

Identification and cholesterol quantification of low density lipoprotein subclasses in young adults by VAP-II methodology

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Abstract Low density lipoprotein (LDL) particles are heterogeneous in size, density, and chemical composition; small, dense LDL may be more atherogenic than large, buoyant LDL. We have developed a rapid microscale method called LDL VAP-II (Vertical Auto Profile-II) for quantification of cholesterol in LDL subclasses. The method is based upon a short (1 h) single vertical spin density-gradient ultracentrifugation and on-line VAP-II analyzer. LDL VAP-II is rapid and reproducible. Using this method five LDL subclasses, designated as LDL-1 (most buoyant) through LDL-5 (most dense), have been identified in a population consisting of 195 medical students (ages, 22–29 years). The R_f (relative position of the major LDL peak in the density gradient; the higher the R_f value, the lower the peak density) was significantly positively correlated with cholesterol levels of high density lipoprotein (HDL) ($r = 0.594$), HDL₃ (0.350) and HDL₂ (0.625), and significantly negatively correlated with triglycerides (TG) (-0.355) and cholesterol levels of very low density lipoprotein (VLDL) (-0.386) and intermediate density lipoprotein (IDL) (-0.432). These results are consistent with those obtained by other investigators. The R_f value was significantly correlated with peak particle diameter as determined by non-denaturing gradient gel electrophoresis ($r = 0.859$). In a forward stepwise multivariate analysis comparing R_f with sex, VLDL, LDL, Lp[a], IDL, HDL₃, HDL₂, and triglyceride, only HDL₂ remained in the model.—Kulkarni, K. R., D. W. Garber, M. K. Jones, and J. P. Segrest. Identification and cholesterol quantification of low density lipoprotein subclasses in young adults by VAP-II methodology. *J. Lipid Res.* 1995. **36**: 2291–2302.

Supplementary key words LDL heterogeneity • single vertical spin density-gradient ultracentrifugation • gradient gel electrophoresis

Elevated plasma concentrations of low density lipoprotein (LDL) cholesterol are strongly and positively linked to increased risk of coronary heart disease (CHD) (1). LDL particles are heterogeneous in their physical and chemical properties, including hydrated density, particle size and shape, and chemical composition. Several subclasses of LDL with differing physico-chemical

properties are known to exist in patients with hypertriglyceridemia (2), as well as in normal subjects (3).

There has been an increasing interest in LDL subclasses recently due to the finding from several case-control studies that small, dense LDL is more prevalent in CHD patients than in control subjects (4–7), thus linking small, dense LDL to increased risk of CHD. The increased presence of small, dense LDL is also associated with insulin resistance states such as are seen in patients with non-insulin-dependent diabetes mellitus (8), abdominal obesity (9) and hypertension (9, 10). Using non-denaturing gradient gel electrophoresis, Krauss and Burke (11) have identified seven LDL size subclasses, and Austin et al. (12) observed two distinct patterns of distribution of these subclasses in human plasma corresponding to two phenotypes: pattern A, comprising mainly large, buoyant LDL particles with peak particle diameter >255 Å, and pattern B, with the predominance of small, dense LDL particles having peak particle diameter ≤ 255 Å. In a case-control study (13), a 3-fold increase in CHD risk was attributed to the presence of LDL pattern B, although the significance of its association with CHD disappeared when contributions due to triglycerides (TG) and high density lipoprotein (HDL) cholesterol were taken into consideration. However, the presence of pattern B has been shown to be strongly associated with the high risk lipoprotein

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; IDL, intermediate density lipoproteins; VLDL, very low density lipoproteins; Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; TC, total cholesterol; TG, triglycerides; VAP, Vertical Auto Profile; NWLRL, Northwest Lipid Research Laboratories; CDC, Centers for Disease Control and Prevention; NHLBI, National Heart, Lung, and Blood Institute; NCEP, National Cholesterol Education Program.

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profile characterized by increased levels of TG, apolipoprotein (apo) B, intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL), and decreased levels of HDL cholesterol and apoA-I (12). Thus, pattern B (small, dense LDL) may serve as a marker of an atherogenic lipoprotein profile.

Recent reports have also suggested that the LDL size distribution is, in part, genetically controlled and that the LDL subclass pattern B may be linked to multiple genes, including the LDL receptor locus on the short arm of chromosome 19p, the apoA-I/C-III/A-IV cluster on chromosome 16, and the manganese superoxide dismutase gene on chromosome 6 (4, 14). However, environmental effects are known to influence the LDL size distribution as well (15). The expression of LDL subclass pattern B is also found to be age-dependent, usually expressed only after the age of 20 in men and after menopause in women.

Among various techniques available for LDL subclass analysis, non-denaturing gradient gel electrophoresis and ultracentrifugation methods, including sequential flotation (16), analytical (17), and various density-gradient methods (18–21), have been widely used. All currently used ultracentrifugation procedures are lengthy and laborious, and thus are restricted to research studies. Non-denaturing 2–16% polyacrylamide gradient gel electrophoresis is the most common method (11, 15, 22, 23) because it requires only a small amount of plasma, can analyze multiple samples on a single gel, and can be performed in most laboratories. However, the lack of availability of commercial gels could make reproducibility a problem for new users of this technique. Thus,

additional methods will be beneficial for the applications involving LDL subclasses.

Here we describe a rapid and reproducible method called LDL VAP-II (Vertical Auto Profile-II) which requires less than 3 h for the complete analysis of eight samples while using only 70 μ l of plasma per sample. Clinical application of this technique to the study of LDL subclass distribution in a healthy population consisting of 195 medical students is demonstrated.

MATERIALS AND METHODS

Study subjects

Blood samples from 195 fasting medical students from three different classes (68 women and 127 men, ages 22–29 years) attending the University of Alabama at Birmingham were drawn into EDTA-containing Vacutainer tubes, and plasma was separated from the blood by low speed centrifugation. As part of education and cholesterol awareness, each year sophomore medical students are provided with their complete lipoprotein cholesterol profile test performed by the Atherosclerosis Research Unit at the University of Alabama at Birmingham using the VAP-I method described previously (24–26). Aliquots from these plasma samples were used for LDL VAP-II analysis and gradient gel electrophoresis.

