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Cardiovascular Disease Lipids and Lipoproteins Biomarker Standardization

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Abstract

Cardiovascular disease (CVD) is the leading cause of mortality in the United States and globally. This review describes changes in CVD lipid and lipoprotein biomarker measurements that occurred in line with the evolution of clinical practice guidelines for CVD risk assessment and treatment and discusses the level of comparability of these biomarker measurements in clinical practice. Comparable and reliable measurements are achieved through assay standardization, which not only depends on correct test calibration but also on factors such as analytical sensitivity, selectivity, susceptibility to factors that can affect the analytical measurement process, and the stability of the test system over time. The current status of standardization for traditional and newer CVD biomarkers is discussed, as are approaches to setting and achieving standardization goals for low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), triglycerides (TG), lipoprotein(a) (Lp(a)), apolipoproteins (apo) A-I and B, and non-HDL-C. Appropriate levels of standardization for blood lipids are maintained by the Centers for Disease Control and Prevention's (CDC) CVD Biomarkers Standardization Program (CDC CVD BSP) using the analytical performance goals recommended by the National Cholesterol Education Program. The level of measurement agreement that can be achieved is dependent on the characteristics of the analytes and differences in measurement principles between reference measurement procedures and clinical assays. The technical and analytical limitations observed with traditional blood lipids are not observed with apolipoproteins. Additionally, apoB and Lp(a) may more accurately capture CVD risk and residual CVD risk, respectively, than traditional lipids, thus prompting current guidelines to recommend apolipoprotein measurements.

This review further discusses CDC's approach to standardization and describes the analytical performance of traditional blood lipids and apoA-I and B observed over the past 11 years. The reference systems for apoA-I and B, previously maintained by a single laboratory, no longer exist, thus requiring the creation of new systems, which is currently underway. This situation emphasizes the importance of a collaborative network of laboratories, such as CDC's Cholesterol Reference Methods Laboratory Network (CRMLN), to ensure standardization sustainability. CDC

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is supporting the International Federation of Clinical Chemistry and Laboratory Medicine's (IFCC) work to establish such a network for lipoproteins.

Ensuring comparability and reliability of CVD biomarker measurements through standardization remains critical for the effective implementation of clinical practice guidelines and for improving patient care. Utilizing experience gained over three decades, CDC CVD BSP will continue to improve the standardization of traditional and emerging CVD biomarkers together with stakeholders.

Keywords

Standardization; lipids; cardiovascular disease biomarkers; apolipoproteins; lipoprotein(a)

1 Introduction: Evolution of CVD Biomarker Use in Clinical Practice

Cardiovascular disease is the leading cause of mortality in the United States and globally. In 2020, 207 of 100,000 people died of heart disease and stroke in the U.S. and an estimated 19.05 million deaths were attributed to cardiovascular disease (CVD) globally, which is an increase from 2010 by 19% [1]. Reducing the risks for cardiovascular diseases is a major focus in public health and patient care.

Blood lipids, such as low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and total cholesterol (TC), are considered modifiable risk factors for cardiovascular diseases [2]. Lipid-lowering therapies, such as statins and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, reduce the risk of atherosclerotic cardiovascular diseases (ASCVD) [3]. A meta-analysis of 27 randomized trials found that in individuals with 5-year risk of major vascular events (MACE) lower than 10%, each 1 mmol/L reduction in LDL cholesterol produced an absolute reduction in MACE of about 11 per 1000 over 5 years [4]. Other studies showed that statin-mediated lipid-lowering therapies reduced MACE in high-risk individuals and in individuals with diabetes mellitus [3]. The success of statins in CVD risk reduction stimulated the development of a range of new drugs and changed how clinicians treat elevated LDL-C and reduce ASCVD risk, as summarized recently [5].

