Evaluation of an immunoseparation method for quantitative measurement of remnant-like particle-cholesterol in serum and plasma

Elizabeth Teng Leary,1* Tao Wang,2 Daniel J. Baker,1 Donald D. Cilla,2 Jianhua Zhong,2 G. Russell Warnick,1 Katsuyuki Nakajima,2 and Richard J. Havel3

Substantial evidence indicates that triglyceride-rich lipoprotein remnants are atherogenic. Additional research has, however, been limited by available methods for separation and quantification of remnants. We have evaluated an immunoseparation assay developed to measure cholesterol in remnant-like particles (RLP-C). This method uses monoclonal antibodies to human apolipoproteins B-100 and A-I to remove most of the apolipoprotein B-100-containing lipoproteins (namely LDL and nascent VLDL) and apolipoprotein A-I-containing lipoproteins (namely chylomicrons and HDL), leaving behind a fraction of triglyceride-rich lipoproteins, including chylomicron and VLDL remnants, both of which are enriched in apolipoprotein E. Cholesterol in the unbound fraction is measured with a sensitive enzymatic assay. The RLP-C concentration was highly correlated with total triglyceride-rich lipoproteins (sum of VLDL-cholesterol and IDL-cholesterol) separated by ultracentrifugation and by polyacrylamide gel electrophoresis (r = 0.86 and 0.76, respectively). The within-run and run-to-run imprecision (CV) of the assay was ~6% and 10%, respectively. The assay was not affected by hemoglobin up to 5000 mg/L (500 mg/dL), bilirubin up to 342 mmol/L (20 mg/dL), glucose up to 67 mmol/L (1200 mg/dL), or ascorbic acid up to 170 mmol/L (3.0 mg/dL). In 726 subjects (men, n = 364; women, n = 362) in the US, the 75th percentiles of RLP-C concentration were 0.17 mmol/L (6.6 mg/dL) and 0.23 mmol/L (8.8 mg/dL) in sera obtained after overnight fasting or randomly, respectively. A group of 151 patients from nine US centers and one Canadian center with coronary artery atherosclerosis established by angiography had higher median RLP-C concentrations than 302 gender- and age-matched controls (P <0.05). We conclude that the RLP-C assay compares favorably to ultracentrifugation and electrophoresis and provides a convenient and economical approach to measure triglyceride-rich lipoprotein remnants in routine clinical laboratories.

The importance of plasma triglycerides (TGs)4 as a risk factor for atherosclerotic disease has been controversial. Whereas some epidemiological studies have suggested that serum TG concentration is an independent risk factor for coronary artery disease (CAD) (1–3), it has often been found to predict CAD in univariate models but not in multivariate models after adjustment for HDL-cholesterol (HDL-C) and other risk factors (4–6). In a recent Framingham study update, TGs were an independent risk factor for women but not for men (7). Metaanalysis of >50 000 subjects in 17 population-based prospective studies indicated that TGs are an independent risk factor for both men and women in the general population (8). One limitation of using plasma TG concentration as a predictor for CAD is that it does not separate nonatherogenic TG-rich lipoproteins, such as chylomicrons and nascent VLDL, from their potentially more atherogenic components, such as chylomicron and VLDL remnants. Studies in which lipoprotein remnants have been estimated have consistently demonstrated that increased plasma remnant concentrations are associated with the presence and progression of CAD (9–13).

1 Pacific Biometrics, Inc., 220 West Harrison Street, Seattle, WA 98119.
2 Otsuka America Pharmaceutical, Inc., Rockville, MD 20850.
3 Cardiovascular Research Institute, University of California, San Francisco, CA 94143.
*Author for correspondence. Fax 206-298-9838; e-mail ETL@PACBIO.COM.
Received May 5, 1998; revision accepted August 25, 1998.

4 Nonstandard abbreviations: TG, triglyceride; CAD, coronary artery disease; HDL-C, HDL-cholesterol; UC, ultracentrifugation; IDL, intermediate-density lipoprotein; PAGE, polyacrylamide gel electrophoresis; apo, apolipoprotein; RLP, remnant-like particle; RLP-C, remnant-like particle-cholesterol; TC, total cholesterol; QC, quality control; VLDL-C, VLDL-cholesterol; IDL-C, intermediate-density lipoprotein-cholesterol; and LDL-C, LDL-cholesterol.
General application of these research findings has been limited by lack of facile methods for quantification of VLDL and chylomicron remnants. Although lipoprotein remnants are adequately defined in functional terms, no straightforward methods have been developed to separate them from other TG-rich lipoproteins (14). Preparative and analytical ultracentrifugation (UC) have been used to separate VLDL and intermediate-density lipoprotein (IDL), designating the small VLDL and IDL as lipoprotein remnants. Smaller TG-rich lipoproteins have also been separated by nondenaturing polyacrylamide gel electrophoresis (PAGE) (15) or by electrophoresis in agarose gels, in which they have reduced mobility (16). These methods have limited applicability for routine analysis in clinical laboratories because they are labor-intensive, complex, and expensive.

