Dense low density lipoprotein subspecies with diminished oxidative resistance predominate in combined hyperlipidemia[1]

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Abstract Patients presenting combined hyperlipidemia (CHL) display an elevated risk of coronary heart disease. The atherogenic lipoprotein particles implicated in this disorder remain ill defined. We determined the qualitative and quantitative characteristics of the density distribution of low density lipoprotein (LDL) particle subspecies in nine subjects defined phenotypically as presenting CHL, and under strict dietary control. Seven CHL patients possessed familial antecedents of premature coronary heart disease; none were E_2E_2 homozygotes. Five LDL subspecies were isolated by density gradient ultracentrifugation in the density range 1.019–1.063 g/ml. In all patients, the LDL profile was skewed towards the dense subspecies (LDL-4, d 1.039–1.050 g/ml and LDL-5, d 1.050–1.063 g/ml), representing 47% of total LDL mass; by contrast, these subspecies accounted for only 30% of LDL mass in five normolipidemic subjects ($P < 0.01$). In addition, plasma LDL mass concentrations were some twofold higher in CHL patients as compared to normolipidemic subjects. The % mass of LDL-4 was positively correlated with plasma triglyceride and apoB levels. LDL-2 and LDL-3 in CHL patients were triglyceride-enriched (11.9 and 7.2%, respectively) as compared to the corresponding subspecies in normolipidemic subjects (6.6 and 3.7%, respectively; $P < 0.05$ in each case). LDL particle size decreased with increase in density in both groups; however, significant differences were found between corresponding LDL subspecies (LDL-1, -3, -4, and -5) in CHL patients and normolipidemic subjects, a finding suggestive of dissimilar molecular organization, despite correspondence in hydrated density. The copper-induced oxidative modification of LDL subspecies was assessed by determination of conjugated diene formation. In both groups, LDL-5 was distinct in exhibiting a marked diminution in oxidative resistance as indicated by a significant reduction ($P < 0.01$) in mean lag time. The oxidative susceptibility of LDL subspecies in both groups was independent of vitamin E content when expressed as the ratio vitamin E/LDL mass, although dense LDL in CHL patients tended to be deficient in this antioxidant. The diminished oxidative resistance of dense LDL subspecies could not be accounted for by enrichment in polyunsaturated fatty acids in either group. These studies suggest that in consequence of their elevated circulating concentration and diminished oxidative resistance, dense LDL subspecies represent putative atherogenic subspecies in combined hyperlipidemia.—**Dejager, S., E. Bruckert, and M. J. Chapman.** Dense low density lipoprotein subspecies with diminished oxidative resistance predominate in combined hyperlipidemia. *J. Lipid Res.* 1993. **34:** 295–308.

Supplementary key words coronary heart disease • density gradient ultracentrifugation • physicochemical properties • apoB-100 • particle heterogeneity • Cu^{2+}-induced oxidation • vitamin E

Cholesterol is principally transported in human plasma in the form of lipoprotein particles of low density. Elevated circulating levels of low density lipoproteins (LDL) are now recognized as a major risk factor for the precocious development of atherosclerosis (1). Two major groups of subjects present increased circulating concentrations of LDL, i.e., those with familial hypercholesterolemia (FH), and those with a combination of hypercholesterolemia and hypertriglyceridemia, the latter disorder being termed combined hyperlipidemia (CHL) (2, 3). Individuals in the latter group are distinguished by the possession of elevated levels of both LDL and of very low density lipoproteins (VLDL) (3, 4).

Despite recent progress (for a review, see ref. 5), our knowledge of the molecular and cellular mechanisms involved in the pathogenic relationship between elevated levels of LDL and atherogenesis remains incomplete. A number of therapeutic studies have, however, clearly demonstrated the beneficial effect of reduction in circulating LDL concentration on both the prevention of coronary heart disease (6, 7), and the regression of atherosclerotic plaques (8). Quantitative elevations in

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Abbreviations: apoB-100, apolipoprotein B-100; BHT, butylated hydroxytoluene; CHL, combined hyperlipidemia; EDTA, ethylenediamine tetraacetic acid; FH, familial hypercholesterolemia; FCHL, familial combined hyperlipoproteinemia; CHD, coronary heart disease; LDL, low density lipoproteins; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; TG, triglyceride; VLDL, very low density lipoproteins; HDL, high density lipoproteins.

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plasma LDL do not alone account for the atherogenicity associated with these particles, and indeed additional factors appear to be involved. Thus, on the one hand, circulating LDL do not appear to be of uniform atherogenic potential in different patients presenting familial hypercholesterolemia, and indeed some 20% of heterozygous FH subjects do not present a myocardial infarction before age 60 despite high LDL concentrations (9). On the other hand, plasma LDL level does not appear to be predictive of the age of coronary death (9). The atherogenic risk associated with these cholesterol-rich lipoproteins therefore appears to be linked to their structure, composition, and metabolism as much as to their plasma concentration.

In plasma, LDL are distributed over the density range from 1.019 to 1.063 g/ml as a continuum of particles. It is, however, relevant that substantial evidence of qualitative heterogeneity within this density interval has now accumulated (10-17). Indeed, the occurrence of multiple subspecies of LDL particles that vary markedly in their physicochemical, hydrodynamic, immunological, and structural properties is typical not only of normolipidemic but also of dyslipidemic subjects (10-24). Thus, analyses of LDL heterogeneity in hypertriglyceridemia and hyperapobetalipoproteinemia have documented the predominance of dense, small LDL particles, the major peak of the LDL density profile being typically situated within the range ~ 1.035-1.058 g/ml (3, 4, 5, 19, 23, 24). Moreover, LDL profiles in hypertriglyceridemia frequently feature a bi- or trimodal aspect (14, 15). By contrast, the LDL density distributions characteristic of normo- and of hypercholesterolemic subjects are usually unimodal, with the major proportion of LDL mass localized to a single, narrow peak within the range d ~ 1.027-1.035 g/ml (10, 12, 13, 15, 16, 18, 21). Within this category, patients with FH are distinct as the bulk of their LDL are present as light, cholesteryl ester-rich particles of large size, whose peak density may be as low as ~ 1.027 g/ml; by contrast, the peak of LDL mass in normolipidemic subjects frequently occurs within the density range from ~ 1.031 to 1.035 g/ml (10, 12, 21).

The qualitative heterogeneity of LDL particles now appears to be intimately linked not only to the metabolism of individual LDL subspecies, but also to their potential atherogenicity. Thus, dense LDL subspecies from normolipidemic subjects display a binding affinity for the cellular LDL receptor that is lower than that of their lighter counterparts (25), thereby favoring a prolonged residence time in plasma. Furthermore, dense LDL in healthy subjects display enhanced susceptibility to oxidation in vitro (26); such modification may potentiate their uptake by the scavenger receptor(s) of macrophages, resulting in cholesterol accumulation and foam cell formation (5).

Combined hyperlipidemia (CHL) is one of the most common forms of dyslipidemia and a major risk factor for coronary disease (27, 28). The pathophysiological mechanisms underlying this disorder involve elevated production rates of VLDL-apoB, or LDL-apoB, or both (29, 30). Paradoxically, however, little is known of the quantitative and qualitative aspects of LDL particle subspecies in CHL, or of the molecular and cellular mechanisms whereby LDL particles might express their atherogenicity. The primary aims of this study were therefore *1)* to define the LDL subspecies profile and the physicochemical properties of individual LDL subspecies in a group of subjects defined phenotypically as presenting CHL; *2)* to evaluate the susceptibility of LDL subspecies in CHL patients to copper-induced oxidation in vitro in relation to the particle content of the principal lipophilic antioxidant, vitamin E; and *3)* to compare these parameters with those of corresponding LDL subspecies in a group of normolipidemic subjects. Our studies reveal that elevated plasma levels of dense LDL with diminished resistance to oxidation are a prominent feature of combined hyperlipidemia.

MATERIALS AND METHODS

Patients and normolipidemic subjects

Nine outpatients of the Department of Endocrinology-Metabolism at the Hôpital de la Pitié were selected. All patients received the same dietary recommendations throughout the study and dietary adherence was assessed by a registered dietician. Patients received a normocaloric diet for a 1-month period prior to the study; this diet contained 30% lipid, 15% protein, and 55% carbohydrate with an average intake of 300 mg per day of cholesterol and a P/S ratio of approx. 1. None were being treated with a drug that was known to affect lipid metabolism for at least 2 months before blood sampling. All patients were Caucasian males 31-58 years old, and displayed a primary combined hyperlipidemia (see below). After informed consent and approval by the Human Subjects Review Committee of the Hospital, patients with a total plasma triglyceride level of 200 mg/dl or more and total cholesterol > 250 mg/dl, and receiving the above diet, were included in the study. No significant changes in body mass index were detected during the study. The absence of dietary vitamin supplementation was rigorously verified. A secondary hyperlipidemia was excluded by the determination of hepatic enzyme activities, the plasma levels of creatinine, glucose, thyroid hormones, and blood proteins, and by evaluation of proteinuria. None were obese, and alcohol abuse was excluded. All patients were ambulatory. Control subjects were five healthy, normolipidemic Caucasian male volunteers aged 27-45 years; their diet contained 2000-2500 kcal/day and 38-42% of calories as fat, corresponding to average calorie intake in France. The diet and BMI of these subjects were stable over the period of several months preceding the study.

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Apolipoprotein E phenotype was determined by isoelectric focusing in one dimension as described by Bouthillier, Sing, and Davignon (31).

The clinical characteristics of the nine CHL patients and of the five control subjects are presented in **Table 1;** values shown are those obtained after diet therapy, and, in the case of lipid levels, are means of up to four determinations. None of the CHL patients were E_2E_2 homozygotes; the most frequent phenotypes were E_3E_2 and E_3E_4 (four subjects each). As there is to date no single genetic or clinical marker allowing identification of CHL patients, our classification is similar to that proposed by Arad, Ramakrishnan, and Ginsberg (27) i.e., a lipid profile including an LDL-cholesterol level greater than the 90th percentile for age and sex, with a concomitant plasma triglyceride level of 200 mg/dl or greater; TG levels in CHL subjects 4 and 5 fluctuated around this latter value.

Mean plasma cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, Lp[a], apoB, and apoA-I concentrations in the CHL group were: 297 ± 43, 235 ± 40, 209 ± 38, 39 ± 5, 17 ± 8, 194 ± 27, and 125 ± 11 mg/dl, respectively. Although most cases were associated with a reported familial history of hyperlipidemia (either type IIb, IIa, or IV), the plasma levels in first degree relatives were often unavailable, thereby preventing us from assessing the diagnosis of familial combined hyperlipidemia on the basis of the strict criteria of FCHL previously described (3). Nonetheless, careful assessment of familial history revealed a high incidence of premature coronary heart disease (seven of nine cases) before the age of 60 years (Table 1). The plasma concentrations of lipids, lipoprotein lipids, and apolipoproteins in healthy subjects are typical of a normolipidemic French population (12).

Plasma lipid, LDL-cholesterol, and apoB levels were significantly elevated in CHL patients as compared to normolipidemic subjects ($P < 0.05$). Although within the normal ranges, levels of both HDL-cholesterol and Lp[a] in CHL patients differed significantly from those in the control group ($P < 0.05$ and < 0.03, respectively).

Preparative methods

Blood samples. Freshly drawn venous blood (30–50 ml) from overnight-fasted CHL patients and normolipidemic subjects was collected into sterile tubes containing EDTA (final concentration, 1 mg/ml), from which plasma was isolated by low speed centrifugation (1000 *g*, 20 min at 4°C). Immediately upon collection of plasma samples, solutions of PMSF, EDTA, sodium azide, and gentamicin were added to final concentrations of 0.1 M, 0.01% (w/v), 0.01% (w/v), and 0.005% (w/v), respectively, in order to inhibit serine protease activity, metal cation-catalyzed peroxidative degradation of lipoproteins, and microbial growth; in addition, a solution of gluthatione, Σ-aminocaproic acid, and BHT was added to give additional protection against lipoprotein oxidation (final concentrations of 50, 5, and 130 μg/ml, respectively). Plasma samples were normally taken for lipoprotein separation within 3 h of blood collection, during which time they were maintained at 4°C.

Subfractionation of low density lipoproteins by isopycnic density gradient ultracentrifugation. For present purposes, low density lipoproteins were defined as lipoproteins of density between 1.019 and 1.063 g/ml. Density gradients were constructed as described earlier (32). In brief, the non-protein solvent density of the plasma samples was first increased to 1.21 g/ml by addition of solid KBr (0.325 g/ml plasma). Discontinuous density gradients were then con-

TABLE 1. Morphometric and clinical characteristics of combined hyperlipidemic patients (CHL) and normolipidemic subjects (controls)

Subject	Cardiac History: Personal	Cardiac History: Familial	Tendinous Xanthoma/ Xanthelasma	Age	Body Mass Index[a]	Total Chol	Total TG	LDL-C	HDL-C	Lp[a]	ApoB	ApoA-I
				yr					*mg/dl*			
CHL patients (n = 9)	2 of 9	7 of 9	3 of 9									
Mean ± SD				39.7 ± 8.9	25.3 ± 3.3	297 ± 43[b]	235 ± 40[b]	209 ± 38[b]	39 ± 5[c]	17 ± 8[c]	194 ± 27[b]	125 ± 11
Controls (n = 5)	0	0	0									
Mean ± SD				32.8 ± 7.1	23 ± 2.5	167 ± 13	57 ± 16	111 ± 12	49 ± 6	7 ± 3	89 ± 1	134 ± 21

Plasma cholesterol (Chol), triglyceride (TG), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), Lp[a], apoB, and apoA-I levels were measured as described in Materials and Methods and were normally determined in duplicate.

[a]Body mass index = wt/h; weight in kg, height in m.

[b]Greater than controls, $P < 0.05$.

[c]Less than controls, $P < 0.03$.

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structed at ambient temperature in Ultraclear tubes (Beckman no. 344059) of a Beckman SW41 swinging-bucket rotor. Salt solutions were made up from NaCl and KBr, and their densities were verified to the fourth decimal place with a precision densitometer (Model DMA 40; Anton Paar, Graz, Austria) at 15°C. The gradients were centrifuged in a Sorvall OTD-50B or a Beckman L8-55 ultracentrifuge for 48 h at 15°C and 40,000 rpm. No braking was used at the end of the runs; the Sorvall instrument was used in the ARC: slow/Reograd mode for maximal gradient stability during acceleration. In order to establish the density profile obtained upon completion of ultracentrifugation, control gradients containing NaCl-KBr solutions were fractionated identically and their densities were determined at 15°C as above. A calibration curve of solvent density as a function of volume was used to determine the density intervals of individual subfractions.

On completion of ultracentrifugation, the top 0.8 ml of each gradient was removed by aspiration with a narrow-bore Pasteur pipette; this fraction corresponded to VLDL of d < 1.013 g/ml. Gradients were then fractionated into successive subfractions of 0.8 ml by upward displacement with a density gradient fractionator (Model 185, Isco), coupled to a spectrophotometer to allow continuous monitoring of the eluate at 280 nm (12).