Lipoprotein analysis

Total and lipoprotein [HDL, HDL₃, HDL₂, lipoprotein[a] (Lp[a]), LDL, IDL, and VLDL] cholesterol con-

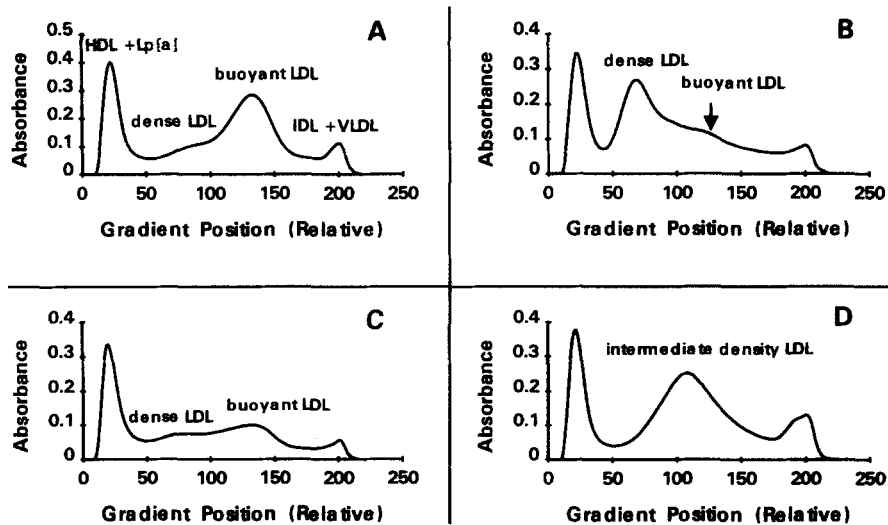


Fig. 1. Representative examples of LDL VAP-II absorbance curves (profiles) as recorded by the chart recorder. LDL subclasses were separated by single vertical spin density-gradient ultracentrifugation, and cholesterol distribution in the gradient was measured using the on-line continuous flow VAP-II analyzer to obtain absorbance curves corresponding to LDL VAP-II profiles.

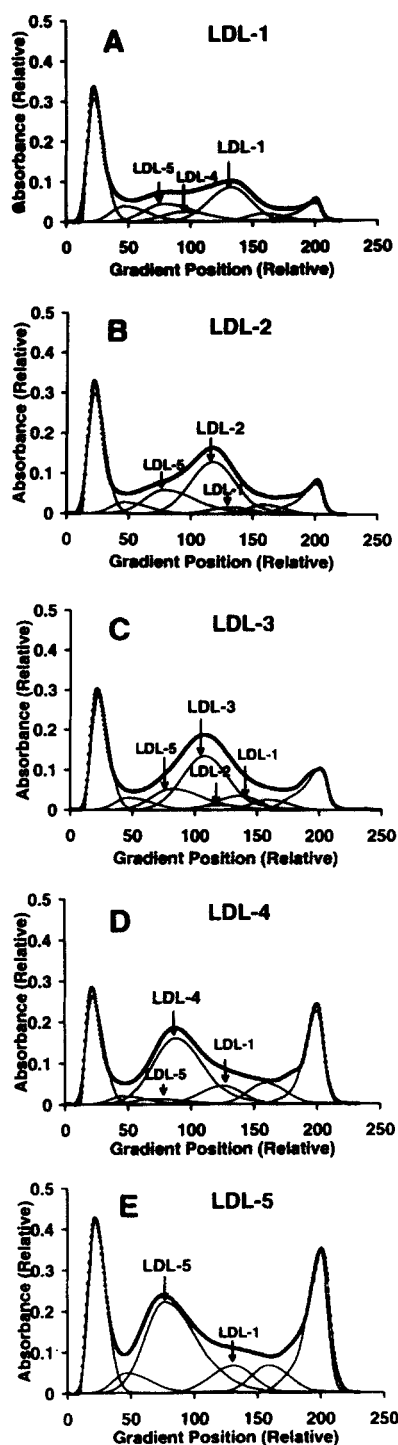


Fig. 3. Computer deconvoluted LDL VAP-II profiles illustrating multiple LDL subclasses. Cholesterol concentrations of all LDL subclasses in each profile are calculated by deconvoluting the digitized output of LDL VAP-II profile as described in Materials and Methods using software developed in this laboratory. Each profile shows the major LDL subclass and minor LDL subclasses that are present. LDL subclasses are shown by solid curves and the parent absorbance curve (the absorbance curve prior to deconvolution) is shown by empty circles (each circle representing a data point) connected by solid line.

The absorbance curve corresponding to the cholesterol distribution in the centrifuge tube was monitored by a strip chart recorder, while digitized absorbance data were collected using an analog-to-digital conversion board and a personal computer. The data points are collected at the rate of 60 points per min. As it requires approximately 6.5 min for the complete analysis of one sample, approximately 400 data points are stored for each profile. However, the number of data points for all profiles is normalized to a predetermined value (usually the data point number for IDL + VLDL peak maximum being adjusted to 200) in order to be able to compare profiles with slightly varying analysis times. A tube containing a plasma sample whose total cholesterol concentration was measured by the Northwest Lipid Research Laboratories (NWLRL) at Seattle, WA was included in each rotor in order to convert the area under each LDL VAP-II absorbance curve into its cholesterol concentration. The digitized and normalized absorbance curve was mathematically deconvoluted into its components (subcurves) using software developed in this laboratory, providing cholesterol values of six LDL subclasses. The software was developed by defining a set of exponential gaussian subcurves for each lipoprotein class. The numbers of subcurves defined for the lipoprotein classes were: LDL, 6; HDL + Lp[a], 3; and IDL + VLDL, 3. The rationale for defining six subcurves for LDL is described in the Results section. The shape parameters (widths at half height and the exponential parameter) of subcurves of all lipoprotein classes were adjusted to provide optimal fit of the lipoprotein class under most conditions. During a profile deconvolution, peak heights for the pre-defined subcurves for all lipoprotein classes were simultaneously varied until the sum of the squared deviations between the sum of the subcurves and the parent absorbance curve was minimized using a linear regression method.

Non-denaturing polyacrylamide gradient gel electrophoresis

Non-denaturing 2–16% polyacrylamide gradient gel electrophoresis was performed on plasma samples obtained from a subset consisting of 32 students at the Donner Laboratory, University of California at Berkeley, CA, as described previously (29). Gels were stained for lipid with 0.1 M Oil Red O, and LDL peak particle diameter was determined using calibration markers (29). LDL subclass patterns were assigned as previously described (12).

Statistical methods

Cholesterol and triglyceride values are expressed as mean (mg/dl) \pm SD. Tests of significance were performed using Student's *t* test (paired or unpaired as

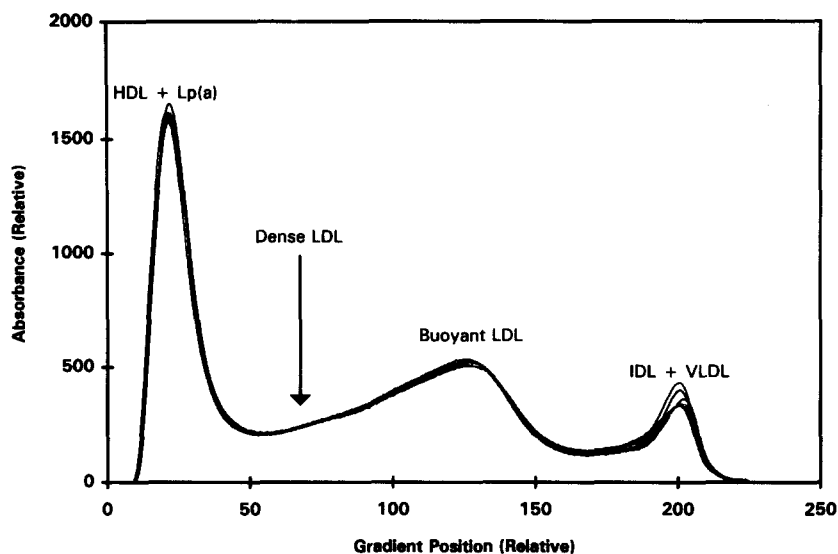


Fig. 4. LDL VAP-II absorbance curves (profiles) demonstrating within-rotor reproducibility of the method. Eight aliquots of the same plasma sample were centrifuged within a single rotor using the conditions described for LDL VAP-II in Materials and Methods. Absorbance curves were obtained by continuous analysis of the contents of the centrifuged tubes for cholesterol using VAP-II analyzer.

required). Simple linear regression was used to calculate Pearson correlation coefficients for the comparison of two measurements or two techniques. Stepwise multivariate regression analysis was used to compare the relative association of various lipoprotein cholesterol, TG, and sex with LDL R_f (relative position of the major LDL peak in the density gradient).

RESULTS

LDL VAP-II and its characterization

Figure 1 shows four representative examples (A–D) of LDL VAP-II cholesterol absorbance curves (profiles) as recorded on the chart paper. Two peaks of LDL, corresponding to dense and buoyant LDL, are apparent in all profiles except profile D; thus, LDL VAP-II is able to differentiate at least two LDL peaks in most cases. Furthermore, profile A is characterized by the predominance of buoyant LDL, whereas profile B is characterized by the predominance of dense LDL. The presence of nearly equal proportions of both the dense and buoyant LDL peaks can occasionally be observed as shown by profile C, while a single broad LDL peak with an intermediate density is seen in profile D.

Although only two LDL peaks are observed in most LDL VAP-II profiles, these peaks do not always occur at the same density positions. LDL subclasses distribute around several density positions, as determined by the R_f distribution. (R_f is the relative position of the major

LDL peak maximum in the density gradient and is calculated by dividing the distance of LDL peak maximum measured from the beginning of the HDL + Lp[a] peak by the distance of IDL + VLDL peak maximum, also measured from the beginning of the HDL + Lp[a] peak.) **Figure 2** shows the frequency distribution of R_f values of the major LDL peak in LDL VAP-II profiles of all 195 medical students. The figure indicates that the R_f values distribute into five clusters in this population, suggesting the existence of at least five LDL subclasses, each cluster corresponding to one LDL subclass. The LDL subclasses are designated as LDL-1 (most buoyant) through LDL-5 (most dense). The underlying reason for relating one cluster to one subclass is the assumption that all subjects with the same major LDL subclass will have similar R_f values, varying slightly from one another due to the experimental variation, and therefore form a cluster around a mid- R_f value. The R_f values corresponding to the mid-positions of these five clusters were, in turn, used to assign peak maximal positions for the five LDL subcurves in LDL VAP-II profile. However, in several profiles a minor but distinct peak (similar to the dense LDL peak seen in profile A of Fig. 1), even denser than LDL-5, was noticed. Therefore, a sixth subcurve (LDL-6) with its peak position corresponding to the position of this minor peak in the main profile was included.

Mathematical deconvolution software was developed utilizing these LDL subclass peak positions and other peak parameters as described in Materials and Methods to determine cholesterol concentrations of LDL sub-

classes. **Figure 3** shows examples of deconvoluted LDL VAP-II profiles displaying both the major LDL subclass and minor LDL subclasses present. The major subclass curve in each example has a corresponding peak in the main profile (absorbance curve). The predominant LDL subclass (the subclass with the highest cholesterol concentration) in each profile was used to classify the profile, thus LDL-1, LDL-2, LDL-3, LDL-4, and LDL-5 are the major subclasses in profiles A, B, C, D, and E, respectively. The cholesterol concentrations of the subclasses are determined by measuring the area under each subcurve and converting it into cholesterol concentration using a calibrator plasma of known cholesterol content. The software also provides a printout with cholesterol values assigned to each of the six LDL subclasses (including the minor LDL-6 subclass) and graphical output of the deconvoluted profile. Absence of any minor subclass results in a subcurve with negative area and thus a less than optimal curve fit. Therefore, subcurves with negative areas are sequentially excluded from the deconvolution process until an optimal fit is obtained. A zero value is assigned to each excluded subclass and thus is not displayed in the graphical output.