As scientific and clinical communities have developed a better understanding of the underlying etiology of CVD, clinical practice guidelines have evolved to incorporate the measurements of new, specific biomarkers central to patient risk assessment and diagnosis. Measurements of blood lipids and lipoproteins have remained central to CVD risk assessment and treatment, but the focus on certain blood lipids and lipoproteins respective concentration targets have changed. The first guideline was developed by National Institutes of Health (NIH) National Cholesterol Education Program (NCEP) in 1985 to create recommendations on the prevention and treatment of CVD [5,6]. In this guideline, known as the Adult Treatment Panel I (ATP I), individuals at higher CVD risk were initially categorized into those with high (≥ 240 mg/dL, 6.2 mmol/L) and borderline (200 – 239 mg/dL, 5.2 – 6.2 mmol/L) total cholesterol [7]. For patients with high or borderline TC and additional risk factors, such as smoking or high blood pressure, additional blood

lipid measurements were recommended, including LDL-C calculation using the Friedewald equation [8]. LDL-C levels were then used as the basis on which drug treatment and/or life-style intervention decisions were made. It was also recommended that patients with LDL-C ≥ 160 mg/dL (4.1 mmol/L), receive cholesterol-lowering therapies with the goal to lower LDL-C below 130 mg/dL (3.4 mmol/L) [9]. Determination of HDL-C concentrations for all individuals were introduced in the 1993 ATP II guidelines as an addition to routine TC measurements [10]. While TC sub-categorization remained unchanged from ATP I, HDL-C categories of ≥ 35 mg/dL (0.91 mmol/L) and < 35 mg/dL were introduced to guide further investigations and treatment decisions. Patients were then subsequently categorized based on LDL-C values as high risk (LDL-C ≥ 160 mg/dL, 4.1 mmol/L), borderline high risk (130 – 159 mg/dL, 3.4 – 4.1 mmol/L) and desirable < 130 mg/dL (3.4 mmol/L). The treatment target was defined at LDL-C ≤ 100 mg/dL [10]. The ATP III guidelines were updated in 2004, and LDL-C remained the primary treatment decision biomarker. The ATP III guidelines recommended, for the first time, that physicians obtain a full lipid profile (TC, HDL-C, LDL-C and TG) on all individuals rather than limiting measurements to TC and HDL-C. While primary treatment decisions continued to center around LDL-C, with lower end goal targets of < 100 mg/dL (2.59 mmol/L), ATP III also suggested treating individuals with TG ≥ 200 mg/dL (2.29 mmol/L). HDL-C decision targets were also changed from < 35 mg/dL (0.91 mmol/L) to < 40 mg/dL (1.03 mmol/L) [11]. Additionally, secondary targets, such as non-HDL-cholesterol (non-HDL-C), were added for those with high TG (≥ 200 mg/dL or 2.29 mmol/L) and metabolic syndrome. The treatment goals for high-risk individuals were set to < 70 mg/dL (1.81 mmol/L) for LDL-C or < 100 mg/dL (< 2.59 mmol/L) for non-HDL-C for individuals with TG > 200 mg/dL (2.29 mmol/L) [12]. The 2006 joint American Heart Association and American College of Cardiology (AHA/ACC) guidelines adopted the recommendations from ATP III, in part, and suggested LDL-C targets of < 100 mg/dL (2.59 mmol/L) for all patients with coronary artery disease (CAD) and other clinical forms of ASCVD, and suggested LDL-C treatment targets of < 70 mg/dL (1.81 mmol/L) in high-risk patients. If attaining LDL-C concentrations of < 70 mg/dL (1.81 mmol/L) is not possible, then one should aim for a LDL-C reduction of $> 50\%$ with lipid lowering drugs [13].