Five subfractions of LDL were collected, in the following density intervals: LDL-1, 1.019-1.023 g/ml; LDL-2, 1.023-1.029 g/ml; LDL-3, 1.029-1.039 g/ml; LDL-4, 1.039-1.050 g/ml; and LDL-5, 1.050-1.063 g/ml. Corresponding LDL subfractions derived from the same starting plasma were pooled; individual subfractions were exhaustively dialyzed at 4°C for 48 h in Spectrapor tubing (exclusion limit ca. 12,000-14,000) against a solution containing 20 mM NH_4HCO_3, 0.01% (w/v) sodium azide, and 0.01% (w/v) EDTA, at pH 7.4. Finally, LDL subfractions were filtered through 0.45-μm filters (Sartorius) into sterile plastic tubes (Corning).

ANALYTICAL METHODS

Chemical analysis

Plasma lipids, lipoprotein lipids, and apolipoproteins. Total plasma cholesterol and triglyceride concentrations were determined by a modification of the Liebermann-Burchard reaction (33) and by the method of Kessler and Lederer (34) after zeolite extraction, respectively. "Seronorm lipid" (Nyegaard AS, Oslo, Norway) was used as the working standard for both assays. Plasma HDL-cholesterol was estimated by the method of Allain et al. (35) using an enzymatic kit (Biotrol, Paris, France). LDL-cholesterol was calculated using the Friedewald formula (36). Plasma apoB, apoA-I, and Lp[a] were measured by a series of immunological assays described earlier (37) and based on a laser immunonephelometer (Immuno AG, Vienna, Austria).

Lipoprotein subfractions. Chemical analyses of LDL subfractions were performed by the series of procedures which we originally described (32), and included protein quantitation by the Lowry procedure (38), measurement of free and esterified cholesterol by the method of Roeschlau, Bernt, and Gruber (39), phospholipid estimation by the procedure of Takayama et al. (40), and triglyceride measurement by the technique of Biggs, Erickson, and Moorehead (41). The appropriate enzymatic assay kits provided by BioMerieux (Marcy L'Etoile, France) were used for cholesterol and phospholipid estimation; cholesteryl ester was calculated as (total cholesterol − free cholesterol) × 1.67, as indicated earlier (32). All analyses were performed in triplicate; the technical errors in these analyses were similar to those determined earlier (32). Calibration curves for lipid assays were established with purified standards. The total LDL mass in each LDL subfraction was calculated as the sum of the concentrations of the lipid and protein components, and allowed determination of the percent chemical composition.

Electrophoretic analyses

Native lipoproteins. Agarose gel. The total (net) electrical charge on both native and copper-oxidized LDL subfractions at neutral pH was estimated by electrophoresis in agarose gel (42). Aliquots of fractions (1 μl containing ~ 0.4 mg/ml protein) were electrophoresed for 30 min on agarose gel films (Universal electrophoresis film agarose; cat. no. 470100, Corning, Palo Alto, CA) using the Corning ACI system. On completion of electrophoresis, sheets were stained for lipid with Fat Red O.

Nondenaturing gradient gel. LDL particle size and heterogeneity were evaluated by electrophoresis in nondenaturing polyacrylamide gradient gels (PAA 2/16; Pharmacia Fine Chemicals, Uppsala, Sweden), in the GE 2/4 LS gel electrophoresis apparatus, under conditions described earlier (12, 43). Standards used for size calibration purposes included latex beads (38.0 nm) (Dow Chemical Company, Indianapolis, IN) and an HMW electrophoresis calibration kit (thyroglobulin, 17.0 nm; apoferritin 12.2 nm, and catalase 10.4 nm; Pharmacia). From the migration distances of the different lipoprotein subfractions and those of the calibration proteins, it was possible to calculate the Stokes diameters of LDL subfractions using the Stokes-Einstein equation (44). A representative correlation coefficient for the regression line of the relationship between the logarithm of the diameter of the calibration proteins and their migration distance was −0.999.

Apolipoproteins. Polyacrylamide gradient gels. In order to evaluate both the quantitative and qualitative aspects of the apolipoprotein content of LDL subfractions, we electrophoresed 20 μg of LDL protein in SDS-gradient poly-

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acrylamide slab gels (5–19% acrylamide) by the method of Irwin et al. (45). Proteins were separated in a single dimension by use of a vertical slab gel electrophoresis cell (13 × 44 × 0.15 cm; model SE 600, Hoefer Scientific, San Francisco, CA). Electrophoresis was carried out at 20 mA/slab at 4°C for approximately 4 h in a buffer containing 192 mM glycine, 5 mM SDS, and 25 mM Tris base at pH 8.4. Apolipoprotein samples were prepared as follows. Aliquots of subfractions were first lyophilized and then delipidated immediately with a mixture of ethanol–diethyl ether 3:1 (v/v). Solubilization of the total apolipoprotein residues was performed with 30 μl of a solution containing 10 mM Tris-HCl-DTT and 0.5% SDS at pH 6.8. Samples were incubated at 100°C for 5 min immediately before electrophoresis. Gels were stained with Coomassie brilliant blue R250. Molecular weights were measured from a calibration curve calculated from a series of polymerized protein standards (56,000–280,000; BDH Biochemicals, Poole, Dorset, U.K.) electrophoresed in parallel.

Determination of lipid hydroperoxides

The total lipid peroxide content of native LDL subfractions was estimated by the tri-iodide spectrophotometric assay recently developed by El-Saadani et al. (46). Values were expressed as nmole relative to LDL protein in mg. In order to eliminate all traces of EDTA and antioxidants in these assays, each subfraction was extensively dialyzed against a 20 mM NH_4HCO_3 buffer made up in degassed, sterile distilled water.

Estimation of vitamin E content

The vitamin E content of native LDL subfractions was estimated by the high performance liquid chromatographic method of Lehman and Martin (47). Briefly, aliquots (~ 100 μg LDL protein) of each subfraction were first mixed with a known amount of the internal standard dissolved in ethanol (100 μl). Neutral lipids and lipid-soluble vitamins were then extracted into hexane; after centrifugation, the supernatant was removed and dried under N_2. The dried residue was subsequently dissolved in methanol, and an aliquot was applied to the chromatographic column (Lichrosorb). Elution was performed with 1% isopropanol in hexane at a flow rate of 1.5 ml/min. For fluorescence detection, an excitation wavelength of 290 nm was used and the emitted fluorescence was measured at 330 nm.

Copper-induced oxidation of LDL subfractions

The chemical modifications induced in LDL particles by copper-induced, free radical-mediated oxidation have been extensively studied by Esterbauer and colleagues (46, 48–50) who have provided evidence that the spectrophotometric detection of conjugated diene formation at 236 nm allows a valid estimate of the degree of lipid peroxidation. We therefore conducted our experiments according to the protocol of these investigators (50), with some modifications. Thus experiments were performed in parallel in duplicate at 37°C at final concentrations of LDL protein and of $CuCl_2$ of 250 μg/ml (i.e., ≈ 1 mg/ml total lipoprotein mass) and 5 μM, respectively, in an NH_4HCO_3 buffer (20 mM) made up in degassed, sterile distilled water at pH 7.4. Samples of LDL were dialyzed prior to assay as above in order to completely remove EDTA, sodium azide, gentamicin, and antioxidants. The kinetics of oxidation (as conjugated diene formation) were monitored continuously for periods up to 10 h by measuring the increase in absorbance at 236 nm; absorbance at time zero in the absence of $CuCl_2$ was taken as the reference value.

For analytical purposes, the 236 nm absorbance profile of conjugated diene formation was separated into lag and propagation phases (48–50). In order to compare the susceptibility of individual LDL subfractions from the same donor with each other, we compared both the duration of the respective lag phases and the rate of oxidation during the propagation phase, the latter being measured as the increase in diene absorption with time (26). The lag phase was determined as the value in minutes of the intercept of the slope of the absorbance curve on the y (time) axis. This procedure proved highly reproducible, giving similar absorbance profiles for the same LDL subfraction at intervals of up to ~ 7 days; during storage both antioxidants and antibacterial agents were present in LDL preparations.

The inclusion of BHT during LDL isolation raises the possibility that this antioxidant might be preferentially enriched in one or more LDL subfractions, and might be incompletely removed by dialysis, thereby protecting against lipid peroxidation. To evaluate this question, we performed control experiments in which a series of LDL subfractions were isolated from the same plasma in the presence and in the absence of BHT. The induction of conjugated diene formation by copper was then evaluated as above; data for the lag phase and rate of propagation for corresponding subfractions from each series closely resembled each other, and no significant differences could be detected (results not shown). These findings suggest that if indeed preferential sequestration and retention of BHT into one or more LDL subfractions does occur, and which our present analyses cannot entirely exclude, then it is without detectable effect on the susceptibility of LDL subfractions to copper-induced oxidation.

The modification in the net electrical charge of LDL subfractions as a result of in vitro oxidation was evaluated at intervals of ~ 3 h (up to 10 h) and expressed as the relative electrophoretic mobility (REM); REM was calculated as the distance of migration on agarose gel of the stained band corresponding to each oxidized LDL subfraction relative to that of the migration of the native LDL from which it was derived.

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Statistical analysis

Comparison of data for clinical and biological parameters in CHL patients and the normolipidemic group was performed by a one-way analysis of variance (ANOVA). Significance was estimated by calculation of the variance ratio (F) and the number of degrees of freedom, and we considered statistical significance to be at the < 0.05 level. Correlation coefficients were determined according to Pearson. The proportions of each chemical component in corresponding LDL subfractions from the CHL patient and normolipidemic groups were compared by the Student's t test.

RESULTS

Mass distribution profile of LDL subfractions

The distribution of the total lipoprotein mass among LDL gradient subfractions as a function of density in the CHL patients and in normolipidemic subjects is shown in **Fig. 1.** These distributions were confirmed upon comparison with the corresponding absorbance profiles recorded at 280 nm (data not shown).

The mass distribution profiles in the combined hyperlipidemic patients and in the normolipidemic subjects were quite distinct. Thus, in the latter, the profile was essentially symmetric, displaying a single, narrow peak in the interval of hydrated density from 1.029 to 1.039 g/ml (LDL-3) in each case, consistent with our earlier findings (12, 32). By marked contrast, the profile in CHL subjects was asymmetric, (Fig. 1B), with a peak density that was significantly shifted to a denser subfraction (LDL-4, d 1.039–1.050 g/ml) as compared to healthy subjects (LDL-3; $P < 0.01$). Furthermore, the overall profile in the CHL group was broader, with a more uniform distribution of mass between subfractions 3 and 4 (mean 30.8 and 35.5%, respectively); equally, the proportions of LDL-1, LDL-4, and LDL-5 were significantly higher, while that of LDL-3 was significantly lower, in the patient group relative to the normolipidemic subjects ($P < 0.01$).

The mean total plasma LDL concentration, determined as the sum of the mass of individual subfractions, was almost twofold higher in CHL patients than in normolipidemic subjects (525.0 ± 89 vs. 271.4 ± 47 mg/dl; $P < 0.001$). Furthermore, it is of note that the mean plasma concentrations of dense subspecies 4 and 5 (186.4 ± 26.1 and 60.4 ± 19.2 mg/dl, respectively) in CHL patients were significantly greater than those of the corresponding subspecies in the normolipidemic group (LDL-4, 61.1 ± 16.0 and LDL-5, 21.7 ± 5.5 mg/dl; $P < 0.01$ for corresponding subfractions). Equally, the concentration of the light subspecies, i.e., LDL-1, in the patient group was significantly elevated as compared to that in the normolipidemic group (47.3 ± 7.2 vs. 10.6 ± 5.0 mg/dl, respectively; $P < 0.01$).

When the possible relationships between plasma lipid and apolipoprotein levels and mass distribution profiles were evaluated by statistical analysis, it became evident that the major subfractions, i.e., LDL-4 in the CHL patients and LDL-3 in normolipidemic subjects, were most influenced by these factors. Overall, apoB concentration was negatively correlated with the proportion of LDL-3

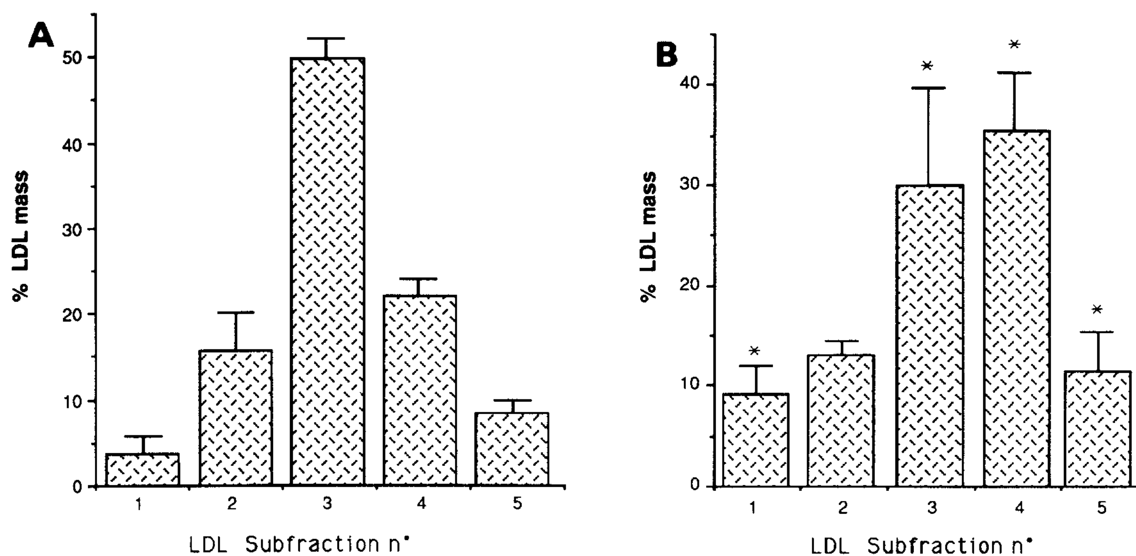

Fig. 1. Distribution of LDL mass between density gradient subfractions in (A) normolipidemic subjects and (B) CHL patients. The % of total mass of LDL (d 1.019 –1.063 g/ml) in individual gradient subfractions is plotted on the ordinate against subfraction number on the abscissa. Data are expressed as the means ± SD; in (A), n = 5, and in (B), n = 9. The mass of each chemical component in individual LDL gradient subfractions was determined by chemical analysis; the total mass of each subfraction was then calculated as the sum of the mass of each component. In (B), * indicates a significant difference relative to the corresponding subfraction in normolipidemic subjects ($P < 0.01$).

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(−0.81; $P < 0.01$) and positively correlated with that of LDL-4 (+0.92; $P < 0.01$). Equally, triglyceride and cholesterol levels were negatively correlated with the % LDL-3 (−0.77 and −0.71, respectively; $P < 0.01$) and positively with that of LDL-4 (+0.82 and +0.85, respectively; $P < 0.01$). Moreover, the concentration of HDL-cholesterol was positively correlated with the % LDL-4 (−0.51; $P < 0.05$). These findings clearly suggest that the degree of elevation in plasma triglyceride, cholesterol, and apoB levels is intimately linked to the extent of the shift in the LDL mass distribution profile towards denser subfractions.

Physicochemical characterization of LDL subfractions

Electrophoretic mobility of native LDL subspecies in agarose gel. The net electrical charge of each LDL subfraction was evaluated at neutral pH by electrophoresis in agarose gel. A single band of β-mobility was detectable in each subfraction upon Fat Red 7B staining (data not shown). Bands of either α or pre-β mobility were undetectable, attesting to the absence of contamination by both VLDL and HDL and, equally, to the absence of contamination by Lp[a]. In both CHL patients and normolipidemic subjects, the subfraction of intermediate density (i.e., LDL-3 of d 1.029–1.039 g/ml) consistently displayed the lowest mobility. Moreover, the lighter (LDL-1 and LDL-3) and denser (LDL-4 and LDL-5) subfractions exhibited mobilities that were from 5–20% greater than that typical of LDL-3. Similar findings were described in our earlier studies in a series of seven LDL subspecies from normolipidemic subjects (12).