Recently, a novel immunoseparation method for remnant lipoproteins was developed by Nakajima et al. (17). This method uses a monoclonal antibody to human apo B-100 that recognizes an epitope near B-51 (18) to remove almost all LDL and most VLDL particles containing apo B-100 together with a monoclonal antibody to apo A-I to remove almost all HDL particles. Remaining unbound to these antibodies are principally all chylomicron remnants and a fraction of VLDL particles, both enriched in apo E, a characteristic of remnant lipoproteins (19). This unbound fraction is designated “remnant-like particles” (RLPs). The monoclonal antibodies are conjugated to Sepharose 4B to facilitate the separation of bound lipoproteins such as LDL and HDL from the RLP fraction. Cholesterol in the unbound fraction (RLP-C) is measured by an enzymatic cholesterol assay. With this immunoseparation method, researchers have found that RLP-C is increased in patients with CAD (20, 21), type III hyperlipoproteinemia (22), and diabetic nephropathy (23) and in patients undergoing hemodialysis (24, 25).

The objectives of this study were to evaluate the technical performance of this RLP-C assay and to assess the relationship between RLP-C and other atherogenic lipoproteins. A reference range of serum RLP-C for the US population was established, and the serum RLP-C concentrations per se were not part of the inclusion or exclusion criteria. No subject took any medication that may alter lipid or lipoprotein metabolism. Each subject was asked to participate in two visits: one after 12 h of fasting and one at random in relation to the last meal (either fasting or nonfasting). Serum concentrations of RLP-C, total cholesterol (TC), TGs, and HDL-C were measured at each visit.

To evaluate the clinical utility of RLP-C in predicting CAD, 203 adult patients with >20% stenosis of at least one coronary artery at coronary angiography were recruited from nine medical centers in the US (Boston, MA; Jacksonville, FL; Lombard, IL; Santa Rosa, CA; Houston, TX; Minneapolis MN; Winter Park, FL; Ocala, FL; and Clearwater, FL) and one medical center in Canada (Montreal, PQ). Control subjects (n = 477) with similar ages to the CAD patients were recruited from the same four US centers that participated in the reference range study. Subjects with serum TG blank values >500 mg/dL (50 mg/dL; because of heparin used during angiography before specimen collection), who did not complete both visits, or who did not have a fasting specimen collected were excluded from the data analysis. The remaining population included 172 CAD patients and 469 controls. All clinical protocols were approved by the Institutional Review Board. Informed consent forms were signed by all participating subjects.

**MATERIALS**

The RLP-C Immunoseparation Reagent kits were provided by Japan Immunoresearch Laboratories. The kit consists of immunoaffinity gel, cholesterol reagent, a cholesterol calibrator, and a Tris-HCl buffer solution (pH 7.4). The immunoaffinity gel consists of monoclonal antibodies to human apo B-100 (JI-H) and apo A-I (H-12) conjugated to Sepharose 4B beads as the solid phase (17). The cholesterol reagent contains ascorbate oxidase, cholesterol esterase, and cholesterol oxidase in R1, and horse-radish peroxidase, 4-amino-antipyrine, and N,N-diethyl-N-(3-methylphenyl)-N’-succinyl-ethylenediamine in R2.

To monitor the imprecision of the RLP-C assay, fresh-frozen serum quality-control pools (RLP QC) were prepared and stored at −70 °C. They were supplemented with potassium EDTA (8 g/L) and sucrose (100 g/L) to preserve lipoprotein integrity during freeze-thaw cycles. Addition of sucrose has been shown to protect lipoproteins from damage during lyophilization (26). Diluted human serum pools were also prepared to monitor the imprecision of the cholesterol assay independent from the
immunoseparation procedure (Low Cholesterol QC). Serum samples were diluted to cholesterol concentrations between 0.13 and 0.65 mmol/L (between 5 and 25 mg/dL), using normal saline with 50 g/L bovine serum albumin. EDTA and sucrose were also added into the diluted serum pools. The EDTA/sucrose-supplemented sera were then further diluted 61-fold with RLP buffer before freezing at −70 °C to simulate the dilution of samples during the RLP immunoseparation step. All frozen-serum QC pools were thawed and mixed on a shaker for 30 min at room temperature before use.

Hemoglobin and bilirubin (conjugated and unconjugated) were obtained from Kokusai Shiyaku Co., Ltd. (Interference Check-A Plus, cat. no. 5186). Glucose and ascorbic acid were obtained from Sigma Chemical Co.