Beginning in 2013, the AHA/ACC guidelines no longer recommended specific LDL-C treatment target goals and suggested that individuals with LDL-C ≥ 190 mg/dL (4.91 mmol/L) aim for a 50% reduction in LDL-C as a primary prevention treatment target [14]. For monitoring therapy, LDL-C reductions of $\geq 50\%$ are anticipated for high intensity treatments in individuals with ≥ 190 mg/dL (4.91 mmol/L) LDL-C or 70–189 mg/dL (1.81–4.89 mmol/L) for individuals who have diabetes mellitus and a high-risk score. The 2018 AHA/ACC guidelines continued risk-based treatment approaches without providing concentration-specific treatment targets. It was also noted that LDL-C levels calculated with the Friedewald equation exhibit increased unreliability at lower levels of LDL-C, more specifically when LDL-C is < 70 mg/dL (< 1.81 mmol/L), and suggested calculation adjustments at very low LDL-C concentrations. Additionally, apolipoprotein B (apoB); also referred to as apolipoprotein B100 or apoB₁₀₀) measurement was recommended for individuals with TG ≥ 200 mg/dL (2.29 mmol/L) [15] as an alternative to LDL-C measurements, and the guidelines noted that an apoB concentration of > 130 mg/dL (~ 1.3

g/L) would correspond to a LDL-C level ≥ 160 mg/dL (4.14 mmol/L). Furthermore, the guideline pointed to lipoprotein(a) (Lp(a)) ≥ 50 mg/dL (≥ 125 nmol/L) as a CVD risk-enhancing factor [15]. Recommendations for measuring apoB and Lp(a) not only represent a means to overcome the limitations of LDL-C measurements but are also a reflection of the scientific community's increased understanding of the roles of these proteins in the atherogenic process. The recent 2022 ACC Expert Consensus Decision Pathway on the Role of Non-statin Therapies for LDL-C Lowering in the Management of Atherosclerotic Cardiovascular Disease Risk categorizes individuals with LDL-C ≥ 100 mg/dL (2.59 mmol/L) and additional conditions as very high risk for future ASCVD events. Furthermore, it suggests that primary hypertriglyceridemia (TG ≥ 175 mg/dL or 1.98 mmol/L), elevated high sensitivity C-reactive protein (hsCRP) (≥ 2 mg/L or 190.48 nmol/L), elevated Lp(a) (≥ 50 mg/dL or ≥ 125 nmol/L), or elevated apoB (≥ 130 mg/dL or ≥ 1.3 g/L) are risk-enhancing factors [16].

Additional guidelines, such as the joint European Society for Cardiology and European Atherosclerosis Society (ESC/EAS) and the Canadian Cardiovascular Society (CCS) guidelines, also recommend LDL-C as primary treatment target with LDL-C treatment goals in line with those in the previous ATP III and AHA/ACC guidelines [17–19]. The 2011 ESC/EAS guidelines pointed out that factors such as lower HDL-C or apoA-I, and higher TG, apoB, Lp(a), or hsCRP are all indicators of higher CVD risk than what is estimated solely using the SCORE algorithm-based risk charts [19]. The 2016 ESC/EAS guidelines recommended measurement of apoB or non-HDL-C in individuals with high TG for risk assessment before treatment [20]. The most recent 2019 ESC/EAS guidelines now recommend Lp(a) testing once in a lifetime to identify those with very high Lp(a) levels (>180 mg/dL or >430 nmol/L) [21,22]. Similarly, the 2021 Canadian Cardiovascular Society guidelines also recommend measuring Lp(a) once in a person's lifetime for screening purposes and recommends earlier and more intensive health behavior modifications in the primary prevention setting for individuals with Lp(a) ≥ 50 mg/dL (or ≥ 100 nmol/L).[23] In patients with familial hyperlipidemia at very high risk, the ESC/EAS guidelines suggested a reduction of $\geq 50\%$ from baseline LDL-C as a treatment target with an LDL-C goal of <55 mg/dL (<1.4 mmol/L). Furthermore, apoB measurements are now recommended for risk assessments in individuals with high TG, diabetes mellitus, obesity or metabolic syndrome, and very low LDL-C. The ESC/EAS guidelines not only provided treatment targets for LDL-C, but also for non-HDL-C (<85 , 100, and 130 mg/dL or <2.2 , 2.6, and 3.4 mmol/L for very high, high, and moderately high risk, respectively) and apoB (<65 , 80, 100 mg/dL or <0.65 , 0.80, 1.0 g/L for very high, high, and moderately high risk, respectively). In addition, the CCS guidelines recommend non-HDL-C or apoB measurements in lieu of LDL-C in patients with TG >1.5 mmol/L[23]. For patients with ASCVD who experience a second MACE within 2 years while taking maximally tolerated statin-based therapy, the EAS/ESC guidelines suggest an LDL-C goal of <40 mg/dL (<1.03 mmol/L) may be considered [21,22].