Chemical composition. The mean weight % chemical compositions of native LDL density gradient subfractions 1–5 in CHL patients and in normolipidemic subjects are presented in **Table 2.** Cholesteryl ester (35.4–40.7%) was the major lipid component in all subfractions from both groups, the proportions in subfractions 2, 3, and 4 tending to be greater than those in either the lightest (LDL-1, d 1.019–1.023 g/ml) or densest (LDL-5, d 1.050–1.063 g/ml) subfractions. By contrast, the contribution of the second neutral lipid class, the triacylglycerols, was maximal at the lightest density (14–16%; LDL-1; $P < 0.001$ vs. LDL-2, 3, 4, and 5 in both groups), decreasing progressively with increase in density to attain a minimum (~5%) in LDL-4 from both CHL patients and normolipidemic subjects. It is noteworthy that a similarly elevated proportion of triglyceride (12.3 ± 3.3%) in an LDL subfraction of d 1.023 g/ml from healthy males was reported recently (51). LDL subfractions 2 and 3 in CHL patients were significantly enriched in triacylglycerols relative to the corresponding normolipidemic subfractions ($P < 0.05$). The total contribution of neutral core lipids to the overall composition of LDL subfractions in both groups varied over a narrow range (44.6–52.1% and

TABLE 2. Mean weight % chemical composition of native human LDL subfractions from combined hyperlipidemic patients (CHL) and from normolipidemic subjects (NLS)

	LDL Subfraction									
	1		2		3		4		5	
	d 1.019–1.023 g/ml		d 1.023–1.029 g/ml		d 1.029–1.039 g/ml		d 1.039–1.050 g/ml		d 1.050–1.063 g/ml	
Component	NLS	CHL	NLS	CHL	NLS	CHL	NLS	CHL	NLS	CHL
Cholesteryl ester	35.4 ± 1.6	35.8 ± 4.0	39.4 ± 1.9	36.5 ± 3.0	40.7 ± 1.5	38.1 ± 3.4	38.5 ± 0.6	39.7 ± 2.9	36.4 ± 3.2	36.6 ± 3.1
Free cholesterol	7.8 ± 2.4	7.9 ± 1.0	9.0 ± 0.8	8.9 ± 0.6	9.8 ± 0.3	9.2 ± 1.3	9.2 ± 0.15	7.9 ± 0.8	7.9 ± 0.5	7.0 ± 0.8
Triglyceride	14.1 ± 1.5	16.3 ± 6.7	6.6 ± 1.9	11.9 ± 4.7[a]	3.7 ± 1.0	7.2 ± 2.0[a]	4.9 ± 1.3	4.9 ± 1.25	5.4 ± 1.2	8.2 ± 3.0
Phospholipid[b]	19.9 ± 2.3	19.5 ± 2.5	20.3 ± 1.5	21.2 ± 1.3	20.8 ± 0.8	21.3 ± 2.3	21.6 ± 1.6	20.0 ± 1.9	21.9 ± 1.9	19.4 ± 2.3
Protein	22.7 ± 2.8	20.4 ± 4.0	23.8 ± 1.8	21.5 ± 2.9	24.9 ± 0.7	24.2 ± 1.5	25.6 ± 3.0	27.3 ± 1.6	28.3 ± 4.1	28.7 ± 2.7
CE/protein	1.56	1.80	1.65	1.70	1.63	1.57	1.50	1.45	1.29	1.28
TG/protein	0.62	0.80	0.28	0.55	0.15	0.30	0.19	0.18	0.19	0.29

Values are means ± SD of duplicate or triplicate determinations of each component; CHL patients, n = 9; NLS subjects, n = 5. Analyses were performed as described in Materials and Methods.
[a]Greater than controls, $P < 0.05$.
[b]Only choline-containing phospholipids were estimated.

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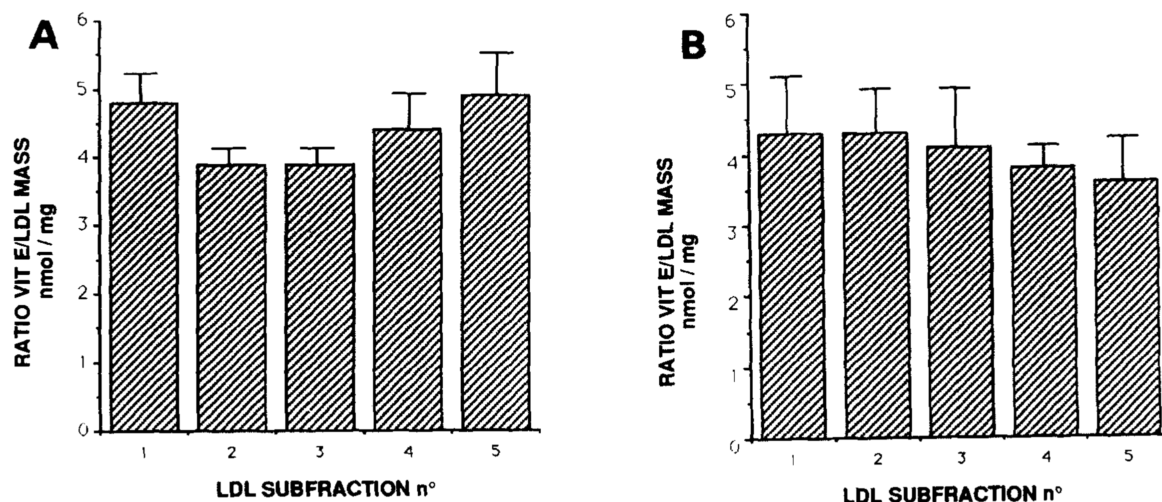

Fig. 2. Vitamin E content of LDL subfractions in (A) control subjects, and (B) CHL patients. Values are expressed as nmol vitamin E/mg total lipoprotein mass. Values are the means ± SD of duplicate determinations in four normolipidemic and nine CHL subjects.

41.8–49.5% in CHL patients and normolipidemic subjects, respectively), diminishing progressively with increase in density in each case. It is equally relevant that the total contribution of these neutral lipids to LDL core composition was consistently greater in each subfraction from CHL patients as compared to that in the corresponding normolipidemic subfraction ($P < 0.05$). With respect to the components of LDL surface structure, the protein content of both series of LDL subfractions increased progressively with elevation in density ($P < 0.001$) and in this respect counterbalanced the opposing evolution in the density profile of the core lipids. The proportions of the second hydrophilic component, the phospholipids, tended to be greatest in LDL-2, 3, and 4 (20.0–21.6%). Like phospholipid, the contribution of free cholesterol varied but little across the density profile.

Calculation of the cholesteryl ester/protein ratios confirmed the above findings, but in addition, revealed that the structural relationship between these components differed in subfractions from the CHL group as compared to normolipidemic subjects. Thus CE/P ratios in subfractions from the latter group were maximal (~ 1.6) in LDL of intermediate density (LDL-2 and 3), whereas this ratio decreased progressively from ~ 1.8 to 1.3 with increase in density in the CHL group (Table 2). The triglyceride/protein ratio was also distinct between the two groups; values were superior in all subfractions in the patient group with the exception of LDL-4, without attaining statistical significance. This ratio tended to diminish with increase in density, although minima were attained in LDL-3 in controls (~ 0.15) and in LDL-4 (~ 0.18) in the patients.

