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Original Research

Interlaboratory comparison of serum lipoprotein(a) analytical results across clinical assays—Steps toward standardization

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Lipoprotein(a);
Apolipoproteins;
Assay comparison;
Interlaboratory comparison;
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Lp(a);
Apo(a)

BACKGROUND: Lipoprotein(a) [Lp(a)] is an independent risk factor for cardiovascular diseases (CVD). Recent clinical guidelines recommend measuring Lp(a); however, the lack of Lp(a) assay standardization presents challenges to using common clinical decision points. Assay standardization may minimize interassay variability. This improves consistency in CVD risk assessment and evaluations of Lp(a) therapeutic efficacy. Genetically determined size variations in the defining apolipoprotein(a) [apo(a)] protein contribute to interindividual Lp(a) heterogeneity. Individuals who express 2 apo(a) isoforms have 2 sizes of apo(a) in circulation, further contributing to Lp(a) heterogeneity.

OBJECTIVE: The Centers for Disease Control and Prevention's Clinical Standardization Programs (CDC CSP) recently launched an Lp(a) standardization program based on the International Federation of Clinical Chemistry endorsed liquid-chromatography mass spectrometry-based reference measurement procedure (RMP). As part of this program, CDC CSP conducted an interlaboratory comparison study to evaluate current Lp(a) interassay variability and to investigate potential factors contributing to measurement variability.

METHODS: Eight clinical laboratories measured Lp(a) in 40 individual donor serum samples and 3 serum pools. Serum samples were immunophenotyped by Western blot analysis to determine Lp(a) isoform sizes. Sample concentrations were measured in duplicate over 2 independent runs.

RESULTS: Assay-specific Lp(a) measurements demonstrated good linear correlation with the RMP. Lp(a) interassay measurement variations ranged from 3.3% to 69.1% across individual samples; however, Lp(a) interassay coefficients of variation did not increase in a concentration-dependent manner and were not correlated with Lp(a) isoform sizes.

CONCLUSION: This study provides new insights into Lp(a) interassay variability and assay performance in clinical laboratories that will guide future standardization efforts.

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Introduction

Despite advances in cardiovascular disease (CVD) diagnosis and treatment, the total CVD prevalence for individuals ≥20 years of age in the United States is 48.6%.[1] Elevated low-density lipoprotein cholesterol (LDL-C) levels contribute to the pathogenesis of atherosclerotic cardiovascular disease (ASCVD) and cholesterol-lowering therapies predominantly target LDL-C, including statins and proprotein convertase subtilisin/kexin-type 9 (PCSK-9) inhibitors.[2,3] However, reports from several clinical trials suggest that a portion of the "residual risk" for adverse CVD outcomes in the setting of controlled LDL-C is associated with elevated lipoprotein(a) [Lp(a)].[4,5] Furthermore, studies have established that elevated Lp(a) is an independent, causal risk factor for the development of ASCVDs, including myocardial infarction.[6,7] Clinical guidelines currently support the measurement of Lp(a) in individuals with a personal or familial history of ASCVD[8] or have advocated for the measurement of Lp(a) at least once for all adults.[9-12] The 2018 American Heart Association/American College of Cardiology Guidelines on the Management of Blood Cholesterol currently state that an Lp(a) ≥50 mg/dL constitutes a risk-enhancing factor, especially at higher Lp(a) concentrations,[13] whereas the 2019 European Society of Cardiology/European Atherosclerosis Society Guidelines for the Management of Dyslipidemias state that Lp(a) concentrations ≥125 nmol/L constitute an increased CVD risk.[11] While guideline recommendations differ with respect to clinical decision cut-off concentrations, units and frequency of measurement, and patient populations for which Lp(a) measurements are recommended, the overarching consensus is that Lp(a) should be measured.[8-12,14] Despite guidelines and scientific statements recommending the use of Lp(a) for CVD risk stratification, Lp(a) is not routinely measured in clinical practice.[4,10,14,15] This is attributed, in part, to a lack of clinical assay standardization, which currently makes comparisons of patient measurements to clinical decision points and across clinical assays and studies challenging.[16,17]

Lp(a) is comprised of an apolipoprotein B (apoB)-containing LDL-like particle that is covalently linked to the apolipoprotein(a) [apo(a)] protein, which contains a plasminogen-like domain, 1 kringle 5 (KV) domain, and 10 kringle 4 (KIV) domains known as KIV_1 to KIV_{10}. The Lp(a) protein is synthesized in the liver and is highly heterogeneous due to apo(a) size polymorphisms that are genetically determined by the number of KIV_2 repeat sequences present, which can range in number from 1 to 40 copies. Depending on the number of KIV_2 repeats, apo(a) molecular weights can range between ~250 and 800 kDa, making the interpretation of Lp(a) concentrations difficult when results are reported in mass units (ie, mg/dL) rather than molar units (nmol/L).[5] Lp(a) measurement and data interpretation are further complicated by the presence of 2 different isoforms in circulation in most individuals (heterozygotes), which is typically not captured with current analytical assays. Also, differences in Lp(a) concentrations across ethnic groups have been described and their relationship to CVD risk has not been fully elucidated.[6,18,19]

Lp(a) measurements performed on the same patient samples by different clinical assays were previously reported to exhibit high variability.[17,20] The consequences of Lp(a) interassay measurement variability include an inability to establish appropriate reference intervals and to universally apply clinical cut-off values. Additionally, a lack of assay agreement impedes Lp(a) patient sample measurement comparisons across clinical assays, interferes with measurement comparisons between clinical studies, and hinders assessments of pharmacologic efficacy, which is important given that several clinical trials are underway for Lp(a) lowering therapeutics. This interassay variability was previously attributed, in part, to assay antibody cross reactivity with the apo(a) KIV_2 domain.[17,21,22] However, multiple factors likely contribute to current Lp(a) interassay measurement variability and must be addressed as Lp(a) assays are standardized. These factors include, but are not limited to, differences in how manufacturers perform assay calibration, how reference materials value assigned in molar units (nmol/L) is used for the calibration of assays reporting in mass units (mg/dL), and limited analytical measurement ranges, which may require the dilution of samples typically observed in the clinical setting.

Lp(a) measurement variability persists, despite some Lp(a) assays being traceable to a common World Health Organization (WHO)/International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference material, known as standard reference material (SRM)−2B,[23,24] with values assigned in nmol/L by an enzyme-linked immunosorbent assay (ELISA)-based, KIV_2 independent reference method.[25] Importantly, this ELISA-based reference method is no longer available and the SRM-2B reference material was depleted, thus establishing the need for a new, more sustainable reference method and materials that can be used in standardization programs. The standardization of Lp(a) assays will help improve measurement agreement across assays and will ensure that measurement results are comparable to clinical decision points, across methods, location, and over time. To date, 2 candidate mass spectrometry-based reference measurement procedures (RMP) have been proposed to replace the original ELISA-based RMP, including one endorsed by the International Federation of Clinical Chemistry and Laboratory Medicine's Working Group for Apolipoproteins by Mass Spectrometry (IFCC WG APO-MS).[17,26] The IFCC WG APO-MS is also working to establish new serum-based reference materials for Lp(a) clinical assay calibration with traceability to the IFCC WG APO-MS RMP.[27,28]

The principal goal of Centers for Disease Control and Prevention (CDC) Clinical Standardization Programs (CSP) is to improve the accuracy and reliability of chronic biomarker measurements used in the patient care, public health, and clinical research settings. The CDC CSP is collaborating with the IFCC WG APO-MS to establish a formal Lp(a) standardization program as part of the IFCC's proposed reference measurement system (RMS). In preparation for this, the CSP

conducted a Lp(a) Interlaboratory Comparison Study with end-user clinical laboratories as participants that focused on Lp(a) clinical assays, to obtain information about current interassay variability, and to guide specific approaches for the implementation of Lp(a) standardization programs. Specifically, this study aimed to obtain information about the analytical performance of clinical assays routinely used in the patient care setting for Lp(a) measurements, to determine the current extent of and potential sources contributing to interassay variability, to assess how Lp(a) clinical assay measurements correlate with mass spectrometry values from the IFCC RMP, and to elucidate if Lp(a) interassay variability is attributable to differences in Lp(a) isoform sizes.