**RLP-C ANALYSIS**

A 3-mm diameter stainless steel bead (Otsuka Electronics) was added to each microsample cup (part no. 140-668-0001, Boehringer Mannheim); 300 μL of the immunoaffinity gel and 5 μL of specimen were then added to each cup. The mixture was incubated at room temperature on a dedicated mixer with build-in magnetic bars effecting mixing by driving the beads up and down in each cup (18). After 2 h, the supernatant (unbound fraction) was transferred for cholesterol analysis in a Cobas MIRA S chemistry analyzer (Roche Analytical Instruments, Inc.). The cholesterol values were multiplied 61-fold to reflect dilution of the specimen at the immunoseparation step (Table 1).

**TECHNICAL EVALUATION OF THE RLP-C ASSAY**

The detection limit of the RLP-C assay was defined as 3 SD above the mean from 10 replicate analyses of the RLP buffer over 2 days. To evaluate linearity, a serum with an increased RLP-C concentration was mixed with a second serum with low RLP-C concentration or with normal saline in various proportions and then analyzed in duplicate. Within-run imprecision (CV) was evaluated by running the two RLP QC pools in 20 replicates. The run-to-run imprecision of the RLP-C assay was evaluated over a 3-month period on the same RLP QC pools by multiple analysts. The CV of the cholesterol assay was evaluated using the two Low Cholesterol QC pools without the immunoseparation step.

To compare the difference between serum and plasma in RLP-C measurement, specimens from 43 subjects were analyzed. Blood samples (fasting or nonfasting) were drawn into various Vacutainer Tubes (Becton Dickinson) in the following order: SST (for serum), EDTA, lithium heparin, EDTA, lithium heparin, SST. After the samples stood at room temperature for 30 min, serum or plasma were prepared by centrifugation to remove blood cells. The same specimen types were pooled and then aliquoted into cryogenic vials for storage at 4 °C or at −70 °C. TC, TGs (corrected for a glycerol blank), and HDL-C were measured in the fresh fasting sera. RLP-C was measured in all specimens within 4 h of collection and at specific intervals after storage.

Interference of hemoglobin, bilirubin, glucose, and ascorbic acid with the RLP-C analysis was evaluated by adding various concentrations of the respective interference substances or saline as control to two serum samples.

**OTHER LIPID AND LIPOPROTEIN ANALYSES**

TC and TGs were measured by CDC-standardized enzymatic methods. HDL-C was measured after precipitation of non-HDL lipoproteins by dextran sulfate (M₄ 50 000)-Ca²⁺. VLDL, IDL, and LDL were separated by UC at 250 000 g rpm using a Beckman Ti 42.2 rotor for 4 h or a Beckman 40.3 rotor for 18 h (27). Serum was centrifuged, and then the centrifuge tube was sliced with a Beckman tube slicer to yield a d <1.006 kg/L fraction (containing chylomicrons and VLDL) and a d ≥1.006 kg/L fraction (containing IDL, LDL, and HDL). The serum was also adjusted to a density of 1.019 kg/L with potassium bromide solution and then centrifuged to yield a d <1.019 kg/L fraction (containing chylomicrons, VLDL, and IDL) and a d ≥1.019 kg/L fraction (containing LDL and HDL). Cholesterol concentrations in each of the four fractions were measured by an enzymatic cholesterol assay. VLDL-cholesterol (VLDL-C) was calculated as [TC − cholesterol in d ≥1.006 kg/L lipoproteins]. IDL-C was calculated as [cholesterol in d ≥1.006 kg/L lipoproteins − cholesterol in d ≥1.019 kg/L lipoproteins]. LDL-C was calculated as [cholesterol in d ≥1.006 kg/L lipoproteins − HDL-C] or measured by the Direct LDL-Cholesterol method (Genzyme Corp.).

Lipoproteins were also separated and measured by PAGE (LipoPhor™ by Quantimetrix) (28). Lipoprotein(a) was measured by an immunoturbidimetric assay from INCSTAR in a Cobas FARA automated chemistry analyzer (Roche Analytical Instruments, Inc.).

**STATISTICAL ANALYSES**

Imprecision of the assays was expressed as CV. Comparison between RLP-C and other lipoproteins was evaluated

<table>
<thead>
<tr>
<th>Table 1. RLP-C test setup on the Cobas MIRA Analyzer.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
</tr>
<tr>
<td>Reaction mode</td>
</tr>
<tr>
<td>Wavelength</td>
</tr>
<tr>
<td>Sample volume</td>
</tr>
<tr>
<td>R1 volume</td>
</tr>
<tr>
<td>R1 addition time</td>
</tr>
<tr>
<td>R2 volume</td>
</tr>
<tr>
<td>R2 addition time</td>
</tr>
<tr>
<td>Final read time</td>
</tr>
<tr>
<td>Conversion factor</td>
</tr>
<tr>
<td>Calibrator concentration, mg/L</td>
</tr>
<tr>
<td>Calibration mode</td>
</tr>
</tbody>
</table>
using the Pearson correlation. The difference among different specimen types was estimated using ANOVA. The Mann–Whitney U-test was used to estimate the statistical difference between CAD patients and age- and gender-matched controls in their lipid and lipoprotein values because some of the values did not fit a gaussian distribution. A P value <0.05 was considered statistically different.