Throughout this remarkable evolution of guidelines from 1988 to present, LDL-C has remained the cornerstone of ASCVD management. Treatment goals changed from LDL-C recommendations of <130 mg/dL (3.36 mmol/L) in ATP I to LDL-C levels <40 mg/dL (1.03 mmol/L) in individuals with certain risk-enhancing conditions. Similarly, the LDL-C levels

for initiating treatment of patients changed from treating patients with LDL-C ≥ 160 mg/dL (4.14 mmol/L) to lower LDL-C levels in patients with additional risk factors. Specific treatment targets for LDL-C are no longer recommended in current guidelines. Also, these guidelines no longer use HDL-C as a biomarker for treatment or diagnostic decisions [24,25] and suggest the use of non-HDL-C, apoB, and Lp(a) as new biomarkers in addition or in lieu of traditional blood lipids.

In addition to directly measured CVD risk biomarkers, there are biomarkers that rely on calculated values derived from traditional blood lipid measurements. For example, LDL-C can be measured directly but is often calculated with the Friedewald equation, which uses TC, TG, and HDL-C values to estimate LDL-C concentrations [8]. The equation assumes that very low-density lipoprotein cholesterol (VLDL-C) concentrations can be estimated as being 1/3 of TG values. The equation was derived using lipids data from healthy individuals and provides reliable estimates in individuals with normal TG values; however, the equation is prone to underestimation of LDL-C in patients with high TG and when the actual LDL-C concentration is low [26]. The limitations of the Friedewald equation, especially at high TG levels, are reflected in the most recent clinical practice guidelines, which now recommend the use of alternate calculation algorithms [15], the calculation of non-HDL-C [11], and the measurement apoB in patients with high TG. Over 20 alternate LDL-C equations have been proposed [27] of which 2 equations are currently used in clinical practice, namely the Martin/Hopkins [28–30] and Sampson equations (also referred to as the NIH equation) [31,32]. These new equations showed a higher accuracy than the Friedewald equation, especially at high TG levels and at LDL-C concentrations < 70 mg/dL (1.81 mmol/L) [27,33]. A detailed discussion of both equations was recently published [34]. These formulas are still subject to further improvements [35,36] and evaluations [37,38]. Several recent guidelines started recommending non-HDL-C as an alternative to LDL-C for CVD risk assessment [39–41]. Non-HDL-C is determined by calculating the difference between TC and HDL-C measurements and therefore includes other atherogenic particles, such as VLDL [42], that LDL-C measurements do not capture. Common to all of these equations is their dependence on accurate and reliable measurements of TC, HDL-C, TG, and other blood lipids.

While measurements of traditional blood lipids (TC, HDL-C, LDL-C, and TG) remain essential for CVD risk assessments and monitoring treatment, measurements of apolipoproteins, such as Lp(a) and apoB, are now established for use in CVD risk management. Despite inclusion of apolipoproteins in clinical practice guidelines, these biomarkers are not yet widely used in patient care and public health. This can be explained, in part, by their structure, role in CVD risk assessment, and in their standardization status not being widely appreciated, which is discussed herein.