Reproducibility of the LDL VAP-II method was assessed using 28 aliquots of the same plasma sample in four rotors, each rotor containing 7 aliquots in addition to an aliquot of a calibration plasma. Within-rotor reproducibility values of the assay, calculated as the coefficient of variation (CV) of the cholesterol concentration of the major LDL subclass, for the four rotors were: 1.9%, 8.3%, 3.6%, and 4.6%, respectively. Excellent within-rotor reproducibility of the method is also demonstrated in **Fig. 4** by the superimposable LDL VAP-II cholesterol absorbance curves obtained from 8 aliquots of the same sample. Between-rotor CV calculated for the above four rotors was 5.2%. Long-term reproducibility of this method studied using 13 aliquots of a single sample in 13 rotors over a period of several days also yielded a highly satisfactory CV of 4.6%. The high reproducibility of this method is due to the continuous nature of the analysis. This considerably reduces the variability usually observed when multiple determinations are made on fractions collected in test tubes.

In order to assess the relationship between the density (as measured by the R_f) and the size of the major LDL peak, 2–16% non-denaturing polyacrylamide gradient gel electrophoresis analyses were performed on a subset consisting of 32 samples at the Donner Laboratory, University of California, Berkeley, CA. R_f of the major LDL peak in LDL VAP-II profile significantly correlated with the peak particle diameter of the predominant LDL peak present in the corresponding densitometric scan of the gradient gel electrophoresis. (**Fig. 5**; $r = 0.859$; P

< 0.0001). In addition, each subject was assigned an LDL size distribution pattern as A, B, or AB (intermediate pattern) by the Donner Laboratory. Of the 32 subjects, 59% had pattern A, 34% pattern AB, and 6% pattern B. The same 32 subjects were also classified based on their major LDL subclass as obtained from LDL VAP-II. Comparison of the two classifications suggested that among all pattern A subjects, 68% had either LDL-1 or LDL-2; 26%, LDL-3; 5%, LDL-4; and 0%, LDL-5 as their major LDL subclass. Among the subjects with pattern AB, 55% had LDL-4, 27% LDL-3, and 18% LDL-5 as their major LDL subclass. All subjects with pattern B had LDL-5 as their major LDL subclass. Thus, LDL-1 and LDL-2 can be considered to be as equivalent to pattern A, LDL-3 and LDL-4 as pattern AB, and LDL-5 as pattern B. It is also noteworthy that subjects with LDL-5 who were classified as having pattern AB by the gel analysis also showed an additional peak with nearly equal peak height and area as that of the predominant LDL peak with its peak particle diameter $< 255 \text{ \AA}$, further suggesting LDL-5 to be more similar to pattern B LDL. These data suggest a reasonable agreement between the two classifications that are based on two different physical properties.

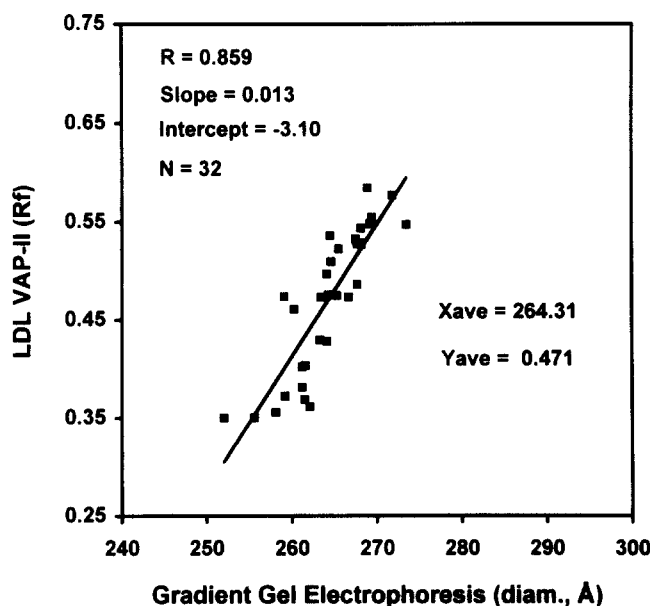


Fig. 5. Comparison of R_f of the major LDL peak in LDL VAP-II profile with the peak particle diameter of the major LDL peak obtained from the gradient gel electrophoresis. Plasma aliquots obtained from a subset consisting of 32 samples were used for the analysis by LDL VAP-II and by 2–16% non-denaturing gradient gel electrophoresis. Gradient gel electrophoresis analysis was performed at Donner Laboratory, University of California, Berkeley. R_f is the relative peak position of the major LDL peak maximum in the density gradient; higher R_f indicates lower peak density.

Clinical application of LDL VAP-II

The mean concentration values of total cholesterol, triglyceride, R_f of the major LDL peak, and various lipoprotein cholesterol fractions obtained for women, men, and for all subjects together, are presented in **Table 1**. As the VAP method separates both Lp[a] and IDL from the "real LDL" (LDL-R), these two lipoprotein measurements were also included in the present study. However, in order to be also consistent with LDL values reported by others, including NCEP, Lp[a] and IDL cholesterol values were added to the LDL-R and the sum is referred to as LDL(NCEP). The mean values of all lipoproteins for both women and men were typical. HDL, HDL₃, and HDL₂ cholesterol values in women were significantly higher than in men, while IDL and VLDL cholesterol were significantly higher in men than in women. Although TG values were higher in men than women, the difference in the mean values did not reach significance ($P > 0.05$). Total, Lp[a], LDL-R, and LDL(NCEP) cholesterol did not significantly differ in the two groups. The mean R_f of the major LDL peak for women was significantly higher than men ($P < 0.001$) suggesting that women generally had more buoyant LDL than men. The frequency distribution diagram of R_f of the major LDL peak for women and men (**Fig. 6**) also suggested that women tend to have more buoyant LDL than men. This is apparent by the skewing of the R_f distribution somewhat towards higher R_f values for women. On the contrary, distribution was skewed towards lower R_f in the case of men. The R_f value for the 50th percentile distribution for women was 0.505 com-

pared to 0.465 found for men ($P < 0.0001$). In addition, LDL subclasses in women appeared to be less dense compared to their respective subclasses in men, as indicated by a shift in the positions of the clusters towards the right in the diagram shown for women. (The respective mid- R_f values for clusters corresponding to LDL-1, -2, -3, -4, and -5 for women are 0.62, 0.555, 0.505, 0.45, and 0.38, while the corresponding values for men are 0.565, 0.515, 0.47, 0.415, and 0.34, respectively.)