Materials and methods

Materials and reagents

The following materials and reagents were used for Western blot analysis: Roche fraction V bovine serum albumin (BSA; Millipore Sigma), phosphate buffered saline (PBS; Bio-Rad Laboratories, Inc), tris buffered saline (TBS; Bio-Rad), Tween 20 (Bio-Rad), agarose (ThermoFisher Scientific, Inc), tris borate EDTA buffer (TBE; Bio-Rad), sodium dodecyl sulfate (SDS; Invitrogen), Laemmli sample buffer (Bio-Rad), dithiothreitol (DTT; ThermoFisher Scientific), goat antimouse antibody (ThermoFisher Scientific), 0.22 uM nitrocellulose membrane (Bio-Rad), and enhanced chemiluminescence reagent (ECL; Pierce Biotechnology).

Individual donor serum samples and quality control serum pools

Commercially prepared, deidentified single donor sera, prepared according to the most recent Clinical and Laboratory Standards Institute C37-A guidance,[29] were procured from Solomon Park Research Laboratories and served as clinical samples. The serum collection protocol was approved by the local institutional review board at Solomon Park and informed consent was obtained for all serum donors by Solomon Park. Research involving human subjects complied with all relevant national regulations, institutional policies, and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013). Samples used in this study were prescreened for Lp(a) concentrations using an immunoturbidimetric assay (Tina-quant Lipoprotein(a) Gen.2 on a Cobas c311 analyzer, Roche Diagnostics). Lp(a) phenotype was determined by Western blot analysis. All individual donor serum units underwent 1 freeze-thaw cycle to create aliquots or to generate quality control (QC) serum pools.

The CDC CSP generated QC serum pools at low, medium, and high Lp(a) concentrations using serum units preselected based on apo(a) isoform content, as determined by Western blot analysis (Fig 1), and Lp(a) screening concentration. Serum units with specific KIV# were selected and used to generate QC serum pools. For each pool, 9 to 10 individual donor serum units were combined in a sterile 2 L glass flask and mixed at a constant, low speed for 5 hours at 4 °C. The homogeneous QC pools remained at 4 °C with low-speed mixing as they were aseptically aliquoted into 2 mL sterile, polypropylene cryovials (Greiner) using a Sci-Print VX2 machine (Scinomix). Mass spectrometry based Lp(a) values in nmol/L were assigned to each serum sample and each QC by the IFCC RMP described below.

Apo(a) phenotyping by Western blot analysis

Agarose gel electrophoresis and Western blot analysis was used to phenotype >200 serum samples to determine apo(a) isoform sizes, as described previously,[30-33] with the modifications described herein. Briefly, serum samples were diluted 1:5 in 1X PBS for samples with <10 mg/dL Lp(a) and were diluted 1:10 for samples with ≥10 mg/dL Lp(a). Laemmli sample buffer (4X; Bio-Rad) containing DTT (ThermoFisher Scientific) as a reducing agent was added to each diluted sample (final concentration: 1X Laemmli buffer, 0.1 M DTT). Samples were denatured at 95 °C for 5 minutes and loaded at a final volume of 18 µL containing approximately 300 ng of Lp(a) or the maximum Lp(a) amount in 18 µL for low concentration samples. An apo(a) isoform size standard, provided by Dr. Florian Kronenberg, with known apo(a) isoforms (13, 19, 23, 27, and 35 KIV repeats, as previously described[34]) was loaded after every 5 to 6 samples. Samples were run on a 1.2% agarose (ThermoFisher Scientific), 0.1% SDS (ThermoFisher Scientific) gel for 6 hours (150 V, constant voltage) using a horizontal gel electrophoresis system (Hoefer Inc). Proteins were transferred for 1.5 hours (100 V, constant voltage) to a 0.22 uM nitrocellulose membrane (Bio-Rad) by wet tank transfer. The membrane was incubated overnight at 4 °C in 5% BSA, 0.2% Tween, 1X TBS containing 210 ng/mL 1A2 mouse monoclonal primary antibody (provided by Dr Florian Kronenberg) and 200 ng/mL goat antimouse secondary antibody (ThermoFisher Scientific). The membrane was washed 6 × 10 minutes in 0.2% Tween, 1X TBS before incubation with ECL reagent (Pierce). Chemiluminescence images were captured using an iBright 1500 (ThermoFisher Scientific). Comparison of this in-house Western blot method with another established method (Dr Kronenberg Laboratory) with 30 samples and independent KIV determination showed an average difference of −1.2 KIVs, confirming excellent agreement between both methods. The CSP immunophenotyped all individual donor serum samples and assigned KIV numbers based on the distance traveled relative to the apo(a) isoform standard prior to selection and use in this study.[34] Individual samples with the same or similar phenotype profiles and similar or different Lp(a) concentrations were specifically selected for study inclusion to assess the potential impact of kringle size on Lp(a) measurement variability. The apo(a) phenotyping results for the 40 selected samples are shown in Figure 1.

