the other IDL particle types were not significantly different among the groups. LDL E-C- particles contributed to 99% of all LDL particles in both nonusers and users. There were no significant differences between the nonusers and users in the abundance of any of the types of LDL particles. The LDL E-C- concentration was 1.0 versus 0.9  $\mu\text{mol/L}$  in the users compared to the nonusers ( $P = \text{ns}$ ). The combination of significantly higher VLDL E-C- and IDL E-C-, and nonsignificantly higher LDL E-C- concentrations accounted for the higher plasma total concentration of apoB in contraceptive users, 87 mg/dl versus 68 mg/dl in nonusers ( $P = 0.02$ ) (Table 1). The contraceptive user with high plasma triglycerides who was excluded from the study had the same pattern of immunofractions as the other users, and including her with the other OC users produced greater mean differences between the user and nonuser groups.

The majority of the triglycerides in the VLDL particles was found associated with the C+ fractions, evenly distributed between the E-C+ and E+C+ fractions (Table 2). Oral contraceptive users had higher plasma VLDL triglyceride

concentrations for all particle types, although most strikingly for VLDL E-C- particles (125  $\mu\text{m}$  for users versus 45  $\mu\text{m}$  for nonusers [ $P = 0.07$ ]). In users, 28% of the VLDL triglyceride was associated with VLDL E-C- particles compared to 15% in nonusers. However, as the difference in concentration of VLDL E-C- particles (apoB) between users and nonusers was greater than the difference in concentration of triglyceride (4-fold for mean E-C- particle concentration versus 3-fold for mean E-C- triglyceride concentration) (Table 2), the content of TG per particle was significantly lower in users ( $P = 0.04$ ) (Table 3). Trends toward lower triglyceride content in the users compared to nonusers were present for the other VLDL fractions but they were not statistically significant (Table 3). The VLDL E-C+ particle was the most TG-rich in content followed in order by E+C+, E+C-, and E-C-. The E-C+ particle had 5- to 9-fold more TG than the E-C- particle.

The pattern of triglyceride distribution among the IDL immunofractions was similar to that of the VLDL fractions. The C+ fractions carried 59% of the IDL triglyceride (Table 2). The IDL E-C+ had the highest whereas IDL E-C- had the lowest triglyceride content (Table 3). There were no significant differences between contraceptive users and nonusers. Among the LDL immunofractions, the E-C- particles carried a majority of the LDL triglyceride (58% nonusers, 73% users), and the C+ particles carried most of the rest (Table 2). However, as the

TABLE 3. Lipid and apoprotein content of VLDL, IDL, and LDL

	VLDL		IDL		LDL	
	Nonusers (n = 7)	Users (n = 6)	Nonusers (n = 7)	Users (n = 6)	Nonusers (n = 7)	Users (n = 6)
E-C-						
TG/apoB	3190 ± 2880	2370 ± 1110 <sup>b</sup>	2500 ± 1060	2040 ± 1609	103 ± 75	153 ± 71
CE/apoB	1700 ± 2000	1350 ± 480	1250 ± 550	1309 ± 570	2510 ± 1280	2000 ± 550
FC/apoB	1880 ± 915	1190 ± 257	977 ± 294	955 ± 263	862 ± 376	715 ± 182
E+C-						
TG/apoB	6610 ± 6970	4413 ± 6164	9019 ± 12098	5136 ± 7550	1849 ± 2189	1440 ± 4160
CE/apoB	86.2 ± 175	738 ± 829 <sup>a</sup>	nd	111.3 ± 273	1073 ± 858	1250 ± 1530
FC/apoB	1320 ± 672	1380 ± 425	765 ± 707	740 ± 610	1960 ± 870	2190 ± 1350
ApoE/apoB	11.7 ± 9.3	5.3 ± 4.4	22.1 ± 16.1	13.6 ± 9.5	12.7 ± 6.2	9.2 ± 6.6
E+C+						
TG/apoB	9910 ± 6450	6760 ± 8520	9870 ± 6340	12600 ± 11120	12920 ± 10870	12560 ± 9660
CE/apoB	1670 ± 890	1260 ± 539	146 ± 289	372 ± 441	2019 ± 1267	1730 ± 1980
FC/apoB	2070 ± 776	1660 ± 401	1050 ± 343	690 ± 469	2860 ± 1120	3220 ± 600
ApoE/apoB	18.8 ± 12.3	10.4 ± 5.2	9.9 ± 12.2	7.6 ± 5.6	16.7 ± 10.3	18.4 ± 13.1
ApoC-III/apoB	18.8 ± 12.1	18.7 ± 4.6	nd	nd	nd	nd
ApoE/apoC-III	0.86	0.41	nd	nd	nd	nd
E-C+						
TG/apoB	28290 ± 15240	11900 ± 27170	18153 ± 12507	14460 ± 11975	7530 ± 8650	10520 ± 8960
CE/apoB	1190 ± 1050	1460 ± 956	321 ± 536	771 ± 745	2440 ± 1290	2510 ± 1480
FC/apoB	2630 ± 523	1860 ± 900	1200 ± 743	1150 ± 315	2620 ± 1690	2620 ± 1350
ApoC-III/apoB	25.1 ± 25.3	36.8 ± 19.5	6.5 ± 6.6	5.5 ± 5.8	nd	nd

Immunofractions separated with affinity resins, as described in Table 2, were analyzed for lipid and apoprotein content. Values are given as mean ± SD; nd, not detectable.