Results

Specimen Type and Stability

Fasting and nonfasting RLP-C concentrations in 43 simultaneously collected samples of serum, EDTA plasma, and heparin plasma were compared. Fasting serum TC, TG (corrected for glycerol blank), and HDL-C concentrations were 5.91 mmol/L (228 ± 58 mg/dL), 2.80 ± 4.33 mmol/L (248 ± 383 mg/dL), and 1.27 ± 0.44 mmol/L (49 ± 17 mg/dL), respectively. To allow the use of ANOVA, a parametric statistical analysis method that is based on the assumption of gaussian distribution of data, five samples with TG concentrations >4.52 mmol/L (400 mg/dL) were analyzed separately. Mean fasting RLP-C concentrations in serum, EDTA plasma, and heparin plasma were 0.17 ± 0.09 mmol/L (6.7 ± 3.4 mg/dL), 0.19 ± 0.08 mmol/L (7.4 ± 2.9 mg/dL), and 0.18 ± 0.08 mmol/L (6.8 ± 3.1 mg/dL), respectively. Mean nonfasting RLP-C concentrations were 0.21 ± 0.11 mmol/L (8.2 ± 4.1 mg/dL), 0.23 ± 0.10 mmol/L (8.7 ± 3.9 mg/dL), and 0.21 ± 0.10 mmol/L (8.3 ± 3.9 mg/dL), respectively, for the three types of specimens. Mean RLP-C concentrations in fasting or nonfasting EDTA plasma samples were slightly higher than in serum or heparinized plasma (P <0.05).

We evaluated the effects of two different storage conditions (4 °C and −70 °C) on RLP-C values. RLP-C concentrations in the nonfasting samples tended to increase when stored at 4 °C (Fig. 1). The increase of mean fasting RLP-C concentrations was <0.03 mmol/L (1.0 mg/dL) within 1 week of sample collection. Freezing at −70 °C prevented the gradual increase seen in both fasting and nonfasting samples with TG concentrations <4.52 mmol/L (400 mg/dL) for up to 3 months (Fig. 1). Fasting RLP-C concentrations rose after freezing in three of the five samples with TG concentrations >4.52 mmol/L (400 mg/dL).

Table 2. Within-run and run-to-run imprecision of RLP-C assay and cholesterol assay alone.

<table>
<thead>
<tr>
<th></th>
<th>RLP-C assay</th>
<th></th>
<th>Cholesterol assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean*</td>
<td>SD*</td>
<td>CV</td>
</tr>
<tr>
<td>Within-run imprecision (n = 20)</td>
<td>0.13 (4.9)</td>
<td>0.01 (0.3)</td>
<td>6.1%</td>
</tr>
<tr>
<td></td>
<td>0.51 (19.8)</td>
<td>0.02 (0.6)</td>
<td>3.5%</td>
</tr>
<tr>
<td>Run-to-run imprecision (n = 36)</td>
<td>0.15 (5.9)</td>
<td>0.02 (0.6)</td>
<td>10.2%</td>
</tr>
<tr>
<td></td>
<td>0.51 (19.7)</td>
<td>0.03 (1.1)</td>
<td>5.6%</td>
</tr>
</tbody>
</table>

* Concentrations in mmol/L (mg/dL).

Technical Performance of the RLP-C Assay

The detection limit, defined as 3 SD above the mean value for the RLP buffer, was 0.08 mmol/L (3.1 mg/dL). The assay was linear up to 3.37 mmol/L (130 mg/dL) when serum was used as the diluent or to 2.33 mmol/L (90 mg/dL) when normal saline was used as the diluent.

The within-run CV of the RLP-C assay was 6.1% at 0.13 mmol/L (4.9 mg/dL) and 3.5% at 0.51 mmol/L (19.8 mg/dL). The within-run CV (n = 20) of the cholesterol assay, independent of immunoseparation, was 4.7% at 0.22 mmol/L (8.6 mg/dL) and 1.6% at 0.67 mmol/L (26.0 mg/dL; Table 2). The run-to-run CV over a representative 3-month period (n = 36) was 10.2% at 0.15 mmol/L (5.9 mg/dL) and 5.6% at 0.51 mmol/L (19.7 mg/dL). The run-to-run CV attributable to the cholesterol assay alone during the same period was 5.7% at 0.23 mmol/L (8.7 mg/dL) and 3.3% at 0.62 mmol/L (23.9 mg/dL; Table 2).