Vitamin E and lipid hydroperoxide content

The content of vitamin E in LDL subfractions from both CHL patients and normolipidemic subjects was expressed in two ways, first as nmol/mg LDL protein (data not shown), and second as nmol/mg lipoprotein mass (**Fig. 2A and B**). When expressed in the former manner, the densest subfraction (LDL-5) in CHL patients contained significantly less vitamin E than the lightest, i.e., LDL-1 (13.2 vs. 23.0 nmol/mg protein, respectively; $P < 0.005$). Indeed, a progressive reduction in vitamin E/protein ratio with increase in LDL subfraction density was observed in the CHL group. In contrast to the CHL patients, however, little variation in the vitamin E/protein ratio (range 15.5:1 to 17.2:1 nmol/mg) was seen among LDL subfractions 2 to 5 in the normolipidemic group. In both the normolipidemic and patient groups, the LDL-1 subfraction displayed the highest vitamin E/protein ratio (~ 22.5:1 as compared to ~ 23:1 in the CHL group).

The simplest explanation for the progressive decrease in vitamin E/protein ratio with increase in LDL density in the CHL group would derive from the effect of an increased proportion of protein per LDL particle in the denser subfractions on a relatively uniform content of vitamin E among LDL subspecies.[3] Indeed this hypothesis was supported by the finding that no significant difference could be detected between the vitamin E contents of individual subfractions in each group when expressed relative to total lipoprotein mass (Fig. 2A and B). Nonetheless, vitamin E content again tended to be lower in the denser subfractions LDL-4 and LDL-5 as compared to LDL-1 in CHL patients (Fig. 2B). By contrast, subfractions LDL-2 and LDL-3 in the normolipidemic subjects were poorest in vitamin E content, independent

[3]It is of note that LDL subspecies across the entire LDL density range contain one apoB-100 polypeptide per particle, and that the mass of lipid relative to apoB-100 decreases with increase in density (see Table 7, ref. 12).

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of the mode of its expression. Clearly then, certain compositional and structural differences among LDL subspecies in the CHL and normolipidemic groups may underlie such distinct distribution profiles of vitamin E (see Table 2).

The total lipid hydroperoxide contents of LDL subfractions were determined immediately after isolation and exhaustive dialysis and were consistently < 5 nmol/mg LDL protein (range 1.3-4.8) in both CHL and normolipidemic groups. These levels are quite comparable to those originally reported by El-Saadani et al. (46) in native LDL (mean 5.4 nmol/mg LDL), and establish that our LDL preparations had not undergone peroxidative damage during isolation. Furthermore, no significant differences were detectable between hydroperoxide levels in individual LDL subspecies of each group.

Particle size and heterogeneity of native LDL subfractions

Both hydrated particle size and particle heterogeneity were evaluated in each series of LDL subfractions from CHL patients and normolipidemic subjects by electrophoresis in 2-16% polyacrylamide gradient gels under nondenaturing conditions; representative gel patterns are shown in **Fig. 3A.** The mean particle diameters of the single major electrophoretic component in the respective subfractions from each group are presented in Fig. 3B; occasionally, minor electrophoretic components were detected that did not amount to more than ~ 10% on a densitometric basis. Particle size showed a marked and progressive diminution as density increased, the densest particles being the smallest. Particle diameters were significantly different ($P < 0.001$) between successive LDL subfractions in both normolipidemics as well as in CHL patients, with the exception of LDL-4 and LDL-5 in each group. Furthermore, the average size decrement between successive subfractions (LDL-1, 2, 3, and 4) was larger in patients (9.3 ± 2.9 Å) than in normolipidemic subjects (4 ± 1.3 Å) (Fig. 3B). Finally, significant differences were detected between the particle diameters of corresponding subfractions in CHL patients and normolipidemics (for LDL-1, LDL-3, LDL-4, and LDL-5; $P < 0.01$ in each case), LDL-1 being larger in patients than normolipidemic subjects, while the reverse was true for LDL-3, -4 and -5, these being smaller in the former group.

As noted above, a twofold elevation in mean plasma Lp[a] level was found in CHL patients as compared to our normolipidemic group (Table 1; 17 ± 8 vs. 7.4 ± 3 mg/dl; $P < 0.05$), although the mean value remained within the normal range (12). It is therefore of relevance that an Lp[a]-sized particle (~ 32 nm; ref. 37) was not detected on nondenaturing gel electrophoresis of LDL subspecies from CHL patients, in which the maximal diameter attained by LDL particles was ~28 nm (Fig. 3).

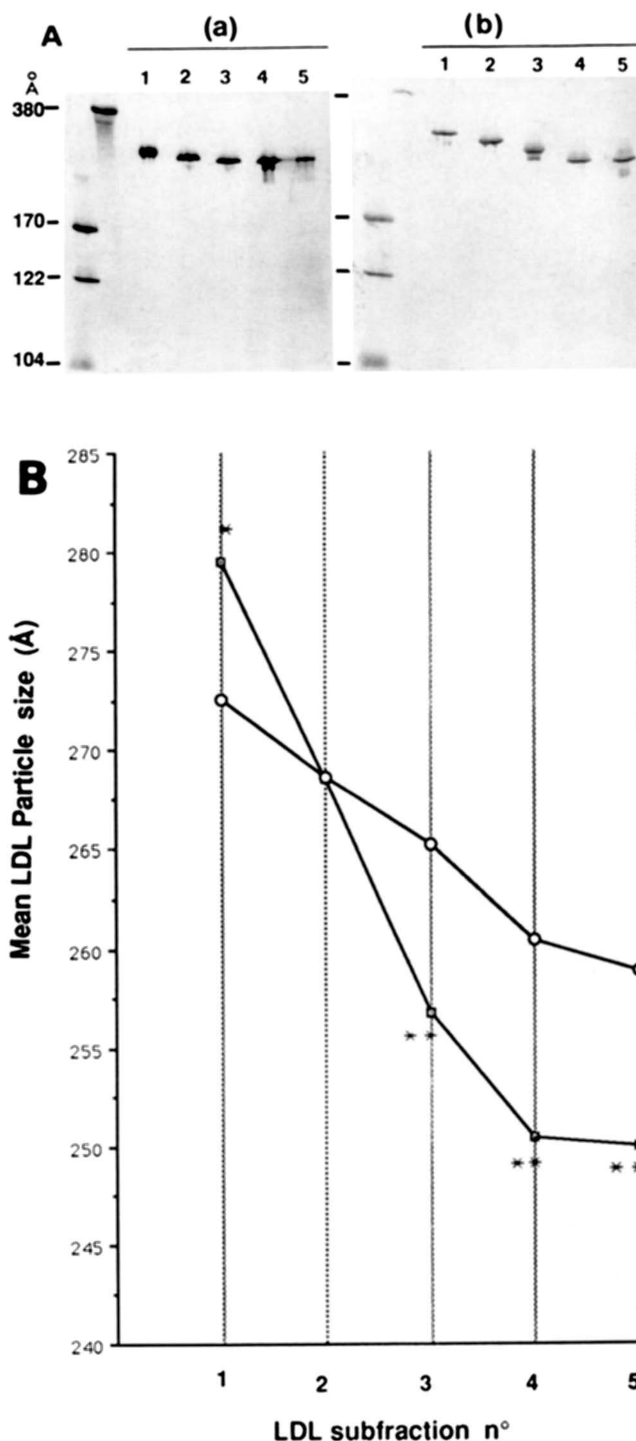

Fig. 3. Evaluation of LDL particle size and heterogeneity in CHL patients and control subjects by electrophoresis in nondenaturing polyacrylamide gradient gels (2-16%). (A) Electrophoretic patterns from a representative series of LDL subfractions from (a) a normolipidemic subject and (b) a CHL patient. LDL subfractions are labeled from 1 to 5 in each case, and from left to right on the photograph. A set of size markers was applied to the left outer lanes on the gel slabs (from top to bottom: latex beads, 380 Å; thyroglobulin, 170 Å; ferritin, 122 Å, and catalase, 104 Å). (B) mean particle sizes of LDL subfractions determined from gel electrophoretic patterns (as in (A)); (○), normolipidemic subjects (n = 5); (□), CHL patients (n = 9); • > controls, $P < 0.01$; •• < controls, $P < 0.01$.