Between nonusers and users, <sup>a</sup>0.05 > P ≤ 0.1; <sup>b</sup>P < 0.05.

LDL E-C- particle comprised 99% of the total LDL particles (Fig. 2, Table 2), the triglyceride content per LDL particle was very low, 100–150 molecules per apoB, compared with approximately 10,000 molecules per apoB in the C+ LDL particles (Table 3). The plasma concentration of LDL E-C- triglyceride in oral contraceptive users versus nonusers was 164 versus 86 μm (P < 0.01) and the triglyceride content was 153 versus 103 molecules per apoB (P = ns). Overall, the higher plasma total triglyceride concentration of users, 119 mg/dl, compared to nonusers, 71 mg/dl (P = 0.01) (Table 1), was the result of higher VLDL and LDL E-C- triglyceride concentrations.

VLDL cholesteryl ester and free cholesterol were distributed primarily between the E-C- and the E+C+ particles (Table 2). Oral contraceptive users had substantially higher plasma VLDL cholesterol concentrations for all particle types compared to nonusers. This increase was significant for free cholesterol and cholesteryl ester in VLDL and IDL E-C- (P < 0.01). There was also a significant increase in the free cholesterol and cholesteryl ester concentration in VLDL E-C+. However, there were no significant differences in the cholesteryl ester and free cholesterol enrichment per particle (Table 3). The major VLDL immunofractions (E-C-, E-C+, E+C+) had similar cholesterol content whereas the E+C- particles had low cholesteryl ester content relative to the other particle types. For IDL and LDL, the cholesteryl ester and free cholesterol was mainly in E-C- particles (Table 2). The IDL particles that contained apoE or C-III had much lower cholesteryl ester content than the E-C- particles (Table 3). For LDL, there were no significant differences in cholesterol concentration or content between contraceptive users and nonusers. The cholesterol content was similar among the particle types.

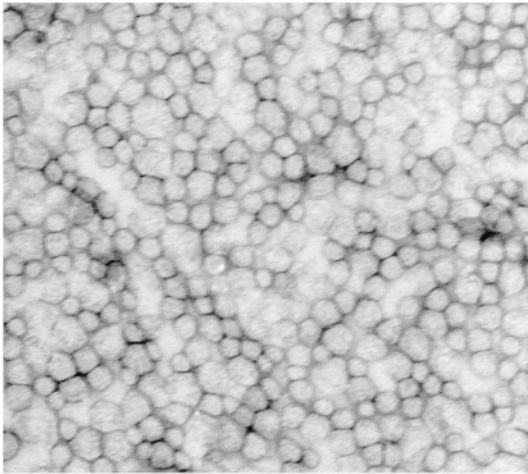
Most of the apoE in the apoB-containing lipoproteins was in VLDL E+C+ particles, 68% in contraceptive nonusers and 62% in users; the rest was distributed between VLDL E+C-, IDL and LDL (Table 2). There were no significant differences between contraceptive users and nonusers in the concentration of apoE in total plasma (Table 1), or in the VLDL, IDL, or LDL particles (Table 2). The lower apoE to apoB ratio in total VLDL of users (2.3 vs. 8.2 in nonusers) was caused by an increase in VLDL E- particles rather than by a decrease in E+ particles or in apoE concentration and enrichment (Tables 2, 3). The number of apoE molecules per VLDL particle was higher in the E+C+ (18.8 and 10.4 in nonusers and users, respectively) than in the E+C- fraction (11.7 and 5.3 in nonusers and users, respectively) (Table 3). The apoE to apoB ratio in the E+C+ and E+C- particles was fairly similar from VLDL to IDL to LDL.

Most of the apoC-III was in VLDL E+C+ particles, 68% in nonusers versus 86% in users; nearly all of the rest was in VLDL E-C+ particles. In users, the apoC-III concentration was significantly higher in VLDL E-C+ particles and tended to be higher in VLDL E+C+ as well (Table 2). The apoC-III concentration and content was very low in IDL and not detectable in LDL (Table 3). The apoC-III enrichment per E-C+ particle tended to be higher in the users (36.8 vs. 25.1 in nonusers), but the enrichment per E+C+ particle was similar for both groups. VLDL E-C+ particles had a higher apoC-III to apoB ratio than the VLDL E+C+ particles.