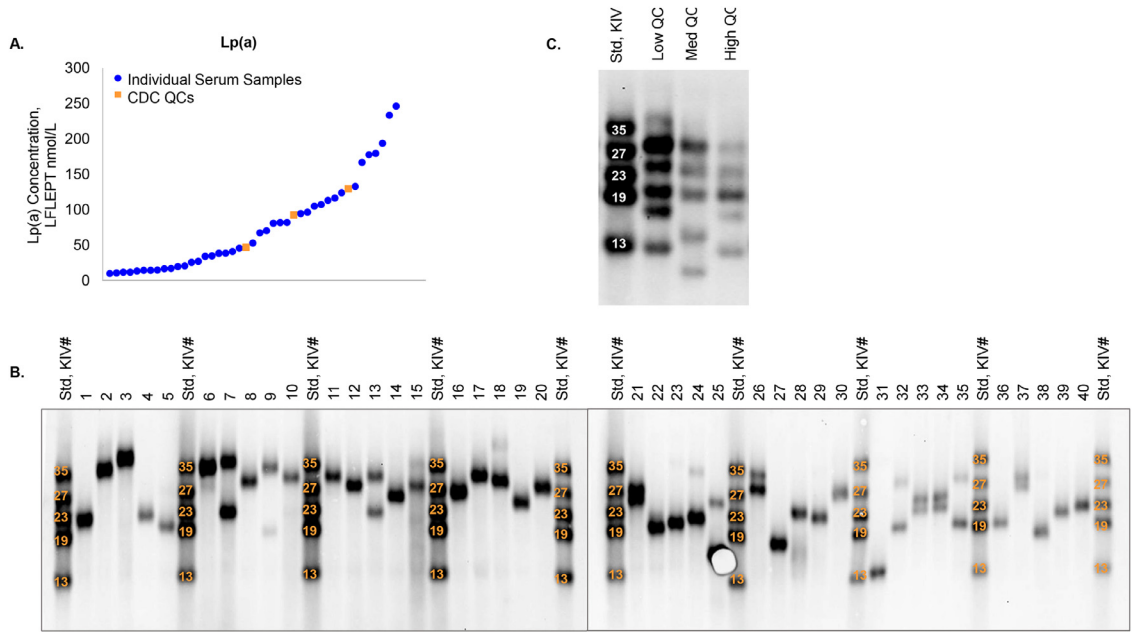

Figure 1. Lp(a) serum sample and QC serum pool characteristics. A, Individual donor serum samples (blue circles) and CDC quality control (QC; orange squares) serum pool concentration distributions in order of lowest to highest mass spectrometry lipoprotein(a) [Lp(a)] concentration. B, All individual samples were immunophenotyped by Western blot analysis and selected based on Lp(a) isoform composition prior to use in this study. Individual samples are loaded in order of arbitrary number assignment. C, Western blot analysis was used to immunophenotype serum samples to identify individual units with specific kringle numbers (KIV#). Serum units with specific KIV# were selected and used to generate QC serum pools at low, medium, and high concentrations that were included in the study. The 3 QC serum pools each contain 6 Lp(a) isoform sizes. The low and high QC pools have the same kringle size profiles.

IFCC-endorsed RMP

The IFCC RMP is a quantitative, bottom-up liquid chromatography mass spectrometry (LC-MS) method and was described previously.[35] Briefly, serum proteins undergo proteolytic digestion using Trypsin and LysC, followed by sample clean-up using solid phase extraction. Three apo(a) peptides from nonrepeat regions (LFLEPTQADIALLK, GISSTTVTGR, and TPENYPNAGLTR) were quantified using isotope dilution (ID) LC-MS to quantify apo(a) protein concentrations in nmol/L.[35] Three transitions were selected for each peptide based on retention times, precursor ion m/*z*, and 3 fragment ion m/*z*, as well as collision energies. One transition serves as the quantifier, while the other 2 serve as qualifying transitions. A single-point calibrator was used to establish apo(a) molar concentrations using a transfer calibrator with a value assignment traceable to WHO/IFCC SRM-2B.[36] The IFCC LC-MS RMP, established in the Leiden Apolipoprotein Reference Laboratory, was used to assign Lp(a) concentration values in nmol/L to 40 serum samples and 3 QC samples.

Lp(a) measurement procedures

Eight end-user laboratory participants measured Lp(a) in samples using measurement procedures (MP) commonly utilized by clinical laboratories (Table 1). Seven laboratories provided results in mg/dL and 1 laboratory provided measurements in nmol/L. The Roche, Randox, and Sentinel Diagnositics Lp(a) assay reagents and calibrators were used by more than 1 laboratory. The use of the same assay reagents and calibrators in unique combination with a different instrument platform was defined as a separate MP. The measurement ranges of the 6 MP are indicated in Table 1. Randox and Sentinel Diagnostics have stated traceability to WHO/IFCC SRM-2B and all MP included in the study use an 5-point calibration system. Samples with Lp(a) concentrations outside the MP measurement range underwent automated dilution into a MP-specific buffer and were remeasured.

Experimental design

Two sets of 40 serum samples and 3 QC samples were provided to each study participant by the CDC CSP. Samples were shipped on dry ice and stored at −80 °C upon arrival. Participants were asked to verify that the analytical system used was appropriately calibrated and performing according to manufacturer's specifications prior to performing experiments. Lp(a) concentrations were measured in duplicate for 2 independent experiments by each MP.

Data and statistical analyses

Data were submitted using an Excel template provided by the CDC CSP. Participants submitted data in the units typi-

Table 1. Measurement procedures surveyed and characteristics of each measurement procedure.

Assay manufacturer [Lab ID]	Instrument platform	Assay name	Calibrator manufacturer	No. of calibrators	Assay Type	Reportable range	LOQ	Stated traceability
Roche [Lp1]	Cobas c501	Tina-quant Lipoprotein(a) Gen.2	Roche	5	IA	6.0 – 240 mg/dL	4.0 mg/L	In-house RM
Roche [Lp2]	Cobas c502	Tina-quant Lipoprotein(a) Gen.2	Roche	5	IA	6.0 – 240 mg/dL	4.0 mg/L	In-house RM
Randox [Lp3]	Beckman AU5800	Lipoprotein(a)	Randox	5	IA	5.2 – 418 nmol/L	5.2 nmol/L	SRM-2B
Randox [Lp4]	Cobas c501	Lipoprotein(a)	Randox	5	IA	3.0 – 180 mg/dL	3.3 mg/dL	SRM-2B
MedTest DX [Lp5]	Alfa Wassermann ACE Axcel	Lipoprotein(a)	MedTest DX	5	IA	2.0 – 180 mg/dL	2 mg/dL	In-house RM
Roche [Lp6]	Cobas c501	Tina-quant Lipoprotein(a) Gen.2	Roche	5	IA	6.0 – 240 mg/dL	4.0 mg/L	In-house RM
Sentinel [Lp8]	Beckman AU5800	Lipoprotein(a) Ultra	Sentinel diagnostics	5	IA	3 – 130 mg/dL	3 mg/dL	SRM-2B
Sentinel [Lp9]	Beckman AU5800	Lipoprotein(a) Ultra	Sentinel diagnostics	5	IA	3 – 130 mg/dL	3 mg/dL	SRM-2B

Abbreviations: IA, immunoassay; LOQ, limit of quantification; RM, reference material; SRM, standard reference material.

cal of their MP. Data were compiled and statistical analyses were performed using Microsoft Excel 2016 and the statistical add-in software Analyse-it version 4.97.4 (Leeds) and R version 4.0.2. The mean, SD, and coefficient of variation (CV) were calculated for each serum sample and QC for each MP. MP-specific sample means were used for subsequent analyses. Apo(a) isoform sizes vary, thus making it difficult to correctly convert Lp(a) measurements from mg/dL into nmol/L; thus, no conversions were used for analyses. For MP comparisons, all sample measurements were compared to the following targets: (i) all-lab mean target values (mg/dL) and (ii) mass-spectrometry assigned target values (nmol/L).

Results

Sample characteristics

The study was designed to evaluate the variability of serum Lp(a) measurements across MP. Nine end-user laboratories running immunoassay-based MP utilized in the US were included in the study. Assay characteristics are outlined in Table 1. The CDC CSP provided 40 single donor serum samples to participants covering Lp(a) concentrations from 10.0 to 246.4 nmol/L (as determined by the IFCC RMP) and Lp(a) isoform sizes ranging from 14 to 40+ total kringle IV domains (Fig 1A,B).[35] Low, medium, and high Lp(a) QC serum pools had Lp(a) concentrations of 46.6, 92.4, and 129.5 nmol/L, respectively. Each QC contains 6 apo(a) isoform sizes, where the low and high QCs have the same apo(a) isoform profiles (low and high QC: 12, 16, 19, 24, 27, 36 KIV domains; medium QC: 9, 14, 19, 24, 27, 36 KIV domains; Fig 1C). Study participants measured all 43 samples in duplicate in 2 independent runs and submitted data to the CDC CSP for analysis. Serum samples with the same kringle sizes and different concentrations, as well as serum samples with the same dominant isoform size and varied secondary isoform sizes, were specifically selected to assess potential effects of kringle size on measurement results.

Initial evaluation of MP-specific measurement results

Precision was assessed for each MP based on replicate concentration measurements for each sample. Median CVs and sample CV ranges for each MP are provided in Supplementary Table S1. Overall, most MPs exhibited good precision with median across sample CVs <4%; however, Lp3, Lp4, and Lp9 exhibited higher median imprecisions, with CVs of 12.1%, 4.8%, and 7.6%, respectively (Supplementary Table S1). While the higher sample CVs for Lp3 and Lp9 were consistent across the full concentration range, higher CVs at lower Lp(a) concentrations (<15 mg/dL) were observed for Lp4.

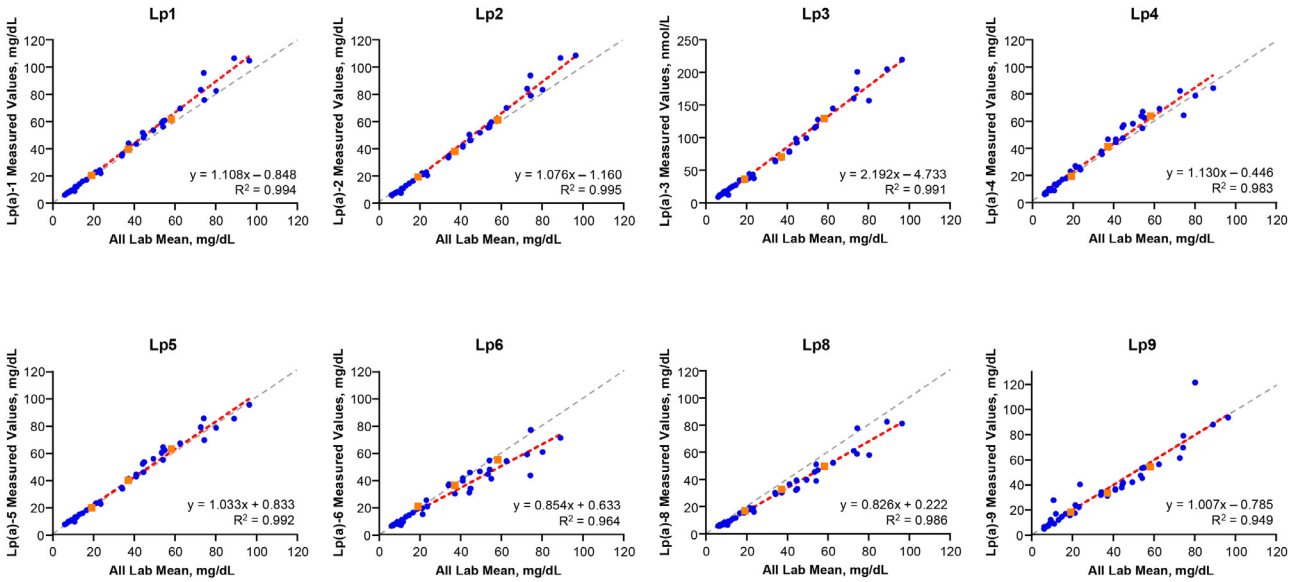

Figure 2. Lp(a) serum samples and QC serum pool concentration distributions across measurement procedures are well correlated with the all-lab mean. Lipoprotein(a) [Lp(a)] was measured in mg/dL (or nmol/L for Lp3) for all individual donor serum samples (blue circles) and for the 3 CDC quality control (QC; orange squares) serum pools using the indicated measurement procedures (MP). MP-specific sample concentration distributions are plotted against the all-lab mean (mg/dL). The dotted grey line is the line of agreement; the dotted red line is the clinical sample regression line. Regression analysis slopes, intercepts, and correlation coefficients are provided in Table 2.

Table 2. Weighted Deming regression analysis comparing MP individual sample results against the all-lab mean concentration.

Assay	Lab ID	Intercept	95% CI, intercept	Slope	95% CI, slope	Correlation coefficient
Roche, Tina-quant Lipoprotein(a) Gen.2 on Cobas c501	Lp1	-0.848	-1.271 to -0.425	1.108	1.076–1.141	0.994
Roche, Tina-quant Lipoprotein(a) Gen.2 on Cobas c502	Lp2	-1.160	-1.842 to -0.477	1.076	1.037–1.114	0.995
Randox, Lipoprotein(a) on Beckman AU5800	Lp3	-4.733	-6.582 to -2.884	2.192	2.091–2.293	0.991
Randox, Lipoprotein(a) on Cobas c501	Lp4	-0.446	-1.245 to 0.353	1.130	1.067–1.194	0.983
MedTest DX, Lipoprotein(a) on Alfa Wassermann ACE Axcel	Lp5	0.833	0.318 to 1.347	1.033	0.995–1.071	0.992
Roche, Tina-quant Lipoprotein(a) Gen.2 on Cobas c501	Lp6	0.633	-0.132 to 1.397	0.854	0.785–0.923	0.964
Sentinel, Lipoprotein(a) Ultra on Beckman AU5800	Lp8	0.222	-0.366 to 0.809	0.826	0.785–0.867	0.986
Sentinel, Lipoprotein(a) Ultra on Beckman AU5800	Lp9	-0.785	-1.834 to 0.265	1.007	0.906–1.107	0.949

Abbreviation: MP, measurement procedure.

Correlation between measurement procedure-specific Lp(a) measurements and the all lab mean concentration

All participants reported Lp(a) results in mg/dL, except participant Lp3, which reported results in nmol/L (**Table 1**). For MPs reporting in mg/dL, the mean of replicate measurements for each MP was compared to the all lab mean concentration (Fig 2). Pairwise comparisons were also performed between each of the MPs (Supplementary Fig S1). As shown in Figure 2, Lp(a) measurements for all individual serum samples and QC pools for each MP were well correlated with the all lab mean, with correlation coefficients ranging from 0.949 to 0.995 (Table 2). Using a weighted Deming regression analysis approach for all MPs reporting in mg/dL, calculated slopes ranged from 0.8260 to 1.130. The MP reporting in nmol/L had a slope of 2.192. Intercepts for MP reporting in mg/dL ranged from −1.160 to 0.8328 and the MP reporting in nmol/L had an intercept of −4.733. While Lp1 and Lp2 showed very similar correlations and patterns, Lp6 was notably different. All 3 laboratories used instruments and assay reagents from the same manufacturer. Similarly, patterns were notably different between Lp8 and Lp9, which used the same assay reagents and the same clinical analyzer platform

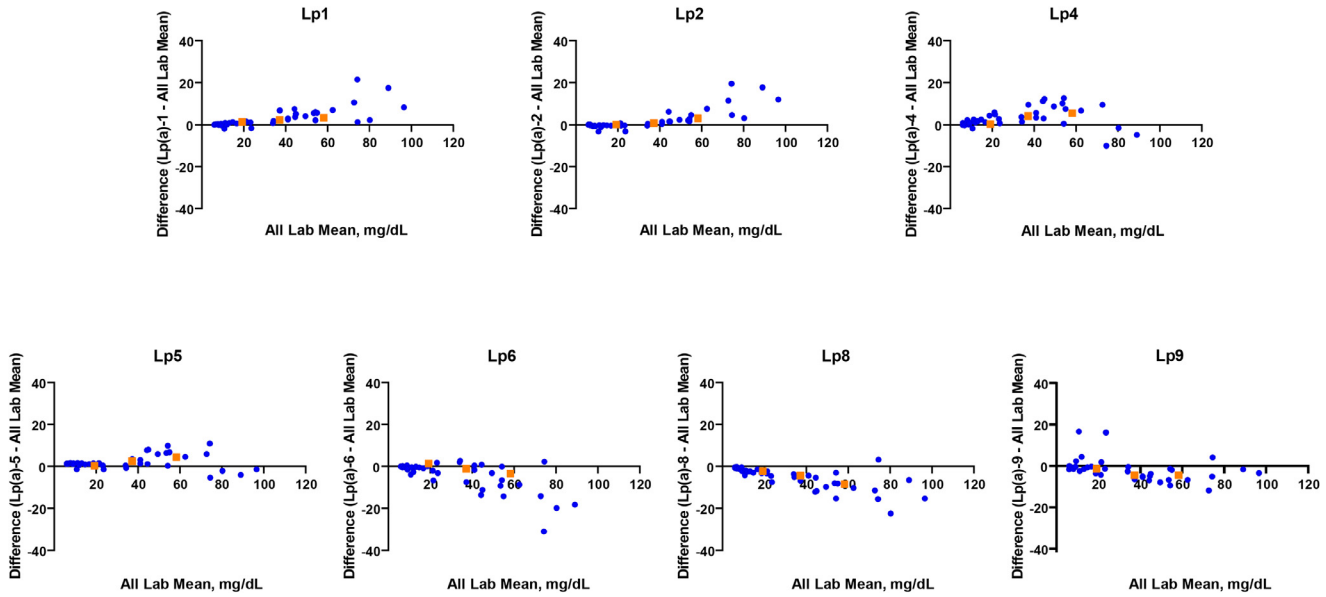

Figure 3. Difference in bias plots for individual serum samples and QC serum pool measurements across measurement procedures exhibit increased bias at higher Lp(a) concentrations when compared to the all-lab mean. Bias plots were generated by plotting the calculated difference between the measurement procedure (MP)-specific mean lipoprotein(a) [Lp(a)] concentrations (mg/dL) and the all-lab mean concentrations (mg/dL) on the y-axis. The Lp3 MP was excluded due to measurements reported in nmol/L. The all-lab mean concentrations (mg/dL) were plotted on the x-axis. Individual serum samples (blue circles) and the 3 CDC quality controls (QC; orange squares) are shown.

for analysis, as well as Lp3 and Lp4, which used the same assay reagents and different instruments. All MPs assessed exhibited a higher degree of scatter at higher Lp(a) concentrations.

Assessment of bias patterns for quality control materials and clinical samples

Bias plots were generated by plotting the calculated difference between the all lab mean concentrations (mg/dL) and the MP-specific Lp(a) concentrations (mg/dL) against the all-lab mean concentrations (mg/dL). The bias plots in Figure 3 illustrate that some MP-specific Lp(a) measurements exhibit a proportional bias that increases with increasing Lp(a) concentration. The overall mean bias for clinical samples across the 0 to 120 mg/dL concentration range was quantitatively assessed for each MP. To establish if or how the mean bias changes with respect to the Lp(a) clinical decision limit of 50 mg/dL, the mean biases were also calculated for 2 concentration ranges centered around the clinical decision point: 0 to 50 mg/dL and 51 to 120 mg/dL (Supplementary Table 3). Lp8 and Lp9 showed no distinct differences in concentration range biases (± 2%) compared to the overall mean bias for each MP. In contrast, Lp1, Lp2, and Lp6 all exhibited increased mean biases at the 51 to 120 mg/dL concentration range compared to the overall mean bias and 0 to 50 mg/dL concentration range. Lp4 and Lp5 showed slightly higher mean biases at the 0 to 50 mg/dL range and slightly lower mean biases at the 51 to 120 mg/dL range when compared to the overall mean bias.

Correlation between measurement procedure-specific Lp(a) measurements and mass spectrometry values

MP-specific Lp(a) concentration distributions in mg/dL (or nmol/L for Lp3) for all individual serum samples and 3 QC pools were compared to LC-MS RMP values in nmol/L, which were generated using the LFLEPTQADIALLK LC-MS peptide for quantitative purposes (Fig 4). MP-specific Lp(a) measurements were well-correlated with LC-MS values across the MP assessed, with correlation coefficients ranging from 0.898 to 0.983 (Table 3). Slopes from weighted Deming regression analyses for MP reporting in mg/dL ranged from 0.3800 to 0.5326 and the MP reporting in nmol/L had a slope of 1.010. Intercepts for MP reporting in mg/dL ranged from 0.8280 to 2.728 and the MP reporting in nmol/L had an intercept of −0.9313 (Table 2). Overall, the concentration distribution patterns for each MP compared to the RMP exhibited patterns similar to those in Figure 1. CDC QC pool Lp(a) measurements were also well correlated with LC-MS values. All MPs exhibited increased scatter at higher Lp(a) concentrations, similar to the results in Figure 3.

Clinical sample and QC interassay variability with increasing Lp(a) concentrations

To assess the impacts of increasing Lp(a) concentrations on interassay variability, individual serum samples and QC pools were plotted from lowest to highest Lp(a) all lab mean concentration moving from left to right (Fig 5). Sample means from each of the 7 MPs reporting Lp(a) in mg/dL were included for each of the 43 samples assessed. Each dot rep-

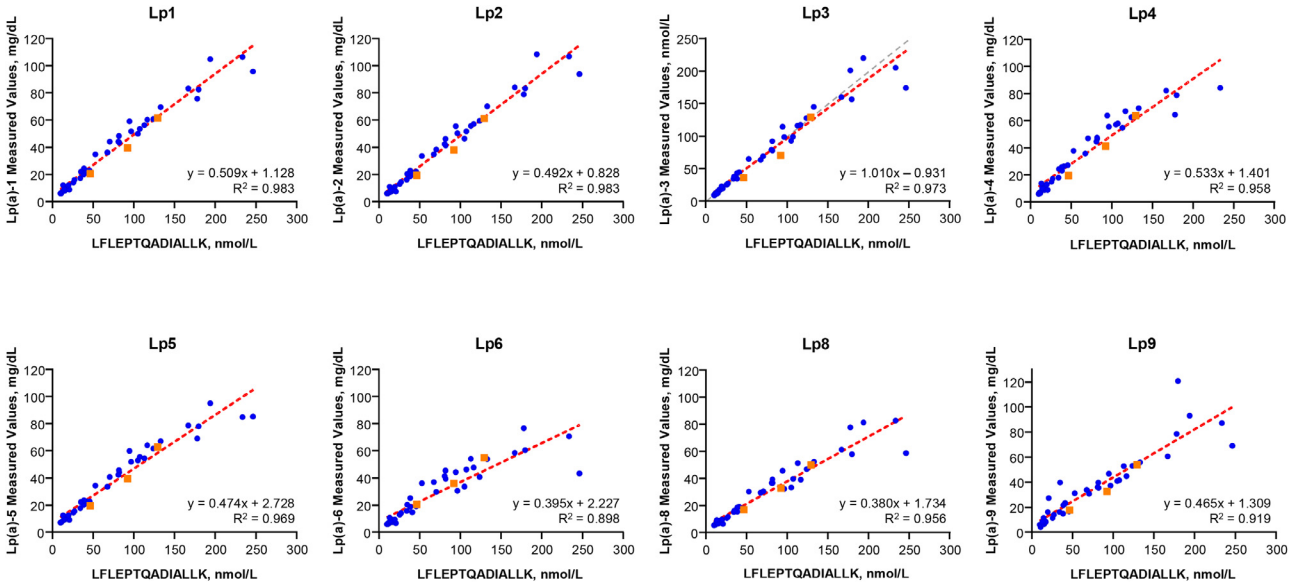

Figure 4. Lp(a) serum samples and QC serum pool measurement distributions for different measurement procedures are well correlated with mass spectrometry-based values. Lipoprotein(a) [Lp(a)] was measured in mg/dL (or nmol/L for Lp3) for all individual donor serum samples (blue circles) and for the 3 CDC quality control (QC; orange squares) serum pools using the indicated measurement procedures (MP). The values from the mass spectrometry method (nmol/L) using the apo(a) LFLEPTQADIALLK peptide for quantification were used as the target. MP-specific sample concentration distributions are plotted against the mass spectrometry target values. The dotted grey line is the line of agreement; the dotted red line is the clinical sample regression line. Regression analysis slopes, intercepts, and correlation coefficients are provided in Table 3.

Table 3. Weighted Deming regression analysis comparing MP individual sample results against mass spectrometry orientation value concentrations from the cRMP.

Assay	Lab ID	Intercept	95% CI, intercept	Slope	95% CI, slope	Correlation coefficient
Roche,Tina-quant Lipoprotein(a) Gen.2 on Cobas c501	Lp1	1.128	0.260–1.997	0.509	0.480-0.539	0.983
Roche, Tina-quant Lipoprotein(a) Gen.2 on Cobas c502	Lp2	0.828	0.049–1.607	0.492	0.467–0.518	0.983
Randox, Lipoprotein(a) on Beckman AU5800	Lp3	-0.931	-2.100–0.238	1.010	0.948–1.071	0.973
Randox, Lipoprotein(a) on Cobas c501	Lp4	1.401	0.132–2.669	0.533	0.485–0.580	0.958
MedTest DX, Lipoprotein(a) on Alfa Wassermann ACE Axcel	Lp5	2.728	1.830–3.627	0.474	0.440–0.508	0.969
Roche, Tina-quant Lipoprotein(a) Gen.2 on Cobas c501	Lp6	2.227	1.179–3.274	0.395	0.344–0.446	0.898
Sentinel, Lipoprotein(a) Ultra on Beckman AU5800	Lp8	1.734	1.007–2.461	0.380	0.348 -0.412	0.956
Sentinel, Lipoprotein(a) Ultra on Beckman AU5800	Lp9	1.309	-0.234 to 2.853	0.465	0.401 -0.530	0.919

Abbreviations: MP, measurement procedure; RMP, reference measurement procedure.

resents the MP-specific mean Lp(a) concentration for each sample, and each dot color corresponds to a different MP. Individual sample SDs increased in a concentration-dependent manner and ranged from 0.54 to 20.96, with a mean SD of 5.36 for all individual serum samples, a mean SD of 3.26 for individual samples 0 to 50 mg/dL, and a mean SD of 10.89 for individual samples 51 to 120 mg/dL. When the all lab mean Lp(a) concentration and 2 times the all lab SD were plotted, an increase in variation was observed with increasing concentrations (Supplementary Fig S2). The all lab mean Lp(a) concentrations and associated CVs for each individual donor serum sample are presented in Supplementary Table 2. CVs across serum samples ranged from 3.3 to 69.1%, with a mean CV of 15.7% and a median CV of 14.6% and did not increase with increasing concentrations (Supplementary Fig S3). Indeed, the level of interassay agreement

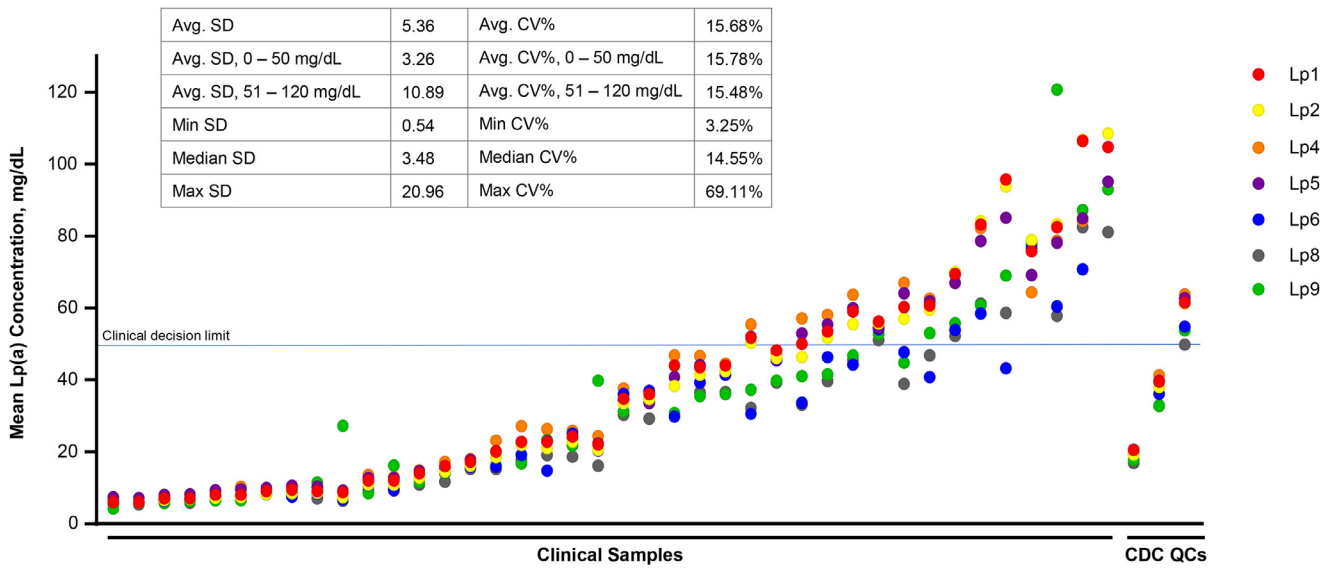

Figure 5. Assay measurements in serum samples exhibit higher measurement variations with increasing Lp(a) concentrations. Individual donor serum samples and CDC quality control (QC) serum pools were plotted from lowest to highest all-lab mean lipoprotein(a) [Lp(a)] concentration. Each dot represents the mean Lp(a) concentration, as determined by duplicate measurements across 2 independent assay runs. Each dot color corresponds to Lp(a) measurements from a different measurement procedure, as indicated in the figure legend. The solid black line for each clinical and QC sample represents the mean.

across MPs for a sample, expressed as %CV (level of disagreement), showed no concentration dependence and was relatively constant across the concentration range with very few exemptions, suggesting variability is not sample dependent. The CDC QC pools at low, medium, and high concentrations exhibited average CVs of 7.1, 9.1, and 9.2%, respectively, which were lower than the average CVs of individual donor samples with comparable concentrations (±4 mg/dL), which were 13.1, 10.4, and 12.8%, respectively.

Increased Lp(a) measurement variability is not Lp(a) isoform size dependent

To determine if or how Lp(a) isoform size contributes to interassay variability, the all-lab MP mean concentration and SD for each sample was plotted relative to each sample's dominant Lp(a) isoform size, as indicated by the number of KIV (KIV#; Fig 6). If samples exhibited equal Lp(a) isoform expression levels, the smaller isoform size was used for assessments. The data in Figure 6A show the means and SDs for multiple individual donor serum samples with the same dominant isoform size, based on KIV number, stacked into columns. As one moves vertically up the 22 KIV column, for example, one sees that SD increases with increasing Lp(a) concentration. Moving horizontally across serum samples at 1 Lp(a) concentration, one observes no appreciable increase in SD with increasing dominant isoform size. Several samples were specifically selected for use in this study because they had the same isoform profile and different concentrations or the same dominant isoform size and varying secondary isoform sizes. Several examples are shown in Figure 6B and illustrate while SDs do increase with Lp(a) concentration, they do not increase with increasing isoform size or with the addition of secondary isoforms. As also indicated in Figure 5 and Supplemental Table S2, QC serum pools created with 6 KIV each (characterized in **Fig 1C**) do not exhibit increased interassay variability as a product of containing >2 isoforms, suggesting isoform size independence. Indeed, the low and high CDC QC serum pools, which have the same isoform composition, have similar CVs of 7.1% and 9.2%, respectively. Altogether, these data suggest that Lp(a) measurement variability does not increase with increasing isoform size.

Discussion

This Lp(a) Interlaboratory Comparison Study was designed and conducted by the CDC CSP to evaluate the accuracy and variability of serum Lp(a) measurements across current clinical assays routinely used in patient care settings, to investigate potential sources of measurement variability, and to inform stakeholders about ongoing Lp(a) standardization efforts. The results of this study demonstrate that interassay variability persists, especially at concentrations used to guide clinical decision-making, despite previous efforts to standardize Lp(a) assays. The previous ELISA-based RMS, to which many assays are currently standardized, is no longer available and reference materials traceable to the previous RMS are now depleted. To fill this critical gap and further improve Lp(a) interassay agreement, the IFCC WG APOMS established a new LC-MS-based RMP and is generating new reference materials value assigned in SI units and anchored to the new RMP for manufacturers to use for calibration purposes. Importantly, assay measurements in mg/dL showed good correlation with LC-MS values in nmol/L, suggesting a change from the previous ELISA-based RMS to

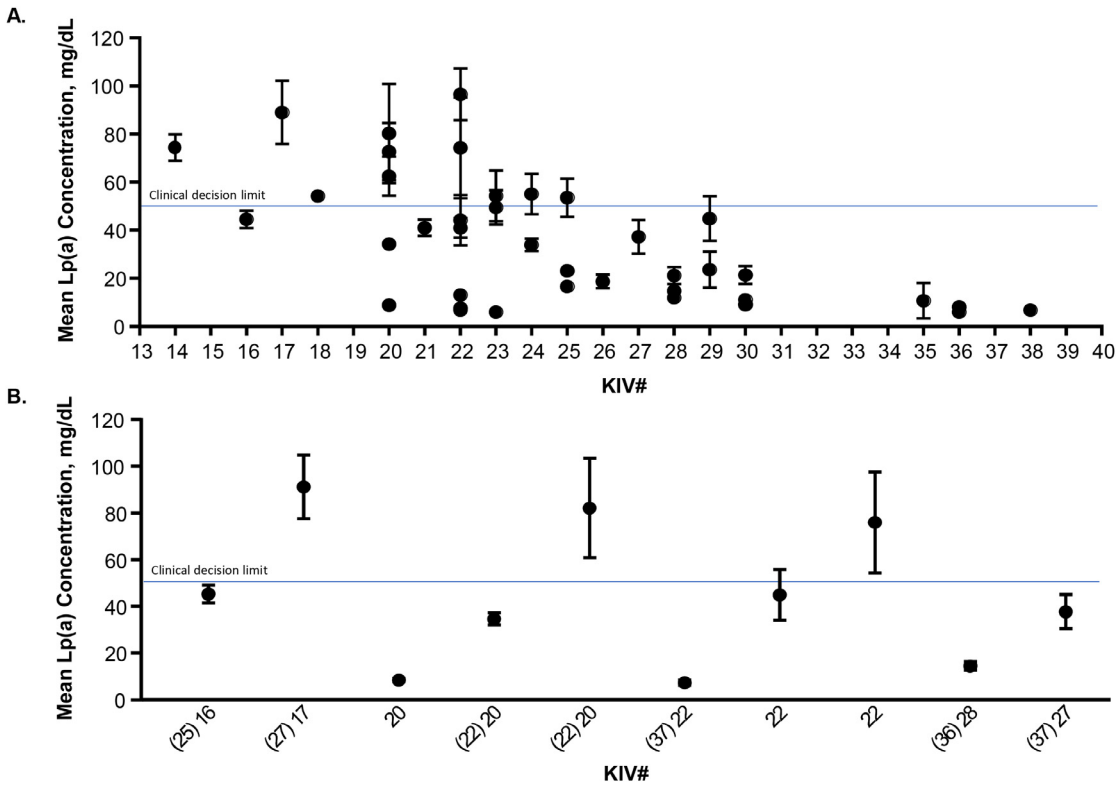

Figure 6. Lp(a) measurement variability increases with Lp(a) concentration and does not increase relative to Lp(a) isoform size, as indicated by kringle number. To determine if or how lipoprotein(a) [Lp(a)] kringle size contributes to interassay variability, we plotted the all-lab measurement procedures (MP) mean concentration and SD for each sample relative to each sample's dominant kringle size. If samples exhibited equal Lp(a) isoform expression levels, we used the smaller isoform kringle number for this assessment.

the new LC-MS-based RMS may have minimal impacts on Lp(a) measurements. Study results also provided important information about factors that do not contribute to interassay variability, such as Lp(a) isoform sizes, as well as factors that may contribute to interassay variability, which will be explored further after assays are calibrated to a new common calibrator. While further improvements in Lp(a) standardization are necessary, the current lack of assay agreement should not preclude the measurement of Lp(a) in patients according to multisociety clinical guidance documents that suggest measuring Lp(a) to identify those with higher CVD risk. Assay standardization takes time; thus, standardization efforts are being conducted in parallel with clinical Lp(a) measurements for risk assessment with the goal to improve Lp(a) measurement agreement over time.

The Lp(a) literature has long attributed interassay Lp(a) measurement variability to the use of polyclonal antibodies and potential antibody cross-reactivity with the KIV_2 sequence in the apo(a) repeat region,[17,37] which was believed to result in Lp(a) concentrations that do not accurately reflect the true Lp(a) concentration. To determine if or how Lp(a) isoform size currently contributes to Lp(a) interassay measurement variability, individual samples were phenotyped and selected based on the total KIV# of the dominant Lp(a) isoform. Samples with the same or similar apo(a) isoform sizes and different Lp(a) concentrations were specifically selected for study inclusion to determine how much variability stems from isoform size. The data from individual samples in this study suggest that Lp(a) measurement variability is not significantly correlated with isoform size, suggesting additional sources of variability should be considered. These same serum units, preselected based on apo(a) isoform content, were used to generate 3 CDC QC serum pools with 6 KIV each. Interestingly, the 3 QCs, 2 with the same isoform sizes, did not exhibit higher interassay variability, again suggesting isoform size independence. The variability observed for the QCs pools did not reflect that observed in clinical samples, where QC variability was lower than individual donor samples with comparable concentrations, suggesting that pooled materials may not provide comprehensive information about measurement variability that occurs in individual donor samples used in patient care and clinical research.

Often, interassay variability is a product of several different factors that must be addressed as assays are standardized. All assays in this study utilized a 5-point calibration system and demonstrated good linearity when compared to the all lab mean concentration (mg/dL). Assays included in this study also exhibited higher sample scatter and biases at higher Lp(a) concentrations, which occurred just below or above the Lp(a) clinical decision point of 50 mg/dL (125 nmol/L). Interestingly, assays included in this study with stated traceability to SRM-2B, a single level RM value assigned in nmol/L by the previous RMS, still exhibit both positive and

negative biases when compared to the all lab mean. Additional factors that may potentially contribute to Lp(a) interassay variability include differences in how manufacturers perform assay calibration and how reference materials, such as SRM-2B, value assigned in molar units (nmol/L) were used for the calibration of assays reporting in mass units (mg/dL). Furthermore, the composition of calibration solutions used by assay manufacturers is unknown and could be a potential contributing factor. One would expect calibration differences to impact sample measurements in a consistent manner and may explain some of the differences in slopes across assays but do not likely explain differences in scatter. The increased scatter may stem from how manufacturers use secondary reference materials value assigned in molar units (nmol/L) to calibrate assays measuring in mass units (mg/dL). This is often done through the application of proprietary mathematical formulas for unit conversions, which could be a potential source of continued interassay variability. Indeed, studies have demonstrated that no clear molar to mass ratio can be universally applied.[37] However, the need for unit conversions would be ameliorated by a transition of all Lp(a) assays to molar unit measurements. Worth noting, a recent scientific statement from the American Heart Association recommended that labs use an "assay that is reported in nanomoles per liter";[14] however, the clinical decision limits currently used in the US are in mg/dL and a switch to molar units requires stakeholder support and time for implementation.

Lp(a) measurement variability may also be due to the potential impacts of sample dilutions, or the diluent used, that are performed when the analytical measurement range does not cover the concentration range observed in patient samples.[22] In this study, some individual samples had concentrations outside the analytical measurement range for 7 out of 8 assays. Preanalytical differences, such as freeze-thawing or sample handling, may also lend to increased Lp(a) measurement variability.[38] Notably, the biases for the same assays run by different laboratories did not always align, where Lp1 and Lp6 exhibited positive and negative biases, respectively. Disparate bias trends were also observed between Lp8 and Lp9, which also exhibited significantly different individual sample average CVs of 4.5% and 11.4%, respectively. These differences occurred despite laboratory participants reporting use of the same assay on the same instrument platform and using calibrators from the same manufacturer. The primary discrepancy noted was a wider degree of sample scatter that is not likely attributable to lot-to-lot reagent variations, which would cause a systematic shift across all measurement results. These results suggest that potential differences in preanalytical sample handling between Lp1 and Lp6, for example, seem to be relevant and require further investigation.

The previous Lp(a) ELISA RMS is no longer available and the SRM-2B reference material is depleted, resulting in the need for a new RMS and new reference materials. The IFCC WG APO-MS is establishing a new LC-MS RMS and reference materials that will be value assigned in nmol/L. To evaluate how moving to the new IFCC-endorsed LC-MS RMS might impact Lp(a) assays currently performing measurements in mg/dL, sample measurements in mg/dL (except Lp3 in nmol/L) were compared to LC-MS values in nmol/L. While assay-specific Lp(a) measurements in mg/dL demonstrated good linear correlation with values from the LC-MS RMP, a comparable degree of scatter was still observed at concentrations $\geq$50 mg/dL (or 125 nmol/L). This suggests that the scatter is not likely attributable to nonspecificity, which would result in higher scatter in measurement comparisons to the LC-MS values than to the all lab mean, which was not the case. The use of Lp(a) assays reporting in 2 different units of measure has hindered the establishment of universal clinical thresholds. Interestingly, a recent publication from Szarek et al.[39] utilized data from the ODYSSEY OUTCOMES trial to investigate if Lp(a) concentration measurements in 11,970 samples by 3 different assays were associated with risk of major adverse cardiovascular events (MACE). The study found that Lp(a) measurements from 2 immunoassays, 1 reporting in mg/dL and 1 in nmol/L, and by the IFCC LC-MS assay were all similarly prognostic for MACE risk.[39] In the study from Szarek, both immunoassays were well-correlated with the LC-MS method ($r > 0.96$), consistent with a previous report.[28,39] In our study, we also found good correlation between immunoassay measurements and the LC-MS RMP values. Research to assess whether a switch to the IFCC LC-MS RMS would affect clinical interpretation is ongoing.

As the data presented in this study suggest, there is clearly a remaining need to standardize Lp(a) assays to (i) allow for clinical trial measurement comparisons and (ii) to help ensure that patient measurement results are comparable across methods, location, and over time. This is important given that there are several small interfering RNA-based pharmacologic therapies in various clinical trial phases (NCT04606602 - SLN360, NCT04023552 - TQJ230 or Pelacarsen, and NCT04270760 - AMG 890 or Olpasiran) that reduce hepatic Lp(a) expression. The standardization of Lp(a) assays will not only allow researchers to appropriately compare clinical trial outcomes, but it will also allow physicians to effectively monitor patient treatment efficacy irrespective of the assay used or laboratory that performs the Lp(a) measurements. While Lp(a) assays are currently used in patient care, these assays exhibit intermeasurement variability despite previous standardization efforts. Therefore, further Lp(a) assay standardization is necessary to improve interassay Lp(a) measurement consistency and, thus, clinical utility, as indicated in a previous study.[27] However, the lack of assay standardization should not preclude the measurement of Lp(a) in patients in line with society clinical guidance documents that suggest measuring Lp(a) to identify those with higher CVD risk. Assay standardization takes time; thus, standardization efforts will be conducted in parallel with continued clinical risk assessments with the goal to improve interassay agreement over time by providing manufacturers with new reference materials anchored to the new IFCC mass spectrometry-based RMS.

The data from this study suggest that Lp(a) measurement variability is not concentration dependent (similar CVs across the concentration range), nor does variability increase with isoform size, necessitating further investigations into

potential sources of assay variability. This study provides new insights into current Lp(a) interassay variability and assay performance that will guide future CDC CSP standardization efforts. Ultimately, Lp(a) standardization, which includes reporting results in SI units, will enable measurement comparisons across clinical assays and between clinical studies, which is currently challenging, thus helping improve patient care and public health by increasing evidence-based clinical decision-making.

CRediT authorship contribution statement

Alicia N. Lyle: Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Elias N. Flores:** Investigation. **Clark C. Coffman:** Investigation. **Alex H. Doty:** Investigation. **Otoe Sugahara:** Validation, Resources. **Florian Kronenberg:** Resources, Investigation. **L. Renee Ruhaak:** Methodology, Investigation. **Christa M. Cobbaert:** Supervision, Methodology. **Hubert W. Vesper:** Writing – review & editing, Visualization, Supervision, Project administration, Conceptualization.

Ethical approval

Informed consent was obtained by Solomon Park for all individuals who donated serum for use in this study. This activity was reviewed by CDC, deemed research not involving human subjects, and was conducted consistently with applicable federal law and CDC policy.

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Declaration of competing interest

Authors have disclosed any financial and/or personal relationships where applicable. *Florian Kronenberg:* honoraria received for lectures, honoraria received from Novartis, Amgen, Silence Therapeutics, and Roche for work on advisory boards; *L. Renee Ruhaak:* Chair of the IFCC WG APO-MS, otherwise none; *Christa M. Cobbaert:* Dr. Cobbaert has received research grants from Roche Diagnostics, which were not used to support this study. All the remaining authors report no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jacl.2025.02.010.

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