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It was of interest to evaluate possible relationships between plasma lipid and apolipoprotein levels and LDL particle size. Thus, both triglyceride and apoB concentrations exerted significant influences on the particle sizes of the densest subfractions, i.e., LDL-4 and LDL-5. Indeed, the higher the triglyceride concentration, the smaller LDL-4 and LDL-5 particles became (correlations: −0.714 and −0.736 for LDL-4 and -5, respectively; $P < 0.01$ in each case). Equally, apoB level was negatively correlated with particle size in LDL-4 and -5 (−0.691 and −0.724 for LDL-4 and -5, respectively; $P < 0.01$ in each case).

Apolipoprotein composition of LDL subfractions

The apolipoprotein contents of the protein moieties of LDL subfractions from CHL patients and normolipidemic subjects were examined by electrophoresis in 5–19% SDS-polyacrylamide gel slabs and consistently revealed a single, high M_r band in each case, whose identity as apoB-100 was confirmed by immunoblotting with a monospecific polyclonal antibody to purified human apoB-100 (12) (data not shown). Trace amounts of polypeptides with lower molecular weight were also detected, notably apoA-I (M_r 26,900 ± 900) in LDL-4 and LDL-5 and apoE (M_r 35,500 ± 620) in LDL-1, -4, and -5 from both groups. On a densitometric basis, apoE represented less than 1% of the protein moiety in each case; by contrast, apoA-I content in LDL-5 typically attained 1–2% of apoLDL.

Oxidation of LDL subfractions

Formation of conjugated dienes was monitored at 236 nm during the copper-induced oxidation of a series of LDL subfractions from nine CHL patients and from five normolipidemic subjects, and the duration of the respective lag phases was calculated as outlined in the Methods section; results are summarized in **Table 3.** In CHL patients, LDL-5 was distinct ($P < 0.001$ vs. LDL-1, LDL-2, LDL-3, and LDL-4) in displaying the most rapid onset of oxidation with the shortest lag phase (91 ± 19 min) (Table 3). Indeed, the lag phases for subfractions 1, 2, 3, and 4 occurred consistently within the range of 110–190 min of oxidation under our experimental conditions.[4] When the rates of oxidation of LDL subfractions from CHL patients were calculated as a function of the molar amounts of conjugated dienes formed per min per mg LDL mass (26), then the mean oxidation rate for LDL-5 (4.8 ± 0.9 nmol diene/min per mg LDL) was greater than that for the remaining subfractions (range for LDL-1, -2, -3, and -4: 3.5–4.7 nmol/min per mg LDL mass), without attaining statistical significance. As in subfractions from the CHL group, the densest LDL subspecies (LDL-5) from normolipidemic subjects was distinct in displaying the shortest lag time (72 ± 23 min; Table 3). These findings, therefore, corroborate those reported recently in which dense, small LDL from healthy volunteers showed an increased oxidative susceptibility in vitro (26, 51). Furthermore, oxidation rates (estimated as conjugated diene formation, see above) in LDL-5 were elevated (5.1 ± 0.7 nmol/min per mg LDL) but not significantly different to those in LDL subspecies 1 to 4 from the same subjects, (range 3.3–4.6 nmol/min per mg protein).

All LDL subfractions from both normal subjects and CHL patients displayed markedly elevated electrophoretic mobility on agarose gel after copper-induced oxidation, a finding indicative of an increase in their net negative charge. The increase in the electrophoretic mobilities of all oxidized LDL subspecies occurred between 8 and 10 h of oxidation, and when expressed relative to their native counterparts (expressed as REM), ranged from 1.6 to 2.8:1; no significant differences were detected between subfractions. In the same electrophoretic system, REM values for acetylated LDL (~2:1–3:1) in our laboratory (54) overlap this latter range; moreover, such acetylated LDL are avidly taken up by the scavenger receptor of human monocyte-macrophages in vitro, resulting in high levels of cellular cholesterol loading (54). These observations are consistent with the suggestion that the present in vitro oxidation conditions give rise to modified form(s) of LDL, certain of whose physicochemical and structural properties resemble those of biologically modified LDL (5).

TABLE 3. Lag phases for the copper-induced oxidative formation of conjugated dienes in LDL subfractions from combined hyperlipidemic patients (CHL) and normolipidemic subjects (NLS)

LDL Subfraction	Lag Phase: CHL (n = 9)	Lag Phase: NLS (n = 5)
	min	
1	151 ± 38	142 ± 25
2	166 ± 34	176 ± 29
3	155 ± 41	183 ± 16
4	133 ± 28	139 ± 18
5	91 ± 19[a]	72 ± 23[a]

Values are means ± SD for duplicate determinations.
[a] $P < 0.001$ vs. LDL-1, LDL-2, LDL-3, and LDL-4.

[4] It is noteworthy that while the lag phase for subfraction LDL-5 in our CHL patients is comparable to those reported elsewhere for native preparations in normolipidemic subjects (26, 48-59, 52, 53), the lag phases found for subfractions LDL-1 to LDL-4 in our patient group were somewhat larger. Such prolongation of the lag phase is probably due to the elevated ratio of LDL mass to copper ion concentration (1 mg LDL Mass/5 μM Cu^{2+}) which we have used (see Methods).

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DISCUSSION

The present studies have identified unique features of lipoprotein profile in primary combined hyperlipidemia (27), and the preponderance of dense, small LDL subspecies (LDL-4 and -5, d 1.039–1.063 g/ml). Indeed, LDL-4 and -5 together represented ~47% of total LDL mass in the CHL group as compared to ~30% in normolipidemic subjects, giving rise to a highly asymmetric profile (Fig. 1). Moreover, the absolute amounts of such dense LDL in CHL patients were some threefold greater than those in controls (247 vs. 83 mg/dl, respectively). The high degree of resemblance in the profile of lipoprotein mass as a function of density among the nine CHL patients was indeed remarkable, and may well reflect the rigorous biological and clinical criteria that were applied in their selection.

It is now well established that a predominance of dense LDL subspecies is associated with an elevated risk for the precocious development of atherosclerosis in certain forms of dyslipidemia, and notably in hyperapobetalipoproteinemia (13, 19, 23), in type IV hypertriglyceridemia (14, 25, 24), and in a small proportion (29%) of family members from large kindreds presenting familial combined hyperlipidemia (22). If the present findings are considered together with those in type IV hypertriglyceridemia, certain FCHL subjects, and patients presenting the hypertriglyceridemic form of hyperapobetalipoproteinemia (13), then it is apparent that elevated levels of triglyceride-rich lipoproteins are intimately associated with such a shift in LDL subspecies profile. Our results reinforce this hypothesis, as a significant correlation was detected between plasma triglyceride levels and the concentration of LDL-4, the major subspecies. Despite qualitative similarities, however, between the LDL subspecies profile in type IV hypertriglyceridemia and that found presently in CHL, the marked quantitative differences in absolute LDL concentrations, which may be up to threefold higher in CHL, should be borne in mind.

The precise mechanisms that underlie the asymmetric LDL profile in CHL and in which dense LDL predominate are as yet largely undefined. Several processes may contribute, and may differ according to the patient considered; these processes include elevated hepatic production rates of apoB-containing lipoproteins (27), modified intravascular lipolysis of VLDL precursors (55), direct production of dense LDL (27, 55, 56), altered rates of catabolism of LDL by the LDL receptor-dependent pathway (24), and diminished rates of transfer and exchange of neutral lipids between VLDL, LDL, and HDL (57). These processes may be loosely grouped into those that might potentially contribute to production of dense LDL subspecies, and those that would enhance reduction in their catabolism; together, such processes would favor accumulation of dense LDL in plasma.

A primary mechanism underlying elevated levels of both VLDL and LDL in CHL patients involves increased entry of apoB-containing lipoproteins into plasma, either as VLDL or directly as LDL (27); indeed, some 75% of LDL-apoB was derived from a source independent of VLDL (27). By contrast, the fractional catabolic rate of LDL in CHL patients is comparable to that in normals (27), tending, therefore, to exclude significant anomalies in LDL receptor activities or in the affinity of LDL particles for this receptor. Nonetheless, in vivo turnover studies were performed on unfractionated LDL (27), and therefore a diminished receptor binding affinity of dense LDL subspecies cannot be entirely discounted; evidence for such a hypothesis has been provided in both normolipidemic and hypertriglyceridemic subjects (24, 25). With respect to processes of intravascular transformation between LDL subpopulations, light LDL are precursors of dense particles, at least in hyperapobetalipoproteinemia (28). If indeed cholesteryl ester transfer from HDL to LDL constitutes a driving force favoring conversion of dense to light LDL via increase in cholesteryl ester core content (58), then the observed tendency to elevated triglyceride content of LDL subspecies (with the exception of LDL-4) in CHL patients might reflect a defect in this key process. Evidence for such a hypothesis derives from our finding that the particle size, and thus molecular weight, of dense LDL (LDL-4 and -5) are significantly smaller in CHL patients than in controls (Fig. 3), implying that their lipid cores contain a reduced number of cholesteryl ester molecules (12), but whose proportion as a wt % is within the normal range (Table 2). Considered together, the above mechanisms may account, at least in part, for formation of increased amounts of dense LDL subspecies in combined hyperlipidemia.

A second significant aspect of these studies concerns the finding that the densest LDL (LDL-5, d 1.050–1.063 g/ml) showed the lowest level of resistance to oxidative stress among the five LDL subspecies. The biochemical processes underlying the copper-catalyzed oxidation of LDL are complex (5, 48–50, 52, 59). Among the multiple parameters that may be measured as indices of LDL oxidation, it has become evident that the continuous monitoring at 234 nm of the conversion of unsaturated fatty acids into fatty acid hydroperoxides with conjugated double bonds is particularly informative (48–50, 52). The time course of such conjugated diene formation involves three phases, an initial lag phase during which time 234 nm absorption is rather stable, followed by a rapid increase in absorption indicating that lipid peroxidation has entered the propagation phase, and finally, a decomposition phase occurring after maximal diene absorption (52).

Initiation of the propagation phase is intimately linked to the antioxidant content of LDL particles, among which vitamin E is a major component (48–50, 53, 60). Indeed, a tendency to a deficiency in antioxidant content with increase in density and decrease in particle size was seen in

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the vitamin E profile of LDL subspecies in the CHL group. The statistical significance of this observation was however restricted to LDL-5 and only in the case when data were expressed as the ratio of vitamin E to particle protein content (results not shown). A similar, but less marked, tendency was also noted upon expression of vitamin E content relative to the mass of each LDL subspecies in the CHL group (Fig. 2B). Clearly then, minor variation in vitamin E content between LDL subspecies may only partially account for the marked reduction in oxidative resistance in the dense LDL subspecies (LDL-5) of CHL patients.

In contrast to our findings in the CHL patients, the content of vitamin E varied but little among LDL subspecies in the normolipidemic group when expressed either as a function of protein content or of LDL particle mass (Fig. 2A). Despite these observations, however, it will be of considerable interest to evaluate the factors that may influence the distribution of vitamin E and other antioxidants among LDL particle species in different dyslipidemic states. Indeed, in the present context, it may be asked whether the distinct vitamin E profiles seen in the CHL and normolipidemic groups reflect differences in the chemical composition and molecular organization of surface and core constituents in corresponding LDL subspecies from each group (chemical composition, Table 2; particle size, Fig. 4). Nonetheless, the densest LDL subspecies in our control subjects, LDL-5, again showed the lowest resistance to oxidative stress induced by copper ions (Table 3), tending therefore to confirm the profile of susceptibility to oxidation seen among LDL subfractions from healthy subjects by both de Graaf et al. (26) and Tribble et al. (51). Furthermore, the present data in both the CHL and normolipidemic groups, together with those of the latter authors (26, 51), strongly imply that the vitamin E content of human LDL is not the sole determinant of oxidative resistance in vitro.

An important question now arises: what features of chemical composition and particle structure render dense LDL so susceptible to oxidative stress? One possibility concerns the small particle size, and thus molecular weight, of dense LDL and suggests that the molecular packing and interactions of lipid and apoprotein components in these particles may be unique. For example, such molecular packing might alter the antioxidant efficiency of vitamin E. Support for an intimate relationship between molecular packing and resistance to oxidative modification has recently been afforded by the data of Tribble et al. (51), in which the free cholesterol content of LDL subfractions was the most closely associated with resistance to oxidative susceptibility. We did not, however, detect such an association in LDL subspecies from either our normolipidemic control subjects or in those isolated from the patient group.

Other compositional and structural factors may exert profound influence on the resistance of LDL to oxidative stress, and prominent among them is the fatty acid content of the lipid esters. Thus, LDL enriched in polyunsaturated fatty acids (two or more double bonds) are readily oxidized (61). The low oxidative resistance of dense LDL (LDL-3, d 1.040–1.045 g/ml) in healthy subjects was significantly correlated with an elevated content of both linoleic and arachidonic acids (50% of total fatty acids; ref. 26). Nonetheless, the lag phase for copper-induced diene formation in LDL-2 closely resembled that of the LDL-3 subfraction (26), clearly suggesting that additional factors contributed significantly to their low resistance to oxidative stress.

Recent gas chromatographic analyses of the fatty acid profiles of LDL subfractions in both the CHL and normolipidemic groups in our laboratory are entirely consistent with the above findings. Thus, dense LDL did not possess a unique fatty acid profile in either group. Rather, LDL subspecies 3, 4, and 5 in both groups were moderately enriched in polyunsaturated fatty acids (mainly 18:2 and 20:4; 46.8 ± 2.5, 46.5 ± 4.2, and 45.3 ± 5.2% of total fatty acids, respectively), as compared to LDL-1 and LDL-2 (33.3 ± 8.5 and 41.5 ± 3.3% of total, respectively); expression of the latter data as μg fatty acid/mg LDL mass confirmed these overall features of fatty acid profile. Finally, it is noteworthy that the total fatty acid pattern of individual LDL subspecies in CHL patients and that of the corresponding subspecies in the control group could not be distinguished, and therefore fatty acid data from both groups (n = 4) were pooled for the above calculations (C. Flament, F. Nigon, and M. J. Chapman, unpublished data).

In light,. then, of our observations on the diminished oxidative resistance of LDL subspecies in both CHL patients and normolipidemic subjects, we conclude that other factor(s), in addition to vitamin E, are implicated in the total antioxidant potential of LDL particles. This finding is entirely consistent with data reported elsewhere (26, 51, 53, 60). It will therefore be of considerable importance to precisely determine both the nature and structural configuration of the chemical constituents of dense LDL particles which render them so vulnerable to free radical-mediated peroxidative modification.

Oxidized LDL occur in vivo in the arterial wall and may contribute to lesion formation via multiple mechanisms (5, 62) including their recognition by scavenger receptors and subsequent uptake by monocyte-derived macrophages to form foam cells (5). Our present studies suggest, therefore, that elevated levels of dense LDL subspecies with diminished oxidative resistance in CHL patients may contribute significantly to the premature vascular disease which they frequently manifest.

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