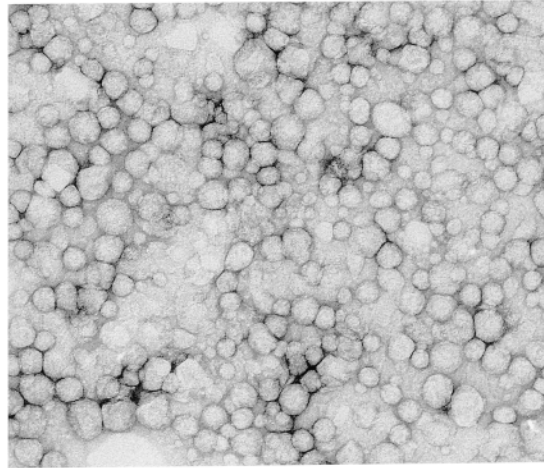
Electron micrographs of the lipoprotein particles separated by immunoaffinity show differences in sizes among the particle types, and heterogeneity within a particle type (Fig. 3). Representative micrographs from the two most



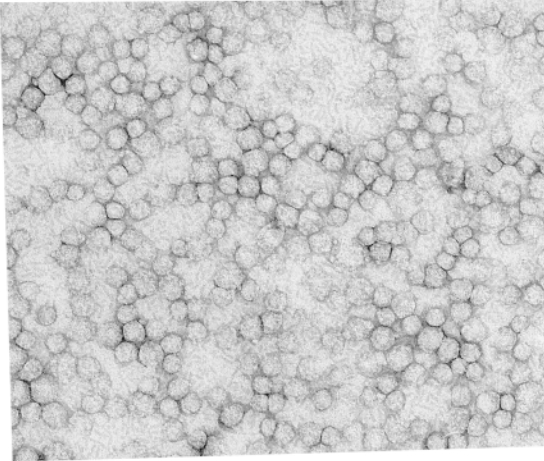
a(i).



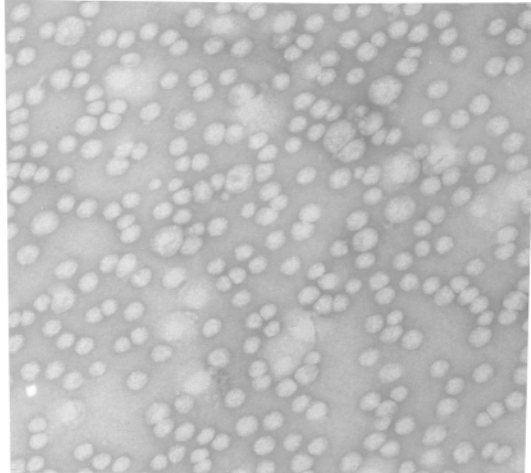
a(ii).



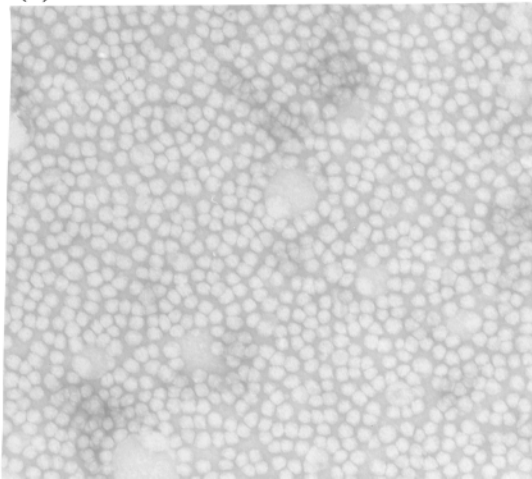
b(i).



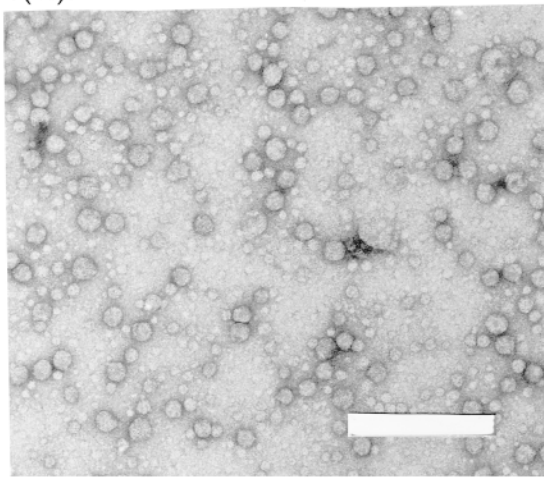
b(ii).



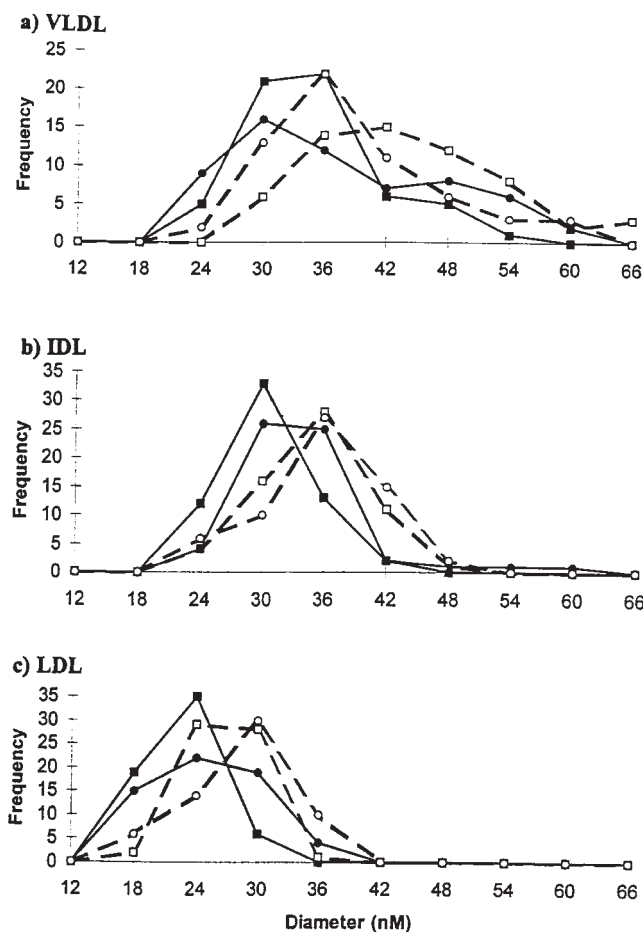
c(i).



c(ii).



**Fig. 3.** Electron micrographs of two major lipoprotein subfractions, from a pool of plasma from 7 normolipidemic persons, 4 female and 3 male, ages 23 to 38 years, separated by the anti-apoE and C-III immunoaffinity resins. The fractions from the two major particle types, E-C- and E+C+, separated as described in Fig. 1, are shown. a) VLDL: i) E-C- ii) E+C+, b) IDL: i) E-C- ii) E+C+, and c) LDL: i) E-C- ii) E+C+. The particles are magnified at 30,000 $\times$  by transmission electron microscopy. The scale at the bottom of the images denotes 200 nm.



**Fig. 4.** Particle size distribution of four types of VLDL, IDL, and LDL immunofractions. The diameter of 60 particles, from a pool of plasma described in Fig. 3, of a) VLDL, b) IDL, and c) LDL E-C-, E+C-, E-C+, and E+C+, were measured from electron micrographs by two independent observers blinded to the fraction category. □, E-C+; ○, E+C+; ●, E+C-; and ■, E-C-.

abundant fractions, E-C- and E+C+ are shown. The relative order of sizes of the VLDL particles is E-C+ > E+C+ = E+C- > E-C-, with the following average diameters E-C+ (44 ± 9 nm), E+C+ (39 ± 9 nm) and E+C- (38 ± 10 nm), E-C- (35 ± 7 nm) (**Fig. 4**, **Table 4**). Among VLDL, IDL, and LDL, the E-C- particles are significantly smaller than the other particles. Within each particle type, the sizes are more homogeneous for IDL and LDL than for VLDL. The triglyceride content is probably the major determinant of the size of the lipoproteins, as the average particle size correlated closely with the triglyceride to apoB ratio.

Correlation analysis of lipoprotein particle concentrations showed significant relationships, particularly among the particles with apoE or C-III, and among those with neither apoE nor C-III. VLDL E+C+ particle concentration was correlated with IDL E+C- ( $r = 0.72$ ,  $P < 0.01$ ), IDL E+C+ ( $r = 0.75$ ,  $P < 0.01$ ), and LDL E+C- ( $r = 0.67$ ,  $P < 0.05$ ). VLDL E-C- correlated with VLDL E-C+ ( $r = 0.87$ ,  $P < 0.001$ ), IDL E-C- ( $r = 0.86$ ,  $P < 0.001$ ) and LDL E-C- ( $r = 0.59$ ,  $P < 0.05$ ).

**TABLE 4.** Average diameters of VLDL, IDL, and LDL subfractions

	E-C+	E+C+	E+C-	E-C-
VLDL (nm)	44 ± 9 <sup>a</sup>	39 ± 9 <sup>b</sup>	38 ± 10 <sup>b,c</sup>	35 ± 7 <sup>c</sup>
IDL (nm)	35 ± 5 <sup>a</sup>	36 ± 6 <sup>a</sup>	34 ± 6 <sup>a</sup>	31 ± 4 <sup>b</sup>
LDL (nm)	27 ± 4 <sup>a</sup>	28 ± 5 <sup>b</sup>	25 ± 5 <sup>a</sup>	23 ± 4 <sup>c</sup>

Electron microscopy was carried out on a pool of control plasma as described in the Methods section to determine particle sizes of the immunofractions. Values are means of 60 representative particles measured by two independent researchers blinded to the particle types. Values are given as mean ± SD.

<sup>a,b,c</sup> Values with different superscript within each density fraction are significantly different at  $P < 0.05$ .

## DISCUSSION

We found that the apoB lipoprotein system in young normolipidemic women consists of a major group of particles poor in apoE and apoC-III (E-C-), and a minor group rich in apoE and/or C-III (E-C+, E+C+, E+C-). Because the VLDL E+C+ particles have a higher plasma concentration than the E+C- particles (4- to 6-fold), most apoE-containing LpB particles in plasma are likely to be LpB:E:C-III, using the nomenclature as defined by Alaupovic (25), with apoE and C-III existing mainly together. Similarly, most apoC-III-containing LpB particles also contain apoE, and they too would be classified as LpB:E:C-III. The particles that do not have apoE or C-III, whether in VLDL, IDL, or LDL, are distinguished by their small size and low triglyceride content from the other relatively large, triglyceride-rich particle types. This suggests that there may be two LpB particle systems, one without apoE or C-III, and the other with either or both of these apolipoproteins.

In young women, the major type of apoB-containing particle, E-C-, was small in size and had low triglyceride content. The E+C- particle was also relatively low in triglyceride compared with the other particles that had apoE or C-III. The relatively low triglyceride content in the particles that do not contain apoC-III, E+C- and E-C-, supports a close relationship between triglyceride and apoC-III content of LpB, and is consistent with the low plasma VLDL-triglyceride concentrations in individuals with apoC-III deficiency (6). The VLDL from these subjects is rapidly metabolized to IDL and then to LDL (6). In the present study, most of the C- particles do not contain apoE (E-C-). Thus without apoE for uptake by cells, and without C-III to inhibit lipolysis, the VLDL E-C- particles may be metabolized to E-C- particles of progressively smaller size and triglyceride content, i.e., IDL and LDL. The high correlations among plasma concentrations of E-C- particles (VLDL, IDL, and LDL) support such a metabolic relationship.

Among the particles that contain apoE or C-III, the E-C+ particles are the largest and carry the most triglyceride. Triglyceride content within VLDL and IDL particle types decreases from that in E-C+ particles to E+C+ and to E+C- particles. The apoC-III to apoB ratio is higher in E-C+ than E+C+ particles, suggesting that apoE may displace apoC-III as shown in *in vitro* studies (2, 26). The con-



centrations of particles that contain apoE and/or apoC-III are strongly correlated with each other. These findings, taken together, suggest that these three particle types are linked in a metabolic sequence in which VLDL E-C+ particles acquire apoE during lipolysis and become smaller VLDL or IDL E+C+ particles. Acquisition of apoE by VLDL E-C+ also may enhance the displacement of apoC-III during lipolysis, producing E+C+ VLDL or IDL that have a lower apoC-III content; continuation of this process eventually produces VLDL or IDL E+C-. Strong correlations among VLDL E+C+ and IDL and LDL E and C-III-containing particles suggest that such a relationship is possible.

Other studies measured apoC-III or apoE in apoB-containing lipoproteins, mainly in patients who had coronary heart disease or who were older than the young women in this study. The plasma concentration of apoE associated with apoB-containing lipoproteins in healthy men ages 25–64 years was 1.38 mg/dl in France (14) and 1.69 mg/dl in Northern Ireland (14), 3.9 mg/dl in healthy men mean age 50 in France (15), 1.4 mg/dl in normolipidemic subjects of unspecified age (20), and 1.0 mg/dl in nonusers and 0.95 mg/dl in users in this study. The plasma concentration of apoC-III associated with apoB-containing lipoproteins was 0.93 mg/dl in France (14), 1.1 mg/dl in Northern Ireland (14), 2.4 mg/dl in France (15), and 0.35 (nonusers) and 0.82 (users) mg/dl in this study. In normolipidemic healthy men, mean age 49, the apoB concentration of lipoprotein particles that had apoC-III (LpB:CIII) was 10.7 mg/dl and of those that had apoE (LpB:E) was 23.5 mg/dl (19). In another study of normolipidemics, mean LpB:E concentration was 12.8 mg/dl (25). Li et al. (27) found that both LpB:CIII and LpB:E concentrations were lower in premenopausal women mean age 41, 5.7 and 14.8 mg/dl respectively, than postmenopausal women mean age 56 years, 9.6 and 23.6 mg/dl, respectively. The young premenopausal women in our study had lower apoB levels than the older premenopausal women (27), 1.6 mg/dl for LpB:E (E+C- and E+C+) and 1.2 mg/dl in LpB:CIII (E-C+ and E+C+) in contraceptive nonusers, and 2.6 and 2.0 mg/dl in users. The young age, female sex, and low plasma lipoprotein concentrations of our subjects together could be the reason for their low concentrations of apoE, apoC-III, and apoB compared to the subjects in previous studies (14, 15, 19, 20, 25). We favor this explanation rather than a difference in methodology as unpublished data from our laboratory using plasma from normolipidemic men, ages 40–70, show a mean LpB:E concentration of 7 mg/dl and a mean LpB:CIII concentration of 9 mg/dl; these concentrations are higher than the values for the women in the present study, and are similar to the results in the other studies (14, 15, 19, 20, 25).

Estrogen, taken orally in contraceptives or in postmenopausal hormonal replacement, raises plasma concentrations of VLDL and LDL triglycerides and VLDL-apoB (28–30). We found that higher concentrations of E-C- particles in VLDL and IDL mainly account for these differences between contraceptive users and nonusers. The increase in VLDL E-C- particles accounts for 51% of the higher VLDL-TG levels in users and 64% of the higher VLDL-apoB concentra-

tions. Although the plasma concentration of VLDL triglycerides was higher in contraceptive users, the VLDL particles did not have a higher triglyceride content, as found in a previous study (31). In fact, the triglyceride was lower in contraceptive users as the VLDL particle concentration increased more than the triglyceride concentration. Thus estrogen does not appear to cause triglyceride enrichment of VLDL but rather stimulates the production of VLDL particles (31) that have a relatively low triglyceride content.

Oral contraceptive users had somewhat higher concentrations of apoE- or C-III-containing VLDL fractions compared to nonusers. However, the higher concentrations of apoE- or C-III-containing VLDL are only a minor aspect of the estrogenic effects on lipoproteins, of which the large increase in the E-C- particles predominates. There were no differences in lipid or apoE or C-III content in these particles between users and nonusers. Importantly, contraceptive users did not have higher enrichment of apoC-III in VLDL, IDL, and LDL particles compared to nonusers except for a trend towards higher enrichment in VLDL E-C+. This may be why OC use does not impair the clearance of these lipoproteins from plasma (31).

This study is limited by its case:control design, which compares two groups of women who are users or nonusers of oral contraceptives. The case:control study has less sensitivity than a randomized intervention trial to detect small differences in the outcome variables. However, there are practical difficulties in conducting a placebo-controlled trial of oral contraceptive use, and most studies of oral contraceptives and plasma lipoproteins used the case:control design. As estrogens strongly affect lipoprotein metabolism, these studies were able to detect differences in lipoprotein concentration and kinetics between users and nonusers (30–32). The present study of estrogen and plasma lipoprotein particles would need to be extended to postmenopausal women to determine the effects of hormonal replacement. Although the effects of estrogen on VLDL apoB and triglyceride concentrations are similar in direction when taken by premenopausal women in an oral contraceptive or when taken by postmenopausal women in hormonal replacement therapy (30, 33–35), it cannot be assumed that a change in estrogen from premenopausal levels to high levels produced by oral contraceptives has precisely the same effect as hormonal replacement. Another limitation is that the plasma that was used for electron microscopy was obtained from a control pool containing plasma from men and women that we were using to develop standards for this methodology. Thus the data on particle lipid and protein composition are not strictly comparable to that on size. Nonetheless, the particle sizes were as expected from the triglyceride content of the particles, suggesting that the overall findings can be generalized to some extent.

The distributions of all VLDL particles are skewed to the right, suggesting some heterogeneity within these subfractions. This could represent metabolically distinct subspecies possibly due to other C apolipoproteins or intermediates in a lipolytic sequence. ApoC-I or apoC-II content could also explain why some denser particles are larger than some buoyant ones. Within each immuno-

fraction, size decreases as density increases. However, all IDL are not smaller than all VLDL fractions as shown in this and other studies (11). Some IDL E-C+ particles were paradoxically larger than VLDL E-C-, probably reflecting the balance between higher TG lowering density and higher apoC-III and other apoproteins raising density.

Estrogenic increases in plasma triglycerides are caused by increases mainly in the concentration of particles that do not have apoE and C-III, and that are relatively low in triglyceride content. For this reason, the hypertriglyceridemia in OC users appears to differ from hypertriglyceridemia of persons with coronary heart disease who have high apoE, apoC-III, LpB:E, and LpB:C-III concentrations and triglyceride-rich lipoproteins (14, 15, 19, 25). The VLDL and IDL particles that are increased most by estrogen in oral contraceptive users (E-C- particles) may have different atherogenicity from particle types that contain apoE and apoC-III which are more prevalent in older patients and in those with coronary heart disease (20, 25, 27). However, there is a trend in the oral contraceptive users toward higher concentrations of LpB:E:CIII and LpB:CIII. It is possible that this trend in these young healthy women may be exacerbated by estrogen in dyslipidemic young women or in older women with other cardiovascular disease risk factors. It will be important to study the LpB particle types in such patients to elucidate further the roles of estrogen and apoE and apoC-III in atherogenesis. **■**

We would like to thank the women in the study for their participation. We would also like to acknowledge Genzyme Corporation for providing the anti-apoE resin and carrying out the coupling of the anti-apoC-III resin. The contribution of Dr. Margaret Yang in the analytical phase of the project and M. Ericsson at the EM core is much appreciated. This research was funded by grants from the National Institutes of Health, Bethesda, MD, National Heart, Lung, and Blood Institute (R01 HL 34980).

Manuscript received 20 January 1998, in revised form 6 July 1998, and in revised form 1 October 1998.