Apolipoproteins are proteins associated with lipoprotein particles, including LDL-C, HDL-C, and others [43]. The surface of the LDL-C particle, for example, is enveloped by a monolayer consisting of phospholipid molecules and apoB. ApoB is a large, hydrophobic protein present as a single copy on LDL particles, VLDL particles, intermediate-density lipoprotein (IDL) particles, as well as Lp(a) particles [44]. Thus, apoB is present in the majority of particles represented by non-HDL. With each particle containing one apoB

copy, the measurement of apoB is a convenient approach to evaluating a patient's total number of circulating atherogenic particles to establish ASCVD risk [45]. Indeed, studies have established that the particle number of apoB-containing, atherogenic lipoproteins is the most important characteristic for determining ASCVD risk [46]. For example, a 2021 study from Johannesen et al. concluded that elevated apoB and non-HDL-C, but not LDL-C, are associated with residual risk for all-cause mortality and myocardial infarction [47].

Lp(a) is a complex protein that consists of an apoB-containing LDL-like particle that is covalently linked to a defining apolipoprotein(a) [apo(a)] protein [48]. Evidence from several high-profile statin and PCSK9 inhibitor clinical trials (4S, AIM-HIGH, FOURIER, among others) previously suggested that when Lp(a) levels are elevated, CVD event rates are higher irrespective of the LDL-C levels achieved through statin and PCSK9 use [49–51]. These findings ultimately led to Lp(a) being recognized as a biomarker of “residual risk” for adverse CVD outcomes in the setting of controlled LDL-C [50,52]. Additionally, Lp(a) is now recognized as an independent, causal risk factor for the development of ASCVD and calcific aortic valvular disease [53,54]. While clinical guidelines have established that elevated Lp(a) levels (≥ 50 mg/dL or ≥ 125 nmol/L) constitute enhanced CVD risk [15,22,23].

The primary structural and functional component of HDL-C is apoA-I. HDL-C is hypothesized to mitigate atherosclerosis via the uptake and clearance of excess lipids/cholesterol from tissues, and this reverse cholesterol transport process is facilitated by apoA-I [55,56]. High levels of apoA-I are associated with a reduced risk of cardiovascular events. In contrast, low levels of apoA-I, in example due to apoA-I mutations, can indicate an increased risk for coronary artery disease [57,58]. Despite its inverse relationship with CVD, HDL-raising therapies have not met clinical expectations, suggesting that lipoprotein biology and/or metabolism are more complicated. Indeed, niacin supplements and cholesteryl-ester transfer protein inhibitors, tested in the HPS2-THRIVE and REVEAL trials, respectively, increased HDL-C [59,60]; however, CVD outcomes trials ended due to non-ideal pharmacokinetics, futility, and serious adverse events. Despite these findings, there is an increasing interest in the measurement of apoA-I in lieu of HDL-C, as apoA-I measurements may allow more direct quantification of HDL-C particle numbers.

For all these biomarkers certain concentrations are stated in guidelines to guide clinical decision making. Therefore, standardization systems need to be created and maintained to ensure the analytical accuracy and reliability of these CVD biomarker measurements and thus to enable correct and consistent clinical decision making using these clinical practice guidelines.

2 Facilitating Guideline Implementation through Coordinated Standardization

Specific concentrations for blood lipids and lipoproteins described in clinical practice guidelines can only be applied when laboratory tests used in patient care have the same analytical accuracy, precision, and selectivity as those used to create these guidelines. Furthermore, to ensure consistency in clinical decision making over time, the analytical performance of laboratory tests needs to be constant over time. Since its inception

in the early 1960s, the Centers for Disease Control and Prevention's (CDC) CVD Biomarkers Standardization Program (CDC CVD BSP), formerly known as the CDC Lipids Standardization Program, has worked with clinical laboratories, researchers, and other key stakeholders to ensure that the CVD biomarker measurements used for research and performed in patient care settings are comparable and consistent over time. This enabled the effective implementation of evidence-based clinical practice guidelines and facilitated the described advancements in patient care and public health [61,62].

The analytical performance of CVD biomarker tests and, thus, the analytical comparability of test results, can differ across testing systems and can change over time if not monitored in a longitudinal manner and corrected appropriately through standