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Lipids, Lipoproteins, and Cardiovascular Risk Factors

Use of a Reference Material Proposed by the International Federation of Clinical Chemistry and Laboratory Medicine to Evaluate Analytical Methods for the Determination of Plasma Lipoprotein(a)

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Background: **As part of the NIH/National Heart, Lung and Blood Institute Contract for the Standardization of Lipoprotein(a) [Lp(a)] Measurements, a study was performed in collaboration with the IFCC Working Group for the Standardization of Lp(a) Assays. The aims of the study, performed with the participation of 16 manufacturers and 6 research laboratories, were to evaluate the IFCC proposed reference material (PRM) for its ability to transfer an accuracy-based value to the immunoassay calibrators and to assess concordance in results among different methods.**

Methods: **Two different purified Lp(a) preparations with protein mass concentrations determined by amino acid analysis were used to calibrate the reference method. A Lp(a) value of 107 nmol/L was assigned to PRM. After uniformity of calibration was demonstrated in the 22 evaluated systems, Lp(a) was measured on 30 fresh-frozen sera covering a wide range of Lp(a) values and apolipoprotein(a) [apo(a)] sizes.**

Results: **The among-laboratory CVs for these samples (6–31%) were, in general, higher than those obtained for PRM (2.8%) and the quality-control samples (14%, 12%, and 9%, respectively), reflecting the broad range of apo(a) sizes in the 30 samples and the sensitivity of most methods to apo(a) size heterogeneity. Thus, although all of the assays were uniformly calibrated through the use of PRM, no uniformity in results was achieved for the isoform-sensitive methods.**

Conclusions: **Linear regression analyses indicated that to various degrees, apo(a) size heterogeneity affects the outcome of the immunochemical methods used to measure Lp(a). We have also shown that the inaccuracy of Lp(a) values determined by methods sensitive to apo(a) size significantly affects the assessment of individual risk status for coronary artery disease.**

Lipoprotein(a) [Lp(a)][10] is a complex macromolecule formed by the assembly of apolipoprotein (apo) B100-containing lipoproteins, composed mainly of LDL with a carbohydrate-rich protein, apo(a), that has a high degree

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[10] Nonstandard abbreviations: Lp(a), lipoprotein(a); apo, apolipoprotein; K4, kringle 4; MAb, monoclonal antibody; NWLRL, Northwest Lipid Research Laboratories; CAD, coronary artery disease; PRM, Proposed Reference Material; SDS, sodium dodecyl sulfate; ITA, immunoturbidimetric assay; INA, immunonephelometric assay; FIA, fluorescence immunoassay; and EID, electroimmunodiffusion.

of structural homology with plasminogen, a key zymogen of the coagulation cascade *(1)*. In Lp(a) particles, one molecule of apo(a) is covalently linked by a disulfide bridge with one molecule of apo B100 *(2)*. The presence of apo(a) imparts unique physicochemical characteristics and metabolic properties to Lp(a) that distinguish Lp(a) from LDL and from all other lipoprotein particles.

In addition to its high carbohydrate content, which accounts for ~30% of the protein mass, another distinct peculiarity of apo(a) is its considerable size heterogeneity. The intra- and interindividual size heterogeneity of apo(a) is genetically determined and is primarily related to differences in the length of the polypeptide chain *(3, 4)*. apo(a) is formed by a variable number of repeats of basic structures called kringles *(5)*, all exhibiting a high homology with plasminogen kringle 4, followed by a single copy of the plasminogen-like kringle 5 and the protease domain *(1)*. On the basis of amino acid differences, the apo(a) kringle 4 (K4) domains are divided into 10 different types. K4 type 1 and types 3–10 are present as a single copy in all apo(a) species, whereas K4 type 2 is present in a variable number of identical repeats, varying from as few as 3 copies to as many as 40. This variable number of K4 type 2 repeats accounts for the apo(a) size variation, from 187 to >662 kDa. This size variation of apo(a) constitutes a serious challenge for the immunochemical measurement of Lp(a) in plasma for the following reasons: (*a*) the choice of apo(a) size in the assay calibrator is arbitrary, and, independent of the choice, the calibrator would not be representative of all apo(a) sizes in plasma samples; and (*b*) the reactivity of the antibodies directed to the repeated antigenic sites of apo(a) K4 type 2 will vary depending on the size of apo(a). As a consequence, it is expected that immunoassays will tend to underestimate the apo(a) concentration in subjects with apo(a) of a size smaller than the apo(a) size present in the assay calibrator, and conversely to overestimate the concentration of larger apo(a) particles. To circumvent this problem and to be able to accurately measure apo(a) in all plasma samples, independently of apo(a) size variations, a monoclonal antibody (MAb) specific to a unique epitope present in apo(a) K4 type 9 was generated and characterized at the Northwest Lipid Research Laboratories (NWLRL), University of Washington. This MAb was then used, as reported previously *(5)*, to develop an enzyme-linked immunoassay for the measurement of Lp(a) in plasma. This assay has been extensively evaluated in a large number of individuals *(5, 6)*, and it was documented that there is no influence of apo(a) size heterogeneity on the accuracy of the measurements. Because the MAb does not interact with any epitope in the variable part of the apo(a) molecule and the assay measures Lp(a) particle number, the assignment of the target value to the assay calibrator is expressed in terms of mole per liter.

Despite poor agreement among Lp(a) values obtained by different methods, Lp(a) has been widely measured in a large variety of clinical studies *(7)*. Although there is a lack of consistency in the conclusions of the studies about the contributory role of Lp(a) to coronary artery disease (CAD), it is widely accepted that Lp(a) is an important risk factor that may contribute to CAD independent of or in cooperation with other lipid or non-lipid risk factors *(7)*. Thus, comparable and accurate Lp(a) values are indispensable to achieve a uniform interpretation of clinical data. At present, common population-based reference values are not available, and results from different clinical studies cannot be combined to establish the cutoff point at which Lp(a) imparts an increased risk for CAD. As was done for other lipid and apolipoprotein markers, a major effort is required to evaluate the various immunoassays for their suitability to measure Lp(a) concentrations and to establish an accuracy-based standardization program.

In 1995, the IFCC Working Group for the Standardization of Lp(a) Assays initiated a project, in collaboration with manufacturers of immunoassays for Lp(a), to select a suitable secondary reference material for Lp(a). The analytical performance of the assays and calibrators was evaluated in the first phase of the study *(8)*. In the second phase, several proposed reference materials were evaluated for their analytical performance and commutability properties *(9)*. On the basis of that work, one of the proposed materials was selected as a common calibrator, designated proposed reference material (PRM), to be used to assign an Lp(a) value to the different assay calibrators. The third phase of this study was organized by the recipients of the NIH/National Heart, Lung and Blood Institute Contract for the Standardization of Lp(a) Measurements. We report here the assignment of an accuracy-based Lp(a) value to the PRM, the transfer of the Lp(a) value to the assay calibrators, the among-laboratory comparability of Lp(a) values, and the degree of apo(a) size dependence of the evaluated methods. Furthermore, we evaluated the impact of the inaccuracy of Lp(a) values on the assessment of individual risk status for CAD.

Materials and Methods

PARTICIPATING LABORATORIES AND TEST SYSTEMS

In this study, organized and coordinated by the NWLRL, 22 Lp(a) test systems were evaluated, involving 16 manufacturers and 6 research laboratories (Table 1). Among the Lp(a) test systems examined, 10 were turbidimetric (ITA), 8 were nephelometric (INA), 2 were fluorescent immunoassays (FIAs), 1 was an electroimmunodiffusion assay (EID), and 1 was an ELISA. Most of the test systems used polyclonal antibodies against the apo(a) moiety to measure Lp(a). Two ITA methods (DiaSorin SPQIII and Daiichi) used latex-bound monoclonal antibodies; one FIA method (DELFIA a/B) used a polyclonal antibody against apo B as the detecting antibody.

REFERENCE MATERIAL AND SERUM SAMPLES

The IFCC-selected material, PRM *(9)*, was used as the common calibrator to assign an Lp(a) value to the calibrators of the different methods. PRM is a human lyophilized

Table 1. Participants in the Lp(a) Standardization Protocol.

Participant	Abbreviation	Location	System	Method
Daiichi Pure Chemicals Co. Ltd.	Daiichi	Ibaraki, Japan	Hitachi 717	ITA
Dako A/S	DAKO	Glostrup, Denmark	Cobas Fara II	ITA
Denka Seiken Co., Ltd.	Denka Seiken	Niigata, Japan	Hitachi 917	ITA
DiaSys Diagnostic Systems	DiaSys	Holzheim, Germany	EPOS 5060	ITA
DiaSorin Inc.	DiaSorin SPQII	Stillwater, MN	Cobas Fara II	ITA
	DiaSorin SPQII		Cobas Fara II	ITA
Nitto Boseki Co., Ltd.	Nitto	Fukushima, Japan	Hitachi 7150	ITA
Orion Diagnostica	Orion	Espoo, Finland	Kone Specific	ITA
Roche Diagnostics GmbH	Roche	Penzberg, Germany	Cobas Integra 700	ITA
	Roche		Hitachi 911	ITA
Baxter Immuno Hyland	Baxter	Wien, Austria	BN100	INA
Beckman Coulter, Inc.	Beckman	Brea, CA	Beckman Array	INA
	Beckman		IMMAGE	INA
Children's Hospital	Children's	Boston, MA	BNII	INA
Dade Behring Marburg GmbH	Dade	Marburg, Germany	BNII	INA
International Enzymes Inc.	IntEnzyme	Fallbrook, CA	Beckman Array	INA
Princess Alexandra Hospital	Queensland	Brisbane, Australia	Beckman Array	INA
Tenon Hospital	Tenon	Paris, France	Beckman Array	INA
Institute of Medical Biochemistry	IMB	Graz, Austria	DELFIA (a/B)	FIA
	IMB		DELFIA (a/a)	FIA
SEBIA	SEBIA	Issy-les-Moulineaux, France		EID
Baylor College of Medicine	Baylor	Houston, TX		ELISA

serum pool preserved by the addition of sucrose, L-lysine monohydrochloride, and sodium azide. The preparation, chemical composition, stability, linearity, and parallelism of PRM have been reported previously *(9)*.

Three fresh-frozen serum samples, designated A01, B01, and C01, respectively, were prepared by the NWLRL as described previously in detail *(10)* to be used as common quality controls in the different test systems. These serum samples were selected to have low, intermediate, and high Lp(a) concentrations and a single medium-sized apo(a) isoform. Following the same procedure used for the preparation of the quality-control samples *(10)*, serum samples were obtained from 30 healthy donors selected to have a large range of Lp(a) values and apo(a) isoforms to be used to compare Lp(a) values obtained by the different methods after common calibration.

Lp(a) PRIMARY CALIBRATOR

Blood, obtained from a healthy adult donor exhibiting a single apo(a) isoform, was collected in 10-mL Vacutainer Tubes containing sodium EDTA to yield a final EDTA concentration of 1 mmol/L. A portion of this plasma was shipped on ice by overnight express mail to the laboratory of Dr. Angelo M. Scanu at the University of Chicago. Lp(a) was isolated from this plasma by two independent procedures in the laboratories of Dr. Marcovina, at the University of Washington, and Dr. Scanu, using the locally established isolation procedures.

The Lp(a) isolation procedure used at the University of Washington is an adaptation of the procedure originally described by Albers and Hazzard *(11)* and involves sequential density ultracentrifugation followed by gel-filtration chromatography. Specifically, the non-protein solvent density (d) of the plasma is adjusted to 1.050 kg/L with solid KBr, and ultracentrifugation is carried out in a 60 Ti rotor at 177 520g at 10 °C for 20 h. The top one-third of each tube is removed, and the bottom fraction is readjusted to 1.090 kg/L with solid KBr and recentrifuged at 177 520g for 20 h. The d = 1.050–1.090 kg/L lipoprotein fraction contained in the top fraction is applied to a 2.5 × 100 cm Sephacryl S-400 column equilibrated with 33 mmol/L sodium phosphate, 0.1 g/L NaN_3, 0.1 g/L sodium EDTA, and 0.2 mol/L proline. Fractions containing only Lp(a) are pooled, dialyzed against the column buffer but without proline, sterilized by filtration through a 0.22 μm filter, and stored at 4 °C under nitrogen.

The Lp(a) isolation procedure used at the University of Chicago is an adaptation of that described by Fless and Snyder *(12)*. The plasma is adjusted with solid NaBr to d = 1.21 kg/L, de-aerated to remove dissolved oxygen, and spun in the 60 Ti rotor at 177 520g for 20 h at 20 °C. The lipoproteins floating at the top of the tube are removed in a volume of 5 mL or less and dialyzed against 33 mmol/L phosphate, 0.1 g/L disodium EDTA, and 0.2 g/L NaN_3, pH 7.4. This fraction is then applied to a lysine-Sepharose column at a flow rate of 12 mL per cm^2 per hour and washed until the absorbance has returned to baseline. A ratio of 1 mL of lysine-Sepharose per mg of Lp(a) protein is usually sufficient and ensures excess capacity. Nonspecifically bound lipoproteins are removed with a column volume of 0.1 mol/L $NaHCO_3$, 0.5 mol/L NaCl, 0.1 g/L disodium EDTA, 0.2 g/L NaN_3, pH 8.3. Lp(a) is then eluted either with 200 mmol/L ϵ-aminocap-

roic acid dissolved in the above phosphate buffer for donors with single apo(a) isoforms or with a 200-mL gradient of 0–200 mmol/L ϵ-aminocaproic acid for donors with two apo(a) isoforms. The fractions containing Lp(a) are pooled consecutively as 40-mL aliquots, which are adjusted with solid CsCl to 75 g/L and subjected to ultracentrifugation in the 50.2 Ti rotor at 20 °C, 24 h, at 197 650*g*. These conditions generate a density gradient in which Lp(a) species with small apo(a) isoforms elute in earlier fractions and Lp(a) with larger apo(a) isoforms elute in later fractions. After the centrifugation step is completed, the tubes are carefully removed from the buckets and placed in the density gradient fractionating system. The tubes are then pierced at the bottom, and the gradient is pushed out of the top at a flow rate of 1 mL/min with the dense fluorocarbon oil Fluorinert FC-40 (ISCO), which has a density of 1.85 kg/L. The chart speed is 1 cm/min, and the fraction collector is set to 0.5 mL/tube. The gradient is monitored at 280 nm. Lipoprotein purity (essentially LDL contamination) is established by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 4–15% polyacrylamide gradient gels (Novex). Lp(a) preparations are dialyzed against 33 mmol/L phosphate, 0.1 g/L disodium EDTA, 0.1 g/L NaN_3, pH 7.4, sterilized into sterile Sarstedt screw cap microtubes in a laminar flow hood, and stored at 4 °C under nitrogen.