The association of R_f of the major LDL peak with various lipoproteins was determined from the Pearson product-moment correlation coefficients calculated from R_f and the respective lipoprotein values. The results are shown in **Table 2**. R_f correlated strongly positively with HDL, HDL₃, and HDL₂ cholesterol, and negatively with IDL and VLDL cholesterol, and with TG when all subjects were considered together. Although LDL-R correlated significantly ($P < 0.05$) with R_f , the association was not as strong as for other lipoproteins. However, LDL(NCEP) correlated strongly ($P < 0.01$) probably due to the strong contribution by IDL. The association of HDL₂ with R_f was the strongest among all measurements. All measurements, except TC, HDL₃ and Lp[a] cholesterol, were correlated with R_f in women. Although TC did not correlate significantly, its association approached significance ($P < 0.1$; the corresponding P value for men, however, is >0.2); this is reflected in the association of LDL-R in women only, as LDL-R is the major fraction of TC ($P < 0.05$). In men, R_f correlated with HDL, HDL₃, HDL₂, IDL, and VLDL cholesterol, and TG, but not with LDL-R cholesterol, Lp[a] cholesterol, LDL(NCEP) cholesterol, or TC. HDL, HDL₃, and

TABLE 1. Mean plasma lipoprotein concentrations in medical students selected for this study

Parameter	All	Women	Men	<i>P</i> (Women vs. Men)
n	195	68	127	
LDL R_f^e	0.475 ± 0.071	0.51 ± 0.064	0.456 ± 0.067	<0.001
TC	171.6 ± 30.6	174.9 ± 27.0	169.8 ± 32.3	>0.2 (n.s)
HDL ^b	45.7 ± 9.8	52.4 ± 9.0	42.1 ± 8.3	<0.0001
HDL ₃ ^b	35.8 ± 5.6	38.4 ± 5.1	34.5 ± 5.4	< 0.001
HDL ₂ ^b	9.8 ± 6.2	14.0 ± 6.6	7.6 ± 4.7	<0.0001
Lp[a] ^b	7.7 ± 4.9	7.4 ± 4.5	7.9 ± 5.2	>0.4 (n.s)
LDL-R ^b	88.0 ± 22.3	88.0 ± 23.4	88.0 ± 21.8	>0.9 (n.s)
IDL ^b	1.5 ± 5.2	10.6 ± 4.2	12.0 ± 5.6	<0.05
LDL(NCEP) ^{b,c}	107.3 ± 26.6	106.1 ± 25.7	107.9 ± 27.1	>0.6(n.s)
VLDL ^b	18.5 ± 9.4	16.0 ± 7.2	19.9 ± 10.1	<0.01
TG	0.1 ± 53.8 (n = 102)	78.5 ± 43.8 (n = 34)	95.9 ± 57.6 (n = 68)	>0.05 (n.s)
Age (y)	24.3 ± 1.6	24.4 ± 1.8	24.3 ± 1.6	>0.6 (n.s)

Values are given as mean ± SD; n.s., nonsignificant (all P values > 0.05 were considered as nonsignificant). Parameter range: TC, 109–269; HDL, 24–78; HDL₃, 21–50; HDL₂, 0–36; Lp[a], 0–30; LDL-R, 44–166; IDL, 3–32; LDL(NCEP), 47–198; VLDL, 5–69; TG (mg/dl), 35–393 (only 8 subjects had >150 mg/dl); and age, 22–29 years. Cholesterol measurements were made using VAP-I method.

^eThe relative position of the major LDL peak maximum in LDL VAP-II profile.

^bCholesterol concentration (mg/dl).

^cLDL(NCEP) = LDL-R + Lp[a] + IDL.

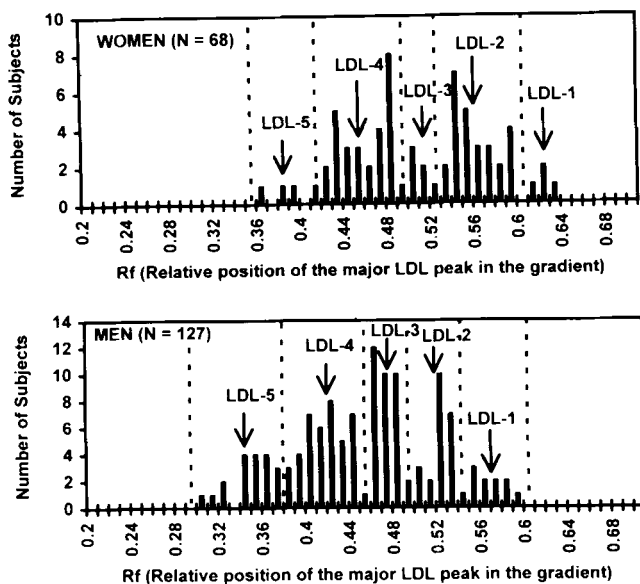


Fig. 6. Frequency distribution diagram showing the distribution of R_f of the major LDL peak maximum in LDL VAP-II density gradient in women and men subjects. R_f was measured as described in Fig. 2. The mid- R_f position of each cluster is indicated by an arrow.

HDL₂ cholesterol correlated with R_f better in men than women, while IDL correlated better in women. Both LDL(NCEP) and LDL-R cholesterol correlated only in women. The age, either of women or of men, did not significantly correlate with R_f in this population.

In a forward stepwise multivariate analysis comparing R_f with sex, VLDL, LDL-R, Lp[a] cholesterol, IDL, HDL₃, HDL₂, and triglyceride, only HDL₂ remained in the model.

To investigate the relationship of LDL subclass distribution with plasma concentrations of various lipoproteins, all subjects were classified into groups based on their major LDL subclass (i.e., the LDL subclass with the highest cholesterol concentration) as obtained from the deconvolution of the parent LDL VAP-II profiles. Thus, five distinct groups, with major LDL subclasses LDL-1 to LDL-5, could be identified (LDL-1 and LDL-2 considered as large, buoyant LDL, LDL-3 and LDL-4 as intermediate density LDL, and LDL-5 as small, dense LDL). There were no subjects with LDL-6 as the major subclass in this population. The mean lipoprotein values obtained for each subclass group are summarized in Table 3. HDL cholesterol values decreased (the decrease in HDL₂ being predominant), while IDL and VLDL cholesterol, and TG increased as the subclass group density increased; i.e., as subclass group number increased from 1 to 5. LDL-R cholesterol and TC values also increased, but the increase was not continuous. The mean LDL-R values of subclass groups 3, 4, and 5 (intermediate and

small, dense LDL) were similar, while the subclass groups 1 and 2 (large, buoyant LDL) had similar mean values. However, LDL-R mean values of subclass groups 3, 4, and 5 were higher than the corresponding mean values of subclass groups 1 and 2. The mean LDL-R value for all women subjects in subclass groups 3, 4, and 5 together was 94.5 mg/dl compared to the mean value of 81.3 mg/dl for all women subjects in subclass groups 1 and 2 together ($P < 0.02$; data calculated from similar tables prepared, but not shown here, for women and men separately). The corresponding values for men in the two groups (i.e., subclass groups 3, 4 and 5, and 1 and 2) were 89.8 mg/dl and 82.6 mg/dl, respectively ($P > 0.05$). Thus, although the differences in mean LDL-R values were observed between the two subgroups within women and men subjects, only the difference within women subgroups was significant. Increases in TC values among subclasses were similar to LDL-R. The data in Table 3 overall suggest that the lipoprotein levels become increasingly atherogenic as LDL subclass group number increases. Thus, mean lipoprotein values for subjects in LDL-5 subclass group are comparatively more atherogenic than the values for subjects in LDL-1 subclass group. Furthermore, 50% of all women were distributed within LDL-1 and LDL-2 subclass groups compared to 25% of all men in the same subclass groups, confirming our observation that compared to men, women generally tend to have more buoyant LDL (Fig. 6) that are accompanied by less atherogenic lipoprotein levels. The progressive changes in the lipoprotein values observed between the subclass groups overall show the effect of LDL subclass distribution on lipoprotein levels.

Figure 7 shows the frequency distribution diagrams of LDL R_f of all five subclass groups plotted separately. With the exception of one subject in the LDL-4 subclass group, the minimal overlap of R_f distribution observed between neighboring subclass groups suggests a high accuracy of our mathematical deconvolution program in identifying the major LDL subclass in each subject.

DISCUSSION

The two most common methods used to study the LDL subspecies distribution have been ultracentrifugation and gradient gel electrophoresis. Currently used ultracentrifugation techniques are lengthy and laborious, and are therefore restricted to research laboratories. Non-denaturing 2–16% gradient gel electrophoresis is a high resolution technique and requires only a small amount of plasma, but the lack of availability of commercial gels could make reproducibility difficult for new users of this technique. As the role of LDL subclasses in the development of atherosclerosis becomes

TABLE 2. Pearson correlation coefficients showing the association of R_f (relative position of the major LDL peak maximum in LDL VAP-II profile; higher the R_f lower the peak density) with lipoprotein concentration in medical students (n = 195)

Parameter	All		Women		Men	
	r	P	r	P	r	P
TC	-0.093	>0.1 (n.s.)	-0.213	<0.1 (n.s.)	-0.098	>0.2 (n.s.)
HDL ^a	0.594	<0.0001	0.472	<0.001	0.532	<0.0001
HDL _s ^a	0.350	<0.001	0.138	>0.2 (n.s.)	0.315	<0.001
HDL ₂ ^a	0.625	<0.0001	0.532	<0.001	0.578	<0.0001
Lp[a] ^a	0.03	>0.6 (n.s.)	0.118	>0.3 (n.s.)	0.021	>0.7 (n.s.)
LDL-R ^a	-0.152	<0.05	-0.265	<0.05	-0.108	>0.2 (n.s.)
IDL ^a	-0.432	<0.0001	-0.528	<0.001	-0.379	<0.001
LDL(NCEP) ^a	-0.206	<0.01	-0.308	<0.01	-0.161	>0.05 (n.s.)
VLDL ^a	-0.386	<0.001	-0.409	<0.001	-0.324	<0.001
TG	-0.355	<0.001	-0.340	<0.05	-0.325	<0.01
Age	0.009	>0.9 (n.s.)	0.105	>0.3 (n.s.)	0.066	>0.4 (n.s.)

^aMeasured as cholesterol concentration (mg/dl); n.s., nonsignificant (all P values >0.05 were considered as nonsignificant).

better understood, demand will increase for methods that can rapidly quantify LDL subclasses in a clinical laboratory. The LDL VAP-II method provides quantification of cholesterol simultaneously in five LDL subclasses using only 1 h centrifugation time; thus eight samples can be completely analyzed in less than 3 h. In addition, it requires only 70 μ l of plasma per sample because of its high sensitivity. With 10-fold dilution, plasma samples with LDL-R cholesterol levels up to 200 mg/dl can be easily analyzed. The method is also highly reproducible, allowing comparisons within and between individuals. Further, this method quantifies cholesterol in LDL density subclasses while the gradient gel electrophoresis method provides sizes of the particles and relative, rather than absolute, concentrations of subspecies. Thus, the two techniques could be complimentary to each other. A good correlation coefficient ($r = 0.859$) observed between R_f measured by LDL VAP-II and peak LDL particle diameter as measured by the gradient gel electrophoresis, an established technique, suggests the reliability of LDL particle distribution measurement by the LDL VAP-II technique.

LDL VAP-II has the potential to become a useful clinical tool. Subjects can be classified as having predominantly small, dense LDL (LDL-5), intermediate density LDL (LDL-4 and LDL-3), and large, buoyant LDL (LDL-2 and LDL-1), similar to the classification made on the basis of the gradient gel electrophoresis patterns. Only one dense LDL subclass (LDL-5) was observed as the major subclass in the present population as none of the subjects were markedly hyper-

triglyceridemic (i.e., with TG values >400 mg/dl; only 8 of the 195 subjects had TG >150 mg/dl, with 393 mg/dl being maximum). However, it is possible that more numbers of small, dense LDL subclasses may be observed when markedly hypertriglyceridemic subjects are included.

Although, as described above, LDL VAP-II compares well with the gradient gel electrophoresis technique, a somewhat greater percentage of subjects were classified as having predominantly intermediate density LDL (LDL-3 and LDL-4) by LDL VAP-II than by the gradient gel electrophoresis (47% vs. 34%). There appear to be two possible explanations to this. First, while the gradient gel electrophoresis classification is based on the measurement of size, our classification is based on the flotation rate, a function of both size and density. Although the density of lipoproteins increases with the decrease in size, the relationship between the two physical parameters may not be perfect, i.e., a 1:1 relationship may not exist. This is because the variation in lipid:protein ratio (which results in density changes) between the subclasses may not result in detectable changes in size or vice versa (11). Thus, it is possible that some subjects classified as having LDL-3 as their density major subclass (i.e., intermediate density LDL) may indeed have major size LDL subclass with particles larger in diameter than assigned to pattern AB (i.e., pattern A by gradient gel electrophoresis). Second, as intermediate density LDL are known to be rich in cholesterol in normal subjects, they more frequently constitute the major subspecies when measured in terms of cholesterol, and this may

TABLE 3. Comparison of mean lipoprotein concentrations among subjects (n = 195) classified based on major LDL subclass

Parameter	LDL Subclass				
	1	2	3	4	5
n	10 (W = 7, M = 3)	56 (W = 27, M = 29)	62 (W = 22, M = 40)	51 (W = 11, M = 40)	16 (W = 1, M = 15)
TC	168.8 ± 22.1	164.2 ± 24.6	175.2 ± 34.3	174.2 ± 32.6	176.7 ± 31.1
HDL ^a	56.8 ± 9.0	51.9 ± 10.3	44.73 ± 6.6	40.2 ± 7.7	38.2 ± 6.9
HDL ₃ ^a	39.2 ± 4.5	37.5 ± 5.8	36.1 ± 4.8	34.1 ± 5.6	32.8 ± 6.0
HDL ₂ ^a	17.6 ± 5.2	14.4 ± 7.1	8.7 ± 3.6	6.1 ± 3.8	5.4 ± 3.2
Lp[a] ^a	9.3 ± 7.5	7.4 ± 5.1	7.3 ± 3.8	8.9 ± 5.5	5.7 ± 3.6
LDL-R ^a	81.3 ± 24.1	81.7 ± 19.1	91.4 ± 25.7	90.8 ± 20.8	92.4 ± 19.3
IDL ^a	6.9 ± 3.0	8.6 ± 3.7	12.6 ± 5.2	13.3 ± 5.1	14.6 ± 4.8
LDL(NCEP) ^a	97.5 ± 23.9	97.7 ± 21.3	111.3 ± 30.5	113.0 ± 25.8	112.7 ± 22.5
VLDL ^a	11.4 ± 3.9	14.7 ± 7.4	19.1 ± 9.6	21.0 ± 8.1	26.2 ± 12.6
TG	54.0 ± 12.2	73.3 ± 33.0	91.9 ± 67.3	103.7 ± 44.5	185.3 ± 55.3
Age (y)	24.7 ± 1.9	24.5 ± 1.6	24.1 ± 1.5	24.4 ± 1.6	24.6 ± 2.1

Major LDL subclass is the LDL subcurve with the highest area as obtained from deconvolution program; W, women; M, men. Subjects were medical students selected for this study.

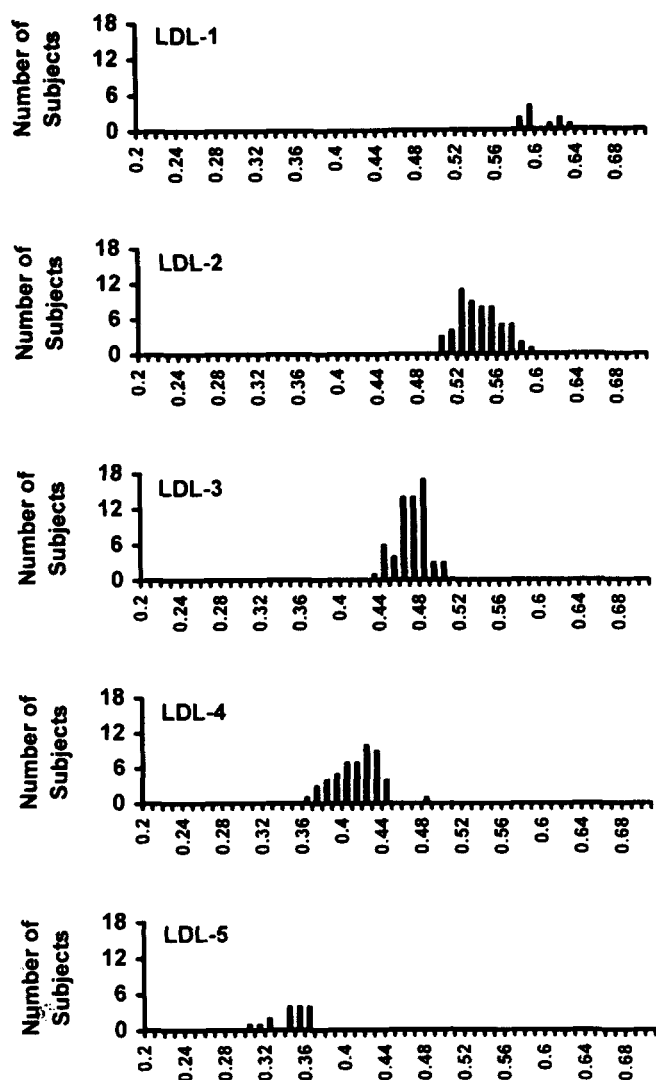
^aCholesterol concentration (mg/dl).