The representative run-to-run CV was ~10% at 85 mg/L (8.5 mg/dL) for VLDL-C plus IDL-C by UC (n = 79) and 13% at 90 mg/L (9.0 mg/dL) for VLDL-C by LipoPhor (n = 64).

Hemoglobin up to 5000 mg/L (500 mg/dL), bilirubin (both conjugated and unconjugated) up to 342 mmol/L (20 mg/dL), glucose up to 66.6 mmol/L (1200 mg/dL), and ascorbic acid up to 170 mmol/L (3 mg/dL) did not interfere with the RLP-C assay. We showed previously that the binding capacity for HDL, expressed as its cholesterol concentration, was 2.59 mmol/L (100 mg/dL) (18). Above this concentration, a substantial amount of HDL appeared in the RLP fraction. By contrast, there was no clear cut point at which a relevant amount of LDL escaped antibody binding. Instead, the monoclonal antibody JI-H gradually lost its binding capacity with increased LDL (measured as LDL-C). Specifically, ~3% and 5% of the LDL were present in the unbound fraction in samples with LDL-C concentrations of 5.18 mmol/L (200 mg/dL) and 7.77 mmol/L (300 mg/dL), respectively (18).

Relationships Between RLP-C and Other Lipids and Lipoproteins

To evaluate the relationship between RLP-C and other lipoproteins, we compared RLP-C with other TG-rich lipoprotein fractions separated by either UC or by PAGE (e.g., LipoPhor) in 157 CAD patients and 314 age- and gender-matched controls. The correlation coefficients (r)
between RLP-C and VLDL-C were 0.88 and 0.77 for the VLDL separated by UC and LipoPhor, respectively (Fig. 2). RLP-C also moderately correlated with serum IDL-C by UC \( (r = 0.46) \) or by LipoPhor \( (r = 0.59; \text{Fig. 2}) \). The correlation coefficients between RLP-C and total TG-rich lipoproteins \( (\text{VLDL-C} + \text{IDL-C}) \) were 0.86 and 0.76 (measured by UC and LipoPhor, respectively). RLP-C was also highly correlated with serum TG \( (r = 0.87) \) but poorly correlated with TC \( (r = 0.36) \) and LDL-C \( (r = 0.18) \). There was no correlation between RLP-C and lipoprotein(a) \( (r = −0.06) \).

The lower correlation between RLP-C and IDL-C than with VLDL-C was supported by the investigation of the distribution of RLP-C in different lipoprotein fractions separated by UC. In 21 sera examined, the concentration of RLP-C in the IDL fraction, calculated as \( \text{RLP-C in the } d < 1.019 \text{ kg/L fraction} – \text{RLP-C in the } d < 1.006 \text{ kg/L fraction} \), was negligible except in samples with TG concentrations >5.65 mmol/L (500 mg/dL; Fig. 3). RLP-C in the \( d < 1.006 \text{ kg/L fraction} \) was highly correlated with serum TG concentrations \( (r = 0.95) \). At higher serum TG concentrations, a larger percentage of RLP-C was in the \( d < 1.006 \text{ kg/L fraction} \).

**REFERENCE RANGES OF RLP-C IN A US POPULATION**

In 364 healthy men and 362 healthy women recruited from four US centers, median fasting TC, TG, LDL-C, and HDL-C concentrations were 5.21 mmol/L (201 mg/dL), 1.10 mmol/L (97 mg/dL), 3.34 mmol/L (129 mg/dL), and 1.22 mmol/L (47 mg/dL), respectively. The 25th through 75th percentiles were 4.48–5.96 mmol/L (173–230 mg/dL) for TC, 0.80–1.63 mmol/L (71–144 mg/dL) for TGs, 2.77–3.99 mmol/L (107–154 mg/dL) for LDL-C, and 1.01–1.45 mmol/L (39–56 mg/dL) for HDL-C. The median

---

**Fig. 1.** Change of mean fasting (●) and nonfasting (□) RLP-C concentrations in serum, EDTA plasma, and heparin plasma from 38 subjects after storage at 4 °C up to 4 weeks (left) and at −70 °C up to 6 months (right).
RLP-C concentrations were 0.13 mmol/L (5.1 mg/dL) in fasting samples and 0.16 mmol/L (6.2 mg/dL) in random samples. The 25th-75th-95th percentile concentrations were 0.10-0.17-0.32 mmol/L (3.9-6.6-12.3 mg/dL) in fasting samples and 0.13-0.23-0.46 mmol/L (4.9-8.8-17.6 mg/dL) in random samples.