chemical analysis of isolated Lp(a)

For amino acid analysis, 75 nmol of norleucine was added to 250 μL of purified Lp(a). Duplicate samples of Lp(a), without lipid extraction, were hydrolyzed in 6 mol/L HCl, 0.5 mL/L mercaptoethanol, 0.2 mL/L phenol for 20 h at 115 °C in sealed evacuated hydrolysis tubes. Analyses were carried out on a Beckman model 7300 amino acid analyzer equipped for single-column methodology using the Beckman sodium buffer system and Beckman System Gold software for data analysis. To compensate for destruction by acid hydrolysis, serine values were increased by 10% and threonine by 5%. All amino acid analysis values were corrected for possible sample transfer losses or possible inaccurate volume measurements during sample application, by calculations taking into account recovery values for the norleucine internal standard *(2)*. Cholesterol, triglycerides, and phospholipid were measured at the NWLRL by highly standardized enzymatic procedures using the Hitachi 917 automated analyzer.

Lp(a) measurements

Lp(a) concentrations were measured by a direct-binding double MAb-based ELISA performed as reported previously *(5)*. The capture MAb (a-6) is directed to an epitope present in apo(a) K4 type 2, and the detection antibody (a-40) is directed to an epitope present in apo(a) K4 type 9. Parallel analyses were also performed with a different detection antibody (a1-) directed to an epitope present in apo(a) K4 type 8. This ELISA method has been evaluated extensively *(5)* and demonstrated to be insensitive to apo(a) isoform size heterogeneity. Lp(a) concentrations are expressed in nmol/L. Fresh-frozen plasma samples from four individuals representing a broad range of Lp(a) concentrations were used as quality controls.

determination of apo(a) isoform size

The apo(a) isoforms were determined by a high-resolution SDS-agarose gel electrophoresis followed by immunoblotting as reported previously *(13)*. We have evaluated the relationship of the number of K4 domains, as determined by pulsed-field gel electrophoresis *(14)*, to the mobility of the isoforms on SDS-agarose gel electrophoresis *(13)* and found that the logarithm of the K4 number is highly correlated with the mobility of the isoforms on agarose gel *(15)*. The relative mobility of the band is used to determine the number of K4 domains and is calculated in comparison to a calibrator with known apo(a) sizes. The calibrator was prepared in-house by combining the plasma of three heterozygous individuals chosen on the basis that they cover a large range of isoforms, 13, 19, 24, 32, 38 K4 domains, as assessed by pulsed-field gel electrophoresis *(14)*. A UMAX Powerlook III Scanner (UMAX Technologies) was used to transform photographic films into image files that were then analyzed with gel analysis software (Sigma Gel, SPSS Application Package). The apo(a) isoforms in the samples were therefore designated by the relative number of K4 domains.

Lp(a) value assignment to PRM

For the preliminary assignment of a Lp(a) target value to PRM, a secondary serum calibrator, designated LL, with a value assigned previously against a primary Lp(a) preparation, was used as interim reference material to calibrate the in-house reference ELISA. Earlier studies by the coordinating laboratory had established that LL had an Lp(a) concentration of 187 nmol/L. Twenty replicate analyses of PRM over a 2-week period in the reference ELISA assay yielded a value of 108.2 ± 3.1 nmol/L for PRM.

The final assignment of a target value to PRM was carried out with the use of two preparations of Lp(a), one isolated in Dr. Marcovina's laboratory at the University of Washington and one in Dr. Scanu's laboratory at the University of Chicago. Each preparation had amino acid analyses performed in duplicate to obtain an accurate absolute mass of the Lp(a) protein expressed in molar units. Each freshly isolated Lp(a) preparation was used to prepare a six-point calibration curve in quadruplicate on multiple plates for each of the two ELISAs based on MAb a-40 or a1-1. PRM was analyzed six times on three separate plates for each ELISA. Additionally, four quality-control samples were analyzed three times on each plate. All analyses were performed in duplicate. The same protocol was carried out for 4 consecutive days, yielding a total of 144 values for PRM.

VALUE TRANSFER PROTOCOL

The coordinating laboratory provided to each participant the PRM; fresh-frozen control serum samples A01, B01, and C01 with low, medium, and high Lp(a) concentrations; and 30 fresh-frozen samples from individual donors to evaluate comparability of the measurements. The 3 quality controls and the 30 samples were analyzed by the coordinating laboratory 320 times in duplicate over a 6-week period by the MAb a-40 reference ELISA, using PRM as calibrator to obtain the assigned value for each sample. All materials were stored at −70 °C until use. Before analysis, each frozen quality-control pool was equilibrated to room temperature. For reconstitution, the lyophilized Lp(a) Reference Material was brought to room temperature, and 1.0 ± 0.005 mL of distilled water at 25 °C was added. The mixture was swirled gently until completely dissolved and then allowed to stand 30 min at room temperature with occasional mixing by inversion. Just before use, the reference material was gently mixed again for 5 min on a rotator or similar device.

The value transfer protocol was carried out in three separate steps. For the first step, each system was calibrated with the Lp(a) PRM according to the assay specifications for each system. The three frozen serum pools were then analyzed in quadruplicate in two analytical runs per day on 3 separate days, with the second run carried out in reverse order. A separate dose–response curve for PRM was prepared for each run. For the second step, each system was again calibrated with PRM according to the usual protocol. The in-house calibrator was run as an unknown in quadruplicate in two analytical runs per day on 5 separate days. The mean of the 40 values was used as the assigned value for the in-house calibrator. Each system was then calibrated with the in-house calibrator with the newly assigned value, and the three frozen serum pools and PRM were run as unknowns in quadruplicate in two analytical runs per day on 2 separate days. For the third step of the protocol, each system was calibrated with the in-house calibrator with the value assigned and validated in step 2. Thirty frozen sera provided by the coordinating laboratory from individual donors covering a wide range of Lp(a) concentrations and sizes were analyzed in duplicate in two different analytical runs along with the three quality-control samples. A separate dose–response curve was prepared for each run. Following the same protocol used by the 22 participants, the 30 samples were also analyzed at the NWLRL with the same ELISA approach used for the assignment of target values except that the detecting MAb, a-40, was replaced by a MAb directed to an epitope present in K4 type 1 and type 2. This ELISA format, as reported previously *(5)*, is highly sensitive to the apo(a) size heterogeneity.

DATA ANALYSIS

The Pearson product–moment correlation coefficient *(2)* between the assigned value and the mean value obtained on each of the 30 samples for each of the analytical systems was computed by linear regression analysis. The mean percent bias and the mean absolute percent bias were calculated according to the approach used for the standardization of methods for the measurement of apo A-I and B *(16, 17)*. The precision of individual assays was evaluated by computing the CV for each sample for the two replicates on 2 separate days and then computing the overall CV as $(\Sigma CV^2/n)^{1/2}$, where n = 30 samples.

The among-method CV for each of the 30 samples was computed from the mean Lp(a) values obtained by each method. The overall among-method CV was computed as $(\Sigma CV^2/n)^{1/2}$, where n = 30 samples.

Results

CHEMICAL COMPOSITION OF THE PRIMARY CALIBRATOR

The Lp(a) to be used as primary calibrator was isolated from a donor exhibiting a single apo(a) isoform containing 19 K4 domains. The chemical composition of the Lp(a) prepared by a combination of sequential ultracentrifugation and molecular sieve chromatography was very similar to the Lp(a) prepared by a combination of lysine-Sepharose and CsCl gradient ultracentrifugation (Table 2). The two Lp(a) preparations contained 25–26% protein,

Table 2. Composition of Lp(a) preparations.[a]

Lp(a) sample	Protein, %	UC,[b] %	CE,[c] %	PL, %	TG, %
Preparation 1[d]	26.5	7.4	38.2	18.4	9.6
Preparation 2[e]	25.2	8.2	38.2	20.1	8.4

[a] Composition expressed as percentage of total weight excluding carbohydrate.

[b] UC, unesterified cholesterol; CE, cholesteryl ester; PL, phospholipids; TG, triglycerides.

[c] CE = (total cholesterol − unesterified cholesterol) × 1.677.

[d] Lp(a) isolated by sequential ultracentrifugation and molecular sieve chromatography at the University of Washington.

[e] Lp(a) isolated by lysine-Sepharose and CsCl density gradient ultracentrifugation at the University of Chicago.

Table 3. Lp(a) concentration of Lp(a) Reference Material PRM when tested against two different Lp(a) preparations in two different ELISAs.

Preparation	Method	Mean, nmol/L	SD, nmol/L	CV, %[a]
UC-Lp(a)[b]	a1-1 ELISA	106.5[c]	8.9	8.3
UC-Lp(a)[b]	a-40 ELISA	107.1[c]	9.8	9.1
UW-Lp(a)[d]	a1-1 ELISA	107.6[c]	6.2	5.8
UW-Lp(a)[d]	a-40 ELISA	107.3[c]	9.5	8.8
All preparations and methods		107.1[e]	8.6	8.0

[a] Within-assay CV = 3.6%; between-assay CV = 7.0%.

[b] Lp(a) isolated by lysine-Sepharose and CsCl density gradient ultracentrifugation at the University of Chicago.

[c] Mean of 36 values from 3 values/plate on 3 plates/day on 4 days.

[d] Lp(a) isolated by sequential ultracentrifugation and molecular sieve chromatography at the University of Washington.

[e] Mean of 144 values.

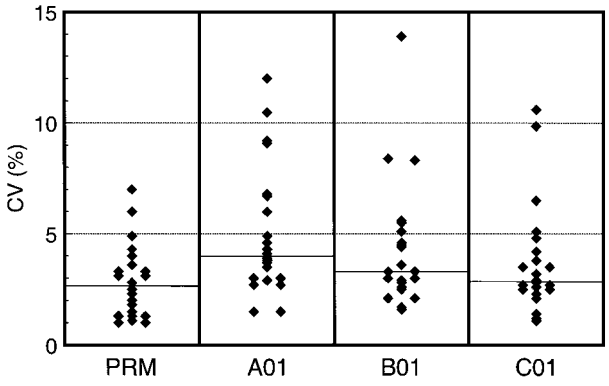

Fig. 1. Within-assay imprecision of the 22 evaluated systems for the PRM and on the fresh-frozen quality-control samples (*A01*, *B01*, and *C01*).

The *solid horizontal lines* indicate the median CVs. The *dashed lines* indicate CVs of 5% and 10%.

7–8% unesterified cholesterol, 38% cholesteryl ester, 18–20% phospholipids, and 8–9% triglycerides, respectively.

ASSIGNMENT OF TARGET VALUE TO PRM

Over a 4-day period, PRM was extensively analyzed by the two different MAb-based ELISAs, yielding a total of 144 values. Very similar Lp(a) values were obtained for PRM regardless of which Lp(a) preparation was used as primary calibrator and regardless of which MAb was used in the ELISA (Table 3). The overall mean ± SD was 107.1 ± 8.6 nmol/L. Thus, the final value assigned to PRM was 107 nmol/L.

DETERMINATION OF APO(A) ISOFORM SIZE

Analysis by SDS-agarose gel electrophoresis followed by immunoblotting evidenced in PRM three predominantly expressed apo(a) isoforms of nearly equal intensity in the gel, containing 16, 17, and 18 K4 domains, respectively, and three minor isoforms formed by 14, 20, and 32 K4 domains, respectively. A similar pattern in apo(a) size distribution but slight difference in the number of K4 domains was obtained when PRM was phenotyped in a different laboratory as reported previously *(9)*. The quality-control samples A01, B01, and C01 contained a single apo(a) isoform size with 18, 21, and 22 K4 domains, respectively.

COMPARISON STUDY

The within-assay imprecision for the quality-control samples and PRM for the 22 systems is illustrated in Fig. 1. For PRM, all systems had good precision with CVs ≤7%. For the low quality-control sample, A01, 5 of the 22 systems had a CV >6%, whereas only 3 systems had a CV >6% for the medium and high Lp(a) samples B01 and C01. Calibration of the systems with PRM at step 1 of the protocol produced reasonably comparable values for the three quality-control pools, the among-method CVs being 12%, 11%, and 9.5% for A01, B01, and C01, respectively. This finding suggests that the among-system matrix effect of PRM is minimal.

In the second step of the protocol, the Lp(a) value was transferred from PRM to the individual calibrators of the systems. When the in-house calibrators with the value assignment traceable to PRM were used, the mean values

Table 4. Relationship of the assigned value to the observed value in 30 samples.

Participant	System	Method	CV, %	*r*	Slope	*y*-Intercept	Absolute bias, nmol/L
Daiichi	Hitachi 717	ITA	4.6	0.989	0.85	4.1	12.4
DAKO	Cobas Fara II	ITA	7.0	0.970	0.82	20.5	18.4
Denka Seiken	Hitachi 917	ITA	2.3	0.999	0.99	1.7	4.4
DiaSys	EPOS 5060	ITA	2.5	0.989	0.81	25.5	19.3
DiaSorin SPQIII	Cobas Fara II	ITA	5.0	0.995	0.86	3.8	13.2
DiaSorin SPQII	Cobas Fara II	ITA	3.1	0.975	0.89	15.8	17.4
Nitto	Hitachi 7150	ITA	2.5	0.985	0.89	22.6	19.6
Orion	Kone Specific	ITA	3.5	0.930	0.78	74.3	59.3
Roche	Cobas Integra 700	ITA	3.3	0.980	0.92	11.1	13.7
	Hitachi 911	ITA	8.2	0.968	0.74	20.0	19.8
Baxter	BN100	INA	16	0.990	0.79	8.9	16.1
Beckman	Beckman Array	INA	2.2	0.980	0.93	10.0	15.8
	IMMAGE	INA	1.6	0.962	0.88	20.5	21.8
Children's	BNII	INA	12	0.969	0.81	15.1	17.1
Dade	BNII	INA	2.5	0.964	0.79	13.6	16.7
IntEnzyme	Beckman Array	INA	10	0.971	0.91	16.1	19.7
Queensland	Beckman Array	INA	4.0	0.988	0.90	7.4	12.6
Tenon	Beckman Array	INA	4.6	0.983	0.90	10.6	15.0
IMB	DELFIA (a/B)	FIA	4.5	0.985	0.78	7.9	16.7
	DELFIA (a/a)	FIA	4.2	0.942	0.66	18.9	23.8
SEBIA		EID	6.5	0.984	0.72	12.1	20.1
Baylor		ELISA	13	0.970	0.77	18.4	19.4

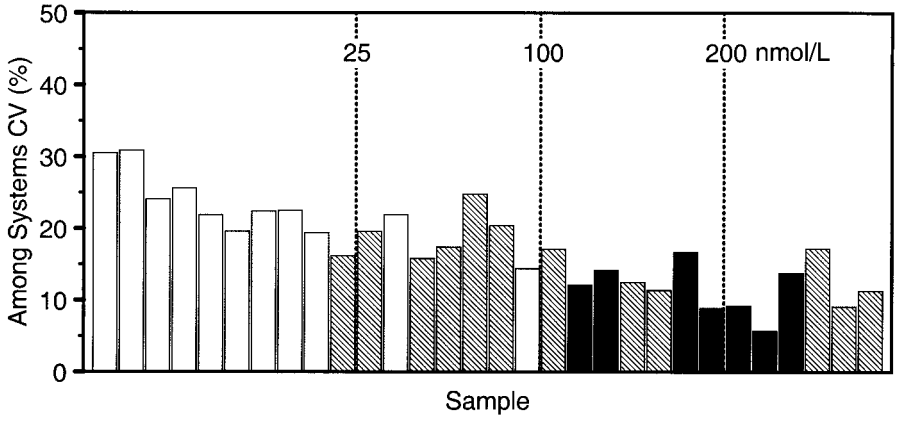

Fig. 2. Among-method CVs for each of the 30 fresh-frozen samples computed from the 21 system means.

The samples are ordered from *left* to *right* by the relative Lp(a) concentration and are grouped into three apo(a) size categories [13–18 (■), 19–24 (▨), and 25–31 (□) K4 domains, respectively].

obtained by the participants on the three quality-control samples were nearly identical to those obtained when PRM was used as calibrator. One analytical system provided Lp(a) values that were two to three times higher than those obtained by the other methods and was then considered an outlier and excluded from the analysis. The remaining 21 analytical methods appeared to be uniformly calibrated at this stage because the among-system CV for PRM was only 2.8% and all but one system had a mean value for PRM within 5 nmol/L of the target value. This again indicates a negligible matrix effect of PRM in the evaluated systems.

To further evaluate the various immunoassays at step 3 of the protocol, each participant analyzed 30 fresh-frozen samples with Lp(a) values of 10–414 nmol/L and predominantly expressed apo(a) size isoforms containing 13–31 K4 domains. Among the 22 systems, 15 had a excellent precision, with overall CVs of 1.6–5.0%. Among the remaining systems, three had CVs of 6.5–8.2%, and four had CVs ≥10% (Table 4). The correlation coefficient between the assigned values and the mean values obtained for each sample varied considerably depending on the system, ranging from a high of 0.999 to a low of 0.930 (Table 4), with 12 of the systems having a correlation of 0.980 or greater. The average absolute bias between the observed and assigned value for each system ranged from a low of 4 nmol/L to a high of 59 nmol/L.

After exclusion of the outlier system, the among-method CV for each of the 30 samples was 6–31% and decreased as the Lp(a) concentration increased (Fig. 2). Thus, for the nine samples with very low Lp(a) values (<25 nmol/L), the CVs were 19–31%, whereas the CVs for the six samples with very high Lp(a) values (>200 nmol/L) were 6–17%. Because of the inverse correlation between Lp(a) concentration and apo(a) size, there was a direct relationship between the CV and the size of apo(a) in the sample (Fig. 2). The overall among-method CV was 18%, and CVs were generally higher for the individual samples than for the quality-control pools (14%, 12%, and 9% for A01, B01, and C01, respectively).

apo(a) size-dependent bias of Lp(a) immunoassays

To further examine the basis for the among-system differences in Lp(a) values, we determined the degree to which the bias of Lp(a) values (percent difference between observed and assigned values) correlated with the apo(a) size of the sample. All systems exhibited a statistically significant ($P < 0.01$) positive correlation between the percent bias for Lp(a) values and the apo(a) size, indicating a general tendency for Lp(a) values to be overestimated in samples with large apo(a) isoforms and underestimated in samples with small apo(a) size. The impact of apo(a) size on the analytical methods was variable, and only three systems exhibited a minimal relationship be-

Table 5. Effect of apo(a) size on the Lp(a) assays.[a]

Participant	System	Method	*r*	Slope	*y*-Intercept
Daiichi	Hitachi 717	ITA	0.812	2.07	−51.2
DAKO	Cobas Fara II	ITA	0.901	10.30	−187.6
Denka Seiken	Hitachi 917	ITA	0.759	2.11	−39.8
DiaSys	EPOS 5060	ITA	0.922	9.99	−178.8
DiaSorin SPQIII	Cobas Fara II	ITA	0.568	2.09	−53.6
DiaSorin SPQII	Cobas Fara II	ITA	0.923	5.92	−107.3
Nitto	Hitachi 7150	ITA	0.910	9.30	−161.4
Orion	Kone Specific	ITA	0.905	25.34	−411.6
Roche	Cobas Integra 700	ITA	0.896	5.82	−106.9
	Hitachi 911	ITA	0.907	8.67	−165.9
Baxter	BN100	INA	0.702	3.73	−81.4
Beckman	Beckman Array	INA	0.693	4.82	−90.8
	IMMAGE	INA	0.921	7.79	−138.7
Children's	BNII	INA	0.932	6.99	−136.5
Dade	BNII	INA	0.855	4.26	−86.7
IntEnzyme	Beckman Array	INA	0.666	5.43	−96.6
Queensland	Beckman Array	INA	0.750	4.23	−84.6
Tenon	Beckman Array	INA	0.719	4.86	−92.8
IMB	DELFIA (a/B)	FIA	0.868	3.30	−77.0
	DELFIA (a/a)	FIA	0.940	7.56	−157.8
SEBIA		EID	0.696	3.66	−82.8
Baylor		ELISA	0.861	7.28	−141.6

[a] Linear regression analysis of the percent bias between the Lp(a) values obtained by the system and the Lp(a) target value vs apo(a) kringle number of the sample.

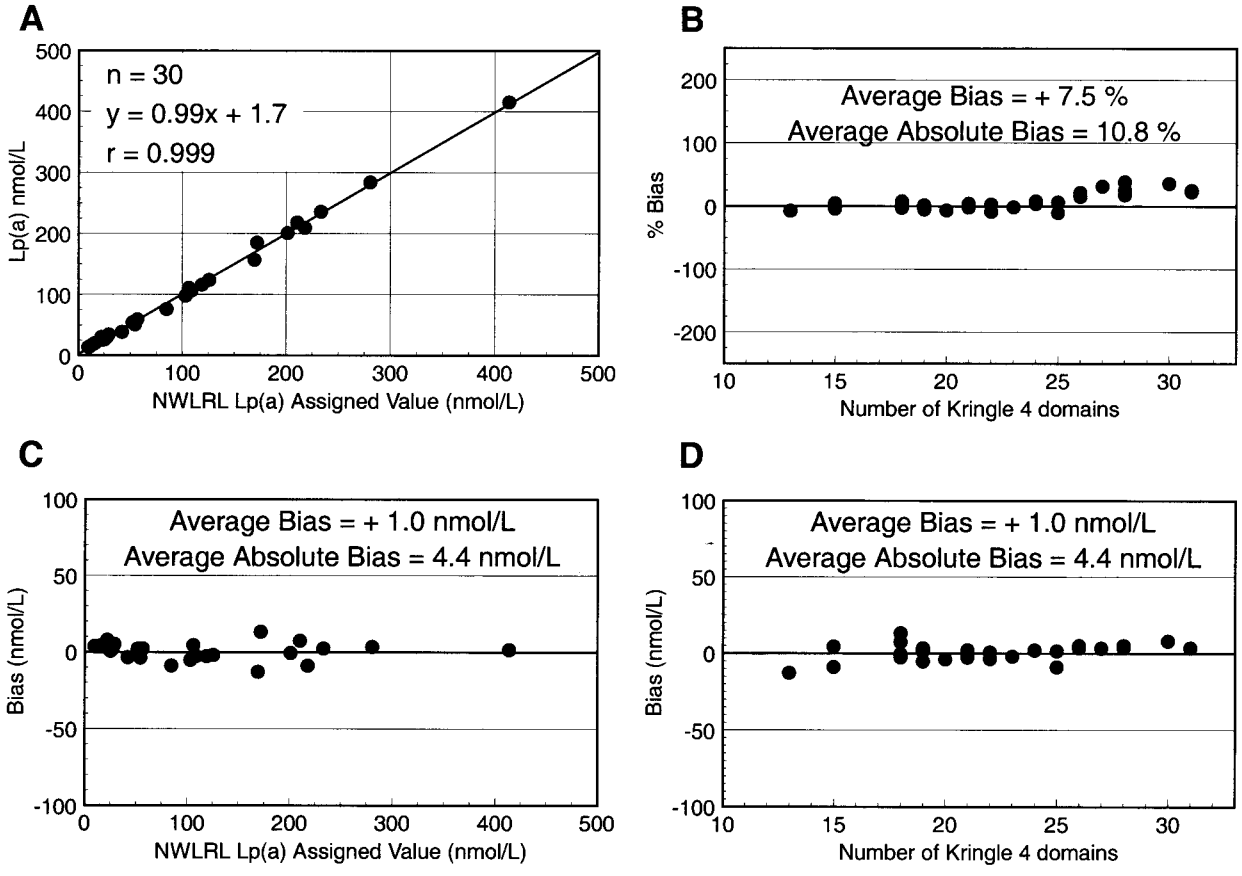

Fig. 3. Evaluation of a turbidimetric method (Denka Seiken reagent on a Hitachi 917 automated analyzer).

(*A*), linear regression analysis of the mean Lp(a) values obtained by the Denka method and the assigned values. (*B*), linear regression analysis of the average percent bias [100 × (Denka value − assigned value)/assigned value] between the values determined by the Denka method and the reference method and the number of apo(a) K4 domains in the samples. (*C*), bias (in nmol/L) between the obtained and the assigned values. (*D*), bias (in nmol/L) between the obtained and the assigned value in samples with defined apo(a) size as indicated by the number of K4 domains.

tween the sample bias and apo(a) size as indicated by both a low slope (<2.2) and a small intercept (less than −55; Table 5). In one system, for two samples with very low Lp(a) values (9.7 and 14.1 nmol/L) and large apo(a) sizes, the percent bias was considerably higher than that obtained for other samples with similar Lp(a) concentrations and apo(a) size. These two samples were therefore considered as outliers and excluded from the statistical evaluation.

In Fig. 3, we show the performance of the test system (Denka Seiken reagent on a Hitachi 917 instrument) that achieved the best concordance with the reference method ($r = 0.999$; $y = 0.99x + 1.7$; Fig. 3A). As evidenced in Fig. 3B, this system exhibited a positive bias for all samples with apo(a) isoforms containing >25 K4 domains. However, because of the low Lp(a) values in these samples (<25 nmol/L), the absolute difference between the observed and the assigned values was negligible (Fig. 3D). Overall (Fig. 3, A and C), superimposable results with the reference method were obtained by this turbidimetric method after calibration with PRM.

The method that exhibited the lowest correlation between the percent bias and apo(a) size ($r = 0.568$; Table 5), indicating a minimal apo(a) size dependence of this system, was a latex-bound MAb-based turbidimetric assay (DiaSorin SPQIII). However, there was less concordance with this method between the obtained and the assigned values as indicated by an absolute bias of 13.2 nmol/L (Table 4). A third system, Daiichi Pure Chemicals, exhibited a good concordance between obtained and assigned values in samples with medium and large apo(a) isoforms, whereas Lp(a) values were underestimated in all samples with apo(a) isoforms containing ≤20 K4 domains. Although the impact of apo(a) size heterogeneity on the accuracy of the values was variable for the remaining methods, eight of the methods had a very similar high degree of apo(a) size dependency as indicated by correlations >0.90 between the number of apo(a) K4 domains and Lp(a) values and similar high slopes. The among-method CVs of these systems for the 30 samples were 5.5–22%, with an overall CV of 13%.

We then computed the mean Lp(a) value obtained by the eight systems for each of the 30 samples. Regression analysis of the percent bias of the mean Lp(a) values vs

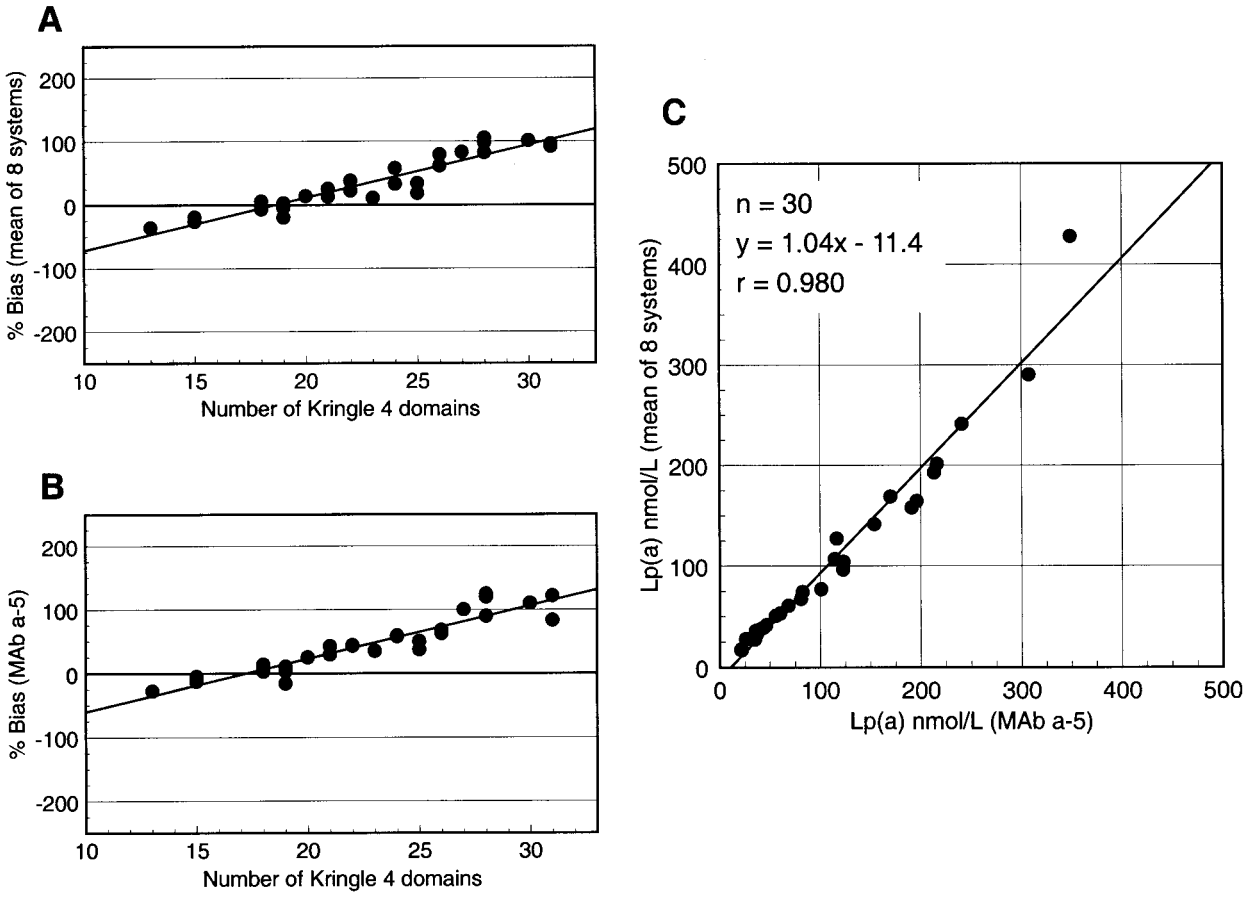

Fig. 4. Linear regression analyses.

(*A*), linear regression analysis of the mean percent bias [100 × (mean of eight systems value − assigned value)/assigned value] between the mean values determined by eight systems and by the reference method and the number of apo(a) K4 domains in the samples. (*B*), linear regression analysis of the average percent bias [100 × (MAb a-5 ELISA value − reference ELISA value) reference ELISA value] between the Lp(a) values determined by the MAb a-5 and the MAB a-40 ELISAs and the number of apo(a) K4 domains in the samples. (*C*), linear regression analysis of the mean Lp(a) values obtained by eight analytical systems and MAb a-5 ELISA values.

the size of apo(a) yielded a line with a slope of 7.75 and a *y*-intercept of −144 (Fig. 4A). Measurement of Lp(a) in these 30 samples by ELISA, using MAb a-5, which is specific for apo(a) K4 type 1 and type 2 repeats, for detection, yielded a slope of 8.31 and a *y*-intercept of −143 (Fig. 4B), which was very similar to that obtained by the eight systems. Additionally, we found a high correlation between the mean Lp(a) values obtained by these systems and those obtained by the MAb a-5-based ELISA (r = 0.980; $y = 1.04x - 11.4$; Fig. 4C). On the basis of the regression line of the percent bias vs the number of K4 domains for the eight systems affected by apo(a) size (Fig. 4A), we calculated the expected percent bias of Lp(a) values as a function of apo(a) size (Table 6). Note that samples with small apo(a) isoforms (<19 K4 repeats) have a negative bias and samples with large apo(a) isoforms (≥19 K4 repeats) have a positive bias, and that the larger the isoform the greater the bias.

We next evaluated the impact of the inaccuracy of the Lp(a) values determined by methods affected by apo(a) size on the assessment of individual risk status for CAD. An Lp(a) value of 75 nmol/L, which approximates the 80th percentile for white Americans *(6)*, was arbitrarily selected as the decision cutpoint. Therefore, from among the 2052 white Americans from the CARDIA study *(6)* whose Lp(a) values were determined by our ELISA reference method, we selected all individuals with values between 50 and 75 nmol/L. From this cohort, 132 individuals, corresponding to 6.3% of the population, fell within this range. Among them, 21 individuals (16%) had a single or a predominantly expressed apo(a) isoform containing <19 K4 domains, whereas 111 (84%) had apo(a) isoforms containing ≥19 K4 domains. The frequency distribution of apo(a) isoforms of these 132 individuals is presented in Fig. 5A. On the basis of the frequency of the isoforms and the regression line depicting the bias (see Fig. 4A), if samples from these 132 individuals, whose correct values were between 50 and 75 nmol/L, were measured by the systems affected by apo(a) size, 63% of the Lp(a) values would be expected to equal or exceed 75 nmol/L (false positive). Therefore, 83 individuals in this group of 132 would be erroneously classified as being at increased risk for CAD, whereas only 49 would be correctly classified. To estimate the number of

Table 6. Percent bias of apo(a) size-sensitive systems and population frequency of predominant apo(a) isoforms in white Americans.

K4 domains	Bias, %	Frequency,[a] % (n = 2052)	Mean Lp(a),[a] nmol/L
13	−43.1	0.4	132.2
14	−35.3	1.0	149.0
15	−27.6	1.1	137.7
16	−19.8	1.5	137.8
17	−12.1	3.8	119.8
18	−4.3	7.6	128.5
19	3.4	6.8	110.8
20	11.2	7.6	64.7
21	18.9	6.7	35.6
22	26.7	4.3	40.8
23	34.4	4.1	45.7
24	42.1	4.7	28.3
25	49.9	5.4	26.8
26	57.6	6.8	26.7
27	65.4	7.3	20.9
28	73.1	6.9	21.3
29	80.9	7.5	14.0
30	88.6	5.2	16.2
31	96.4	3.6	9.7
32	104.1	3.7	9.3
33	111.9	1.6	6.4
34	119.6	1.1	6.7
35	127.4	0.4	3.2
36	135.1	0.3	4.4
37	142.8	0.1	2.5
38	150.6	0.3	3.9

[a] Frequency and mean Lp(a) value of apo(a) isoforms of white Americans taken from Marcovina et al. *(6)*.

potential false negatives, we selected from the CARDIA study participants those whose Lp(a) values were between 75 and 100 nmol/L. In this range, there were 106 individuals, representing 5% of the population. The frequency distribution of apo(a) isoforms of these 106 subjects is presented in Fig. 5B. Following the same approach used for the previous group, we found that nine individuals (8.5%), who based on their Lp(a) values would be considered at increased risk for CAD, were misclassified by the systems affected by apo(a) size (false negative).

Discussion

It is well known that to correctly classify subjects in terms of their risk status for CAD, the analytical methods for measuring the humoral risk factors should be validated in terms of accuracy and precision so that comparable results can be obtained across methods. Well-documented approaches have been used to successfully standardize the measurement of cholesterol in plasma and lipoprotein fractions *(18)* and the measurement of apo A-I and B *(16, 17)*. However, standardization of the analytical methods for the determination of the protein component of Lp(a) is further complicated by the size heterogeneity of apo(a), and no data are available on the impact of the inaccuracy of Lp(a) methods on the assessment of individual risk status for CAD.

Supported by the NIH/National Heart, Lung and Blood Institute Contract for the Standardization of Lp(a) Measurements, and in collaboration with the IFCC Working Group for the Standardization of Lp(a) Assays, an accuracy-based target value of 107 nmol/L was assigned to PRM. The assignment of a target value in nmol/L is an important step toward a scientifically sound and standardized approach in reporting Lp(a) values. The aim of this study was to evaluate to what extent the use of a common reference material would influence plasma Lp(a) values obtained in different laboratories. For this purpose, we used an established and documented approach, similar to that developed for the standardization of apo A-I and B *(16, 17)*, to transfer the accuracy-based value from PRM to the master calibrators of 22 analytical systems. By following this protocol, we found that all systems were uniformly calibrated as demonstrated by the fact that the among-system CV for PRM was only 2.8%. The finding that the among-system CV was significantly higher (6–31%) in the 30 fresh-frozen samples clearly indicates that factors other than method calibration significantly contribute to differences in Lp(a) values.

Among the methods evaluated, two exhibited a very high correlation with our reference method ($r = 0.999$ and 0.995, respectively) with minimum bias between the obtained and the assigned value related to apo(a) size. In contrast, a large apo(a) size-dependent bias was observed in most systems. The high concordance in Lp(a) values obtained by the two methods minimally affected by apo(a) size variability and the very low among-method CV for PRM clearly indicate a lack of significant matrix effect and the suitability of PRM as a reference preparation. However, the use of PRM did not produce concordance in Lp(a) values obtained by isoform-sensitive methods. This study has clearly confirmed that a suitable reference material can reduce the variability related to the calibration component of the different analytical systems but does not produce accurate values. The major problem in the lack of accuracy in Lp(a) values is represented by the over- or underestimation of Lp(a) values as a result of apo(a) size heterogeneity. An additional confounding factor in analyzing the comparability of Lp(a) values obtained by different systems is the variable degree of dependence of the evaluated methods on apo(a) size. This variability is most likely attributable to differences in the reactivity and affinity of the antibodies for the variable part of apo(a) molecule, differences in precision and robustness of the assays, and differences in the system design that can either minimize or maximize the effect of apo(a) heterogeneity.

An additional important component of our study was the possibility, using the data obtained, of determining the extent to which the inaccuracy of Lp(a) values derived from the methods affected by apo(a) size would impact

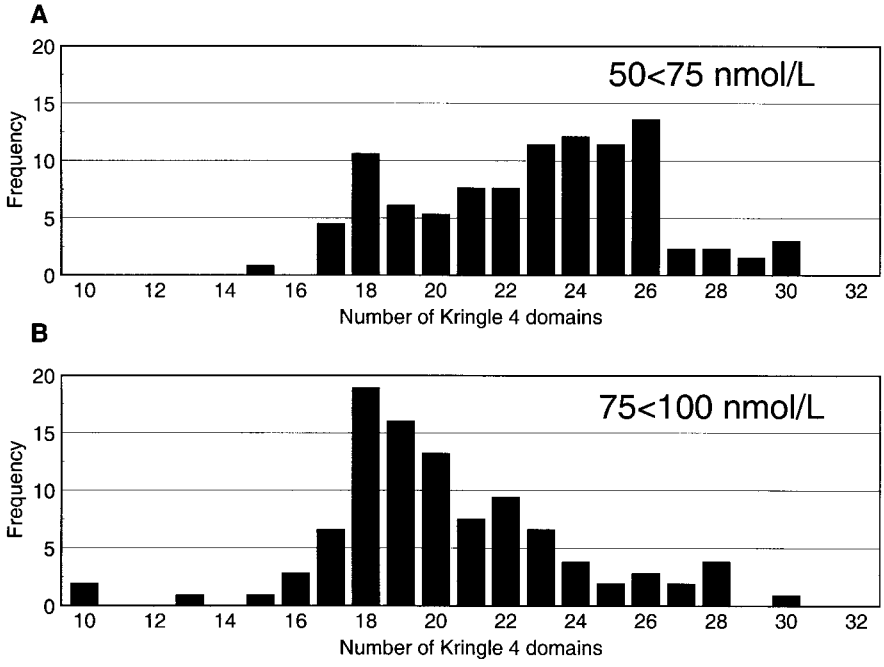

Fig. 5. Frequency distribution of the predominantly expressed apo(a) isoforms, designated by the number of K4 domains, in white Americans *(6)* selected to have Lp(a) values between 50 and 75 nmol/L (*A*) and between 75 and 100 nmol/L (*B*).

the assessment of an individual's risk status for CAD. To this end, among the methods evaluated in this study, we selected eight systems that had a very similar degree of dependence on the apo(a) size heterogeneity with an overall among-method CV for the 30 samples of 13%. Using the regression line for the percent bias of the mean Lp(a) values obtained by these eight systems and the apo(a) isoforms expressed in terms of the relative number of K4 domains, we calculated the expected percent bias of Lp(a) values as a function of apo(a) size, as illustrated in Table 6. We therefore evaluated to what extent the analytical inaccuracy of methods sensitive to apo(a) size would impact the correct classification of subjects as having or not having increased risk for CAD based on their Lp(a) values. To calculate the number of false positives, we selected, from a large cohort of white individuals, those whose Lp(a) values were below the cutoff value of 75 nmol/L which closely corresponds to the 80th percentile of a white population *(6)*. A group of 132 individuals had Lp(a) values that were between 50 and 75 nmol/L. On the basis of the frequency distribution of the apo(a) isoforms in these 132 samples and the regression line expressing the bias, we calculated that 63% of the values originally between 50 and 75 nmol/L would equal or exceed the 75 nmol/L cutoff value (false positive). Therefore, 83 individuals would be erroneously classified as being at increased risk for CAD. To evaluate the number of false negatives, from the same cohort *(6)* we selected 106 white individuals whose Lp(a) values were between 75 and 100 nmol. In this group, 8.5% of the values were estimated to be <75 nmol/L (false negative). Therefore, nine individuals originally at increased risk for CAD would be misclassified if their Lp(a) values were determined by methods that are affected by apo(a) size heterogeneity.

It needs to be emphasized that the number of misclassified individuals can dramatically increase or decrease depending on the specific method used to measure Lp(a) and depending on the frequency distribution of apo(a) isoforms in the studied population. However, it is clear from these data that in studies aimed at evaluating the clinical significance of Lp(a) and the power of Lp(a) values as predictors of risk for CAD, Lp(a) concentration should be determined only by methods that are validated as not affected by apo(a) size heterogeneity. We found in our study that the number of false positives was negligible in samples with Lp(a) values below 50 nmol/L (data not shown). Therefore, it seems to be safe at this point in time to suggest that commercially available methods sensitive to apo(a) size be used only for screening purposes. On the basis of the skewed distribution of Lp(a) values, >60% of Caucasians and a higher proportion of Asians would be expected to have Lp(a) values <50 nmol/L. Therefore, a large proportion of individuals would be correctly classified in terms of their risk status by the currently available methods. Clearly, all of the samples exceeding 50 nmol/L should be remeasured by a reference laboratory using a validated method. It should be emphasized here that the above statements are not valid for the black population because the Lp(a) concentrations in the black population are both substantially higher and differently distributed than in Caucasians *(6)*. Manufacturers of Lp(a) tests should include as one of their primary goals the development of new analytical methods for the measurement of Lp(a) that are demonstrated

to be unaffected by apo(a) size heterogeneity and therefore able to accurately measure Lp(a).

In conclusion, from the results of our current study, it appears that the IFCC PRM has the characteristics of a suitable reference material and that its availability will play an important role in the standardization process by providing accuracy-based calibration of those assays that are validated to be unaffected by apo(a) size heterogeneity. On the basis of the results of this study, the IFCC will seek recognition of PRM as an international reference material for Lp(a). However, it is obvious that no reference material, either primary or secondary, would be able to eliminate the substantial difference in Lp(a) values obtained by different analytical methods that are affected by apo(a) size heterogeneity. A major educational effort is required to make clinical chemists, clinicians, and epidemiologists aware of the significant problems related to the immunochemical measurement of this complex lipoprotein particle.

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