not be true when measured in terms of other properties, such as size. However, the percentages of prevalence of intermediate density LDL obtained in the present study by both the techniques were much higher than the corresponding reported value of 15% in normolipidemic subjects (30).

As observed in other studies, the gender differences in the distribution of LDL subclasses were also evident in the present study (Table 3). Small, dense LDL was substantially less prevalent in women subjects compared to men subjects. Only 1 of the 68 (1.5%) women subjects studied here showed LDL-5 (small, dense LDL) as the major LDL subclass compared to 15 of the 127 (12%) men subjects. Similarly, fewer number of women subjects (n = 11, 16%) had LDL-4 (an intermediate density LDL subclass) compared to men subjects (n = 40, 31.5%). However, the prevalence of LDL-3 (also an intermediate density LDL subclass) was similar in both women and men subjects (32.5% and 31.5%, respectively). On the other hand, a greater percentage of women subjects had LDL-1 and LDL-2 (large, buoyant LDL) as their major LDL subclass compared to men subjects (40% and 10% of LDL-2 and LDL-1, respectively, in women compared to 23% and 2.5%, respectively, in men). Thus, women subjects had increased prevalence of large, buoyant LDL compared to men subjects, and both groups had significantly increased prevalence of intermediate density LDL.

In the present work, the relative position of the major LDL peak maximum in the density gradient (R_f ; a higher R_f indicates more buoyant LDL) was found to associate

strongly and positively with HDL, HDL₃, and HDL₂ cholesterol, and negatively with VLDL and IDL cholesterol and TG. LDL(NCEP) cholesterol, which was derived by adding Lp[a] and IDL cholesterol to LDL-R, correlated better than LDL-R with R_f in women. This increased association is due most likely to the contribution by IDL, which also correlated better in women than in men. While these results are generally consistent with the results obtained from other investigators (5, 6, 22, 23, 31, 32), some notable differences were observed which appear to be due to the specific age range of this population (22–29 years). The association of TG with R_f was not as strong as found in other studies. This appears to be primarily due to the lower TG values in this population; the mean TG value being only 90 mg/dl, a value smaller than the cross-over value (95 mg/dl) between patterns A and B, suggested by Austin et al. (12). On the other hand, HDL and HDL₂ cholesterol showed strong association with R_f especially in men. Although association of HDL₃ was not as strong as HDL or HDL₂ in men, it was highly significant ($P < 0.001$). However, HDL₃ did not correlate with R_f in women. The age, within the selected narrow and lower range (22–29 years), was not correlated with R_f in both women and men subjects. The association of Lp[a] cholesterol with LDL size or density has not been previously studied in detail. The only report available in the literature suggests a significant positive association of Lp[a] mass with dense LDL in women only (33). However, our data suggested no such association, at least in the present population. The overall agreement of our results with



Rf (Relative position of the major LDL peak in the gradient)

Fig. 7. Frequency distribution diagram showing the distribution of R_f of the major LDL peak maximum in LDL VAP-II profiles of subjects classified based on their major LDL subclass. All subjects ($n = 195$) were classified into five groups (LDL-1 through LDL-5) based on their major LDL subclass as determined by the mathematical deconvolution software. R_f of the major LDL peak maximum was determined as described in Materials and Methods and the frequency distribution diagram of R_f for each group was plotted. Minimal overlap of the R_f distribution between neighboring groups suggests a high accuracy of the mathematical deconvolution in the identification of major LDL subclass.

those previously reported again suggests the reliability of the LDL VAP-II.

Another important feature of LDL VAP-II is its ability to classify subjects based on the atherogenicity of various lipoprotein values. For example, subjects classified in LDL-5 subclass group had all lipoproteins at more atherogenic levels than the lipoprotein levels in subjects in LDL-1 subclass group (Table 3). In addition, the

progressive changes in atherogenicity of all lipoprotein levels, except LDL-R, can be seen from one to the next subgroup. The changes in LDL-R and LDL (NCEP) were not continuous, as also observed by Campos et al. (15). The mean LDL-R and LDL(NCEP) cholesterol values of both men and women subjects in LDL-3 and LDL-4 subclass groups (intermediate density LDL subclass) were higher than the corresponding mean values observed for subjects with LDL-1 and LDL-2 (large, buoyant LDL subclass) as their major LDL subclass (data not shown). These observations are similar to the observations made by Campos et al. (15). However, only the difference between LDL-R mean values of LDL-2 and LDL-3 subclass groups in women and the differences between mean values of LDL(NCEP) of LDL-2 and LDL-3 subclass groups in women and LDL-2 and LDL-4 subclass groups in men were significant ($P < 0.05$). In addition, in our study the mean LDL-R and LDL(NCEP) values in subjects (both men and women) with LDL-5 (small, dense LDL) as the major subclass were similar to the corresponding values in subjects with LDL-4 and LDL-3 as their major LDL subclass, while LDL cholesterol values decreased for subjects with small, dense LDL (except in men with LDL-5) in the study by Campos et al. (15).

Thus, our results overall suggest that LDL VAP-II can also serve as a screen for atherogenic lipoproteins. In summary, we have developed a simple and rapid method for the determination of LDL subclasses that can be utilized for clinical studies. The LDL VAP-II method can be used to study the LDL subclass cholesterol distribution in small animals and infants where the blood volume obtained is limited. ■

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