There was a significant difference in RLP-C concentrations between men and women and between different age groups (Table 3). In fasting samples, there was no difference in RLP-C concentrations among the three age groups tested for the men (18–34, 35–54, and ≥55 years). RLP-C concentrations increased with age in women. The median RLP-C concentration in women 55 and older was no different from that in men of the same age group. In contrast, random RLP-C concentrations increased with age in both men and women. In addition to the effects of gender and age, fasting and random RLP-C concentrations were higher in Hispanics (n = 173) and lower in African Americans (n = 67) than in Caucasians (n = 475). The median fasting RLP-C concentrations in the three populations were 0.14 mmol/L (5.4 mg/dL), 0.10 mmol/L (4.0 mg/dL), and 0.13 mmol/L (5.1 mg/dL), respectively.

**Table 3. Comparison of RLP-C concentrations in different gender and age groups.**

<table>
<thead>
<tr>
<th>Age, years</th>
<th>Men (n = 364)</th>
<th>Women (n = 362)</th>
<th>Men (n = 362)</th>
<th>Women (n = 362)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18–34b</td>
<td>0.124 (0.10, 0.17)</td>
<td>0.101 (0.09, 0.13)c</td>
<td>0.158 (0.12, 0.21)</td>
<td>0.130 (0.10, 0.16)c</td>
</tr>
<tr>
<td></td>
<td>[4.8 (3.8, 6.7)]</td>
<td>[3.9 (3.3, 5.0)]</td>
<td>[6.1 (4.5 8.1)]</td>
<td>[5.0 (3.9, 6.2)]</td>
</tr>
<tr>
<td>35–54b</td>
<td>0.153 (0.11, 0.20)c</td>
<td>0.119 (0.10, 0.16)c</td>
<td>0.184 (0.13, 0.28)c</td>
<td>0.158 (0.13, 0.21)c</td>
</tr>
<tr>
<td></td>
<td>[5.9 (4.3, 7.6)]</td>
<td>[4.6 (3.9, 6.1)]</td>
<td>[7.1 (5.0, 10.9)]</td>
<td>[6.1 (5.1, 8.1)]</td>
</tr>
<tr>
<td>≥55b</td>
<td>0.142 (0.12, 0.19)c</td>
<td>0.150 (0.12, 0.18)c</td>
<td>0.197 (0.15, 0.25)c</td>
<td>0.188 (0.14, 0.22)c</td>
</tr>
<tr>
<td></td>
<td>[5.5 (4.5, 7.2)]</td>
<td>[5.8 (4.8, 7.1)]</td>
<td>[7.6 (5.6, 10.8)]</td>
<td>[6.5 (5.5, 8.5)]</td>
</tr>
</tbody>
</table>

* All results are in mmol/L [mg/dL] and presented as median (25th percentile, 75th percentile).

b Each subgroup consisted of ~120 subjects.

c–f Compared with men of the same age group in the same visit: c P < 0.001; d P < 0.01; e P = 0.05; f P < 0.05.

g–i Compared with 18–34 age group of the same gender in the same visit: g P < 0.05; h P < 0.01; i P < 0.001.

**Fig. 3. Distribution of RLP-C in different hydrated densities (kg/L) in serum samples segregated by TG concentration.**

Serum samples were centrifuged at densities of 1.006 kg/L and 1.019 kg/L, respectively, to obtain \( d < 1.006 \text{ kg/L} \), 1.006 kg/L < \( d < 1.019 \text{ kg/L} \), and \( d > 1.019 \text{ kg/L} \) fractions. RLP-C was measured in each of the four fractions. RLP-C concentrations in the 1.006–1.019 kg/L fraction were calculated by subtracting the RLP-C in the \( d < 1.006 \text{ kg/L} \) fraction from the RLP-C in the \( d > 1.019 \text{ kg/L} \) fraction.

**RLP-C in CAD Patients and Controls**

Of the 172 eligible CAD patients, 161 (94%) were Caucasians, 7 (4%) were African Americans, 2 (1%) were Hispanics, and 2 (1%) were other races. In comparison, Caucasians accounted for only 58% of the control population of the clinical utility study. To provide vigorous comparison between the CAD and control groups, we matched each Caucasian CAD patient with two Caucasian controls, either from the reference range study or the clinical utility study, by their gender and age (±1 year). The average age was 63.4 ± 8.6 years for both groups; 77% were men and 23% were women.

Median fasting and random RLP-C concentrations were significantly higher in CAD patients than in controls (\( P < 0.05 \) and \( P < 0.001 \), respectively; Table 4). Serum TG concentrations, fasting or random, were also significantly higher in CAD patients. HDL-C concentrations were...
significantly lower in CAD patients. LDL-C concentrations were slightly lower in CAD patients. There were no differences between the two groups in serum TC concentrations.