## REFERENCES

- Aalto-Setälä, K., P. H. Weinstock, C. L. Bisgaier, L. Wu, J. D. Smith, and J. L. Breslow. 1996. Further characterization of the metabolic properties of triglyceride-rich lipoproteins from human and mouse apoC-III transgenic mice. *J. Lipid Res.* **37**: 1802-1811.
- Sehayek, E., and S. Eisenberg. 1991. Mechanisms of inhibition by apolipoprotein C of apolipoprotein E-dependent cellular metabolism of human triglyceride-rich lipoproteins through the low-density lipoprotein receptor pathway. *J. Biol. Chem.* **266**: 18259-18267.
- Ebara, T., R. Ramakrishnan, G. Steiner, and N. S. Shachter. 1997. Chylomicronemia due to apolipoprotein CIII overexpression in apolipoprotein-null mice. Apolipoprotein CIII-induced hypertriglyceridemia is not mediated by effects on apolipoprotein E. *J. Clin. Invest.* **99**: 2672-2681.
- Ito, Y., N. Azrolan, A. O'Connell, A. Walsh, and J. L. Breslow. 1985. Hypertriglyceridemia as a result of human apoCIII gene expression in transgenic mice. *Science*. **249**: 790-793.
- Malmendier, C. L., J. Lontie, C. Delcroix, D. Y. Dubois, T. Magot, and L. De Roy. 1989. Apolipoproteins C-II and C-III metabolism in hypertriglyceridemic patients. *Atherosclerosis*. **77**: 139-149.
- Ginsberg, H. N., N. Le, I. J. Goldberg, J. C. Gibson, A. Rubinstein, P. Wang-Iverson, R. Norum, and W. V. Brown. 1986. Apolipoprotein B metabolism in subjects with deficiency of apolipoprotein CIII and AI. *J. Clin. Invest.* **78**: 1287-1295.
- Maeda, N., H. Li, D. Lee, P. Oliver, S. H. Quarfordt, and J. Osada. 1994. Targeted disruption of the apolipoprotein C-III gene in mice results in hypotriglyceridemia and protection from postprandial hypertriglyceridemia. *J. Biol. Chem.* **269**: 23610-23616.
- Dergunov, A. D., and M. Rosseneu. 1994. The significance of apolipoprotein E structure to the metabolism of plasma triglyceride-rich lipoproteins. *Biol. Chem. Hoppe-Seyler*. **375**: 485-495.
- Friedman, G., D. Gavish, D. Vogel, and S. Eisenberg. 1990. Cellular metabolism of human intermediate-density lipoprotein (IDL). *Biochim. Biophys. Acta*. **1044**: 118-126.
- Bradley, W. A., and S. H. Gianturco. 1986. ApoE is necessary and sufficient for the binding of large triglyceride-rich lipoproteins to the LDL receptor; apoB is unnecessary. *J. Lipid Res.* **27**: 40-48.
- Schaefer, E. J., R. E. Gregg, G. Ghiselli, T. M. Forte, J. M. Ordovas, L. A. Zech, and H. B. Brewer. 1986. Familial apolipoprotein E deficiency. *J. Clin. Invest.* **78**: 1206-1219.
- Zhang, S. H., R. L. Reddick, B. Burkey, and N. Maeda. 1994. Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. *J. Clin. Invest.* **94**: 937-945.
- Ehnholm, C., R. W. Mahley, D. A. Chappell, K. H. Weisgraber, E. Ludwig, and J. L. Witztum. 1984. Role of apolipoprotein E in the lipolytic conversion of beta VLDL to LDL in type III hyperlipoproteinemia. *Proc. Natl. Acad. Sci. USA*. **81**: 5566-5570.
- Luc, G., C. Fievet, D. Arveiler, A. E. Evans, J. Bard, F. Cambien, J. Fruchart, and P. Ducimetiere. 1996. Apolipoproteins C-III and E in apoB- and non-apoB-containing lipoproteins in two populations at contrasting risk for myocardial infarction: the ECTIM study. *J. Lipid Res.* **37**: 508-517.
- Chivot, L., F. Mainard, E. Bigot, J. M. Bard, J. L. Auget, Y. Madec, and J. C. Fruchart. 1990. Logistic discriminant analysis of lipids and apolipoproteins in a population of coronary bypass patients and the significance of apolipoproteins CIII and E. *Atherosclerosis*. **82**: 205-211.
- Alaupovic, P., H. N. Hodis, C. Knight-Gibson, W. J. Mack, L. LaBree, L. Cashin-Hemphill, C. N. Corder, D. M. Krams, and D. H. Blankenhorn. 1994. Effects of lovastatin on apoA- and apoB-containing lipoprotein families in a subpopulation of patients participating in the Monitored Atherosclerosis Regression Study (MARS). *Arterioscler. Thromb.* **14**: 1906-1914.
- Blankenhorn, D. H., P. Alaupovic, E. Wickham, H. P. Chin, and S. P. Azen. 1990. Prediction of angiographic change in native human coronary arteries and aortocoronary bypass grafts. *Circulation*. **81**: 470-476.
- Syvänne, M., M. Rosseneu, C. Labeur, H. Hilden, and M. Taskinen. 1994. Enrichment with apolipoprotein E characterizes postprandial TG-rich lipoproteins in patients with non-insulin-dependent diabetes mellitus and coronary artery disease: a preliminary report. *Atherosclerosis*. **105**: 25-34.
- Genest, J. J., Jr., J. M. Bard, J. Fruchart, J. M. Ordovas, P. F. W. Wilson, and E. J. Schaefer. 1991. Plasma apolipoprotein AI, AII, B, E and CIII-containing particles in men with premature coronary artery disease. *Atherosclerosis*. **90**: 149-157.
- Cohn, J. S., M. Tremblay, M. Amiot, D. Bouthillier, M. Roy, J. J. Genest, Jr., and J. Davignon. 1996. Plasma concentration of apolipoprotein E in intermediate-sized remnant-like lipoproteins in normolipidemic and hyperlipidemic subjects. *Arterioscler. Thromb. Vasc. Biol.* **16**: 149-159.
- Gianturco, S. H., A. H. Lin, S. C. Hwang, J. Young, S. A. Brown, D. P. Via, and W. A. Bradley. 1988. Distinct murine macrophage receptor pathway for human triglyceride-rich lipoproteins. *J. Clin. Invest.* **82**: 1633-1643.
- Getz, G. S., T. Mazzone, P. Soltys, and S. R. Bates. 1988. Atherosclerosis and apoprotein E. *Arch. Pathol. Lab. Med.* **112**: 1048-1055.
- The Lipid Research Clinics Program Epidemiology Committee. 1979. Plasma lipid distribution in selected North American populations: The Lipid Research Clinics Program Prevalence Study. *Circulation*. **60**: 427-439.
- Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345-1353.
- Alaupovic, P. 1991. Apolipoprotein composition as the basis for classifying plasma lipoproteins. Characterization of apoA- and apoB-containing lipoprotein families. *Prog. Lipid Res.* **30**: 105-138.
- Kowal, R. C., J. Herz, K. H. Weisgraber, R. W. Mahley, M. S. Brown, and J. L. Goldstein. 1990. Opposing effects of apolipoprotein E and C on lipoprotein binding to low density lipoprotein receptor-related protein. *J. Biol. Chem.* **265**: 10771-10779.

27. Li, Z., J. R. McNamara, J. Fruchart, G. Luc, J. M. Bard, J. M. Ordovas, P. W. F. Wilson, and E. J. Schaefer. 1996. Effects of gender and menopausal status on plasma lipoprotein species and particle sizes. *J. Lipid Res.* **37**: 1886–1896.
28. Stampfer, M. J., W. C. Willett, G. A. Colditz, F. E. Speizer, and C. H. Hennekens. 1990. Past use of oral contraceptives and cardiovascular disease: a meta-analysis in the context of Nurses' Health Study. *Am. J. Obstet. Gynecol.* **163**: 285–291.
29. Schaefer, E. J., D. M. Foster, L. A. Zech, F. T. Lindgren, H. B. Brewer, Jr., and R. I. Levy. 1983. The effects of estrogen administration on plasma lipoprotein metabolism in premenopausal females. *J. Clin. Endocrinol. Metab.* **57**: 262–267.
30. Wahl, P., C. Walden, R. Knopp, J. Hoover, R. Wallace, G. Heiss, and B. Rifkind. 1983. Effect of estrogen/progestin potency on lipid/lipoprotein cholesterol. *N. Engl. J. Med.* **308**: 862–867.
31. Walsh, B. W., and F. M. Sacks. 1993. Effects of low dose oral contraceptives on very low density and low density lipoprotein metabolism. *J. Clin. Invest.* **91**: 2126–2132.
32. Godsland, I. F., D. Crook, R. Simpson, T. Proudler, C. Felton, B. Lees, V. Anyaoku, M. Devenport, and V. Wynn. 1990. The effects of different formulations of oral contraceptive agents on lipid and carbohydrate metabolism. *N. Engl. J. Med.* **323**: 1375–1381.
33. Campos, H., B. W. Walsh, H. Judge, and F. M. Sacks. 1997. Effect of estrogen on very low density lipoprotein and low density lipoprotein subclass metabolism in postmenopausal women. *J. Clin. Endocrinol. Metab.* **82**: 3955–3963.
34. Sacks, F. M., H. Campos, H. Judge, and B. W. Walsh. 1997. Estrogen treatment and human lipoprotein metabolism. In *Hormonal, Metabolic, and Cellular Influences on Cardiovascular Disease in Women*. T. M. Forte, editor. Futura Publishing Company, Inc., Armonk, NY. 229–246.
35. Walsh, B. W., I. Schiff, B. Rosner, L. Grenberg, V. Ravnkar, and F. M. Sacks. 1991. Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. *N. Engl. J. Med.* **325**: 1196–1204.