**Discussion**

We have evaluated the technical performance and clinical utility of the RLP-C assay. The assay in principle is the same as described previously by Nakajima et al. (17); however, the immunoseparation step and cholesterol assay have been modified to be easier to use in routine laboratories (18). Historically, lipoprotein remnants have been estimated mainly after separation of TG-rich lipoproteins by UC or PAGE. UC is considered the reference method for isolating lipoprotein classes. This method, however, requires instrument not readily available in routine clinical laboratories and does not specifically separate lipoprotein remnants. Furthermore, the procedure is skill-dependent, labor-intensive, and has slow throughput. Although the instruments needed for PAGE are relatively inexpensive and available in laboratories performing electrophoresis, the ability to detect low concentrations of lipoprotein remnants or small changes in their concentrations is limited by the poor sensitivity of the method (28). Subjective interpretation of the electrophoretic pattern and identification of “IDL” bands (a broad “mid-band” between VLDL and LDL) may cause large variations in quantifying this putative remnant fraction, which may also contain lipoprotein(a). By contrast, the RLP-C assay requires only a small mixing device for the isolation of lipoprotein remnants (18), and the cholesterol concentration in the RLP fraction can be measured on any automated chemistry analyzer with an “open design” system and with a precise optical system. Analyzers with a “closed design” system (such as Johnson & Johnson Vitros series chemistry analyzers) are not suitable for RLP-C measurement because the cholesterol measurement with these analyzers lacks the sensitivity required. In comparison with the UC and PAGE methods, the RLP-C assay has a higher throughput and lower complexity. The run-to-run precision of the RLP-C assay is comparable with the UC or PAGE methods.

Lipoprotein remnants are a group of highly heterogeneous particles varying in size, hydrated density, electrophoretic mobility, chemical composition, and recognition by receptors (14). Each of the available separation methods measures lipoprotein remnants on the basis of different physical and chemical properties. UC separates lipoproteins by their hydrated density. Researchers typically have used IDL [hydrated density between 1.006 and 1.019 kg/L, or Svedberg flotation rates (Sv) of 12–20 units] as a surrogate to reflect remnant status because sequential hydrolysis of VLDL leads to a group of smaller, more dense, and cholesteryl ester-rich lipoprotein remnants. Chylomicron remnants, with a size and density generally larger than those of VLDL, do not appear in appreciable amounts as lipoproteins in the 1.006–1.019 kg/L density range. Some investigators have suggested that lipoproteins of Sv between 12 and 60 units reflect remnant status better than lipoproteins of Sv between 12 and 20 units (29). This range of lipoproteins includes both IDL (1.006 < d < 1.019 kg/L) and smaller VLDL particles found in the d < 1.006 kg/L fraction.

In contrast to UC, PAGE separates lipoproteins by their molecular sizes. With this method, lipoprotein remnants are often measured as “IDL”, which includes any lipoprotein particles with the a size between VLDL and LDL (28). In the population studied, IDL measured by LipoPhor correlates only moderately with IDL isolated by UC, with a Pearson correlation coefficient of 0.57 (data not shown). In samples from the type III hyperlipoproteinemic patients, IDL-C measured by the two methods are highly correlated, with a Pearson correlation coefficient of 0.93 (data not shown).

The RLP-C assay separates lipoprotein remnants on the basis of their surface apo components. apo A-I-containing lipoproteins (namely HDL and chylomicrons) are removed by anti-apo A-I; most apo B-100-containing lipoproteins (namely LDL and most VLDL) are removed by anti-apo B-100 (JI-H) (17). This assay measures mainly

### Table 4. RLP-C and other lipid/lipoprotein concentrations in patients with CAD and their age- and gender-matched controls.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 302)</th>
<th>CAD (n = 151)</th>
<th>Control (n = 302)</th>
<th>Random (n = 151)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLP-C</td>
<td>0.168 (0.13, 0.22)</td>
<td>0.184 (0.15, 0.26)</td>
<td>0.210 (0.16, 0.29)</td>
<td>0.241 (0.19, 0.33)</td>
</tr>
<tr>
<td>TC</td>
<td>[6.5 (5.1, 8.5)]</td>
<td>[7.1 (5.7, 10.2)]</td>
<td>[8.1 (6.2, 11.1)]</td>
<td>[9.3 (7.2, 12.8)]</td>
</tr>
<tr>
<td>TG</td>
<td>5.63 (4.97, 6.19)</td>
<td>5.39 (4.82, 6.01)</td>
<td>5.54 (4.82, 6.06)</td>
<td>5.39 (4.61, 6.00)</td>
</tr>
<tr>
<td>[218 (192, 239)]</td>
<td>(208 (186, 232)]</td>
<td>[211 (186, 234)]</td>
<td>[208 (178, 232)]</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>1.28 (0.94, 1.85)</td>
<td>1.62 (1.05, 2.36)</td>
<td>1.56 (1.08, 2.18)</td>
<td>2.03 (1.39, 2.90)</td>
</tr>
<tr>
<td>[113 (83, 164)]</td>
<td>[143 (93, 209)]</td>
<td>[138 (96, 193)]</td>
<td>[180 (123, 257)]</td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.17 (0.98, 1.40)</td>
<td>1.01 (0.85, 1.22)</td>
<td>1.14 (0.96, 1.40)</td>
<td>0.96 (0.83, 1.20)</td>
</tr>
<tr>
<td>[45 (38, 54)]</td>
<td>[39 (33, 47)]</td>
<td>[44 (37, 54)]</td>
<td>[37 (32, 47)]</td>
<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>3.81 (3.30, 4.33)</td>
<td>3.58 (3.08, 4.09)</td>
<td>3.60 (3.03, 4.15)</td>
<td>3.35 (2.88, 3.96)</td>
</tr>
<tr>
<td>[147 (127, 167)]</td>
<td>[138 (119, 158)]</td>
<td>[139 (117, 160)]</td>
<td>[130 (111, 153)]</td>
<td></td>
</tr>
</tbody>
</table>

*All results are in mmol/L [mg/dL] and are presented as median (25th percentile, 75th percentile).

**b** Compared with the controls at the same visit; **c** P < 0.05; **d** P < 0.001; **e** P < 0.01.
chylomicron remnants and larger VLDL remnants (19). These particles are rich in apo E as compared with the bound VLDL (19, 30). However, in samples from patients with diabetic (18) and type III hyperlipoproteinemia (22), particles of IDL size are found in the RLP fraction. In our nondiabetic subjects who did not have type III hyperlipoproteinemia, the IDL fraction (1.006 kg/L < d < 1.019 kg/L) contained little RLP-C (Fig. 3). RLP-C is thus better correlated with total TG-rich lipoproteins (VLDL + IDL) than with IDL-C alone (r = 0.86 vs 0.46 by UC or r = 0.76 vs 0.59 by Lipophor (Fig. 2).

Although IDL concentrations have been shown to predict the presence (10) and progression (31) of CAD in some studies, evidence that these particles are more atherogenic than other TG-rich lipoproteins is limited. Lipoprotein remnants with d <1.006 kg/L, on the other hand, have been found to possess proatherogenic characteristics: they increase permeability of the endothelial barrier (32) and are taken up by macrophages by receptor-mediated mechanisms (33, 34). Furthermore, lipoproteins isolated from human atherosclerotic plaque structurally resemble TG-rich lipoprotein remnants (35, 36). Clinical studies have also suggested that the concentration of remnants in the VLDL plus IDL range predict coronary atherosclerosis lesion progression (11, 13) or clinical coronary heart disease events (11) better than LDL.

RLPs isolated by the immunoaffinity mixed gel have been demonstrated to have biological properties of atherogenic lipoprotein remnants, including inhibition of endothelium-dependent vasorelaxation (37–39), enhancement of platelet aggregation in whole blood (40, 41), and uptake by macrophages without modification (42, 43). All of these properties of RLPs are potentially proatherogenic. In addition to in vitro evidence, clinical studies have also demonstrated that RLP-C concentrations are higher in CAD patients (17, 20, 21). Serum RLP-C concentrations have also been found to be substantially higher in patients with non-insulin-dependent diabetes mellitus and microalbuminuria (23), undergoing chronic hemodialysis (24), or with intermittent claudication (44).

To help us evaluate the clinical utility of the RLP-C assay in North American populations, we conducted a multicenter program to establish reference ranges and to compare RLP-C concentrations in patients with CAD and in controls with similar ages. Consistent with other studies (17, 20, 21), RLP-C concentrations, particularly in random samples, were higher in Caucasian CAD patients than in age- and gender-matched Caucasian controls (Table 4). Because of the large size of chylomicron-derived particles in nonfasting samples, VLDL-C cannot be calculated with reasonable accuracy in the postprandial state. To permit estimation of LDL-C, plasma lipid analyses are ordinarily performed on fasting samples in the clinical laboratory. A potential disadvantage of this practice is that it may reduce the opportunity to detect defects in lipoprotein remnant removal. It has been hypothesized that atherosclerotic disease can result from postprandial accumulation of remnant particles (45, 46). Previous studies have shown that postprandial RLP-C concentrations are substantially increased above fasting concentrations in CAD patients but not in healthy controls, where the fasting concentrations were considered within reference values in both groups (47, 48). Random sampling provides an alternative to cumbersome fat-loading studies in measuring postprandial RLP-C concentrations.

In summary, we have evaluated a novel immunoseparation method for the quantitative estimation of a lipoprotein remnant fraction. The RLP-C immunoseparation method is faster and easier to use than laboratory methods based on UC or electrophoresis. Additional clinical studies are needed to establish the usefulness of this novel assay.

References

13. Alaupovic P, Mack WJ, Knight-Gibson C, Hodis HN. The role of triglyceride-rich lipoprotein families in the progression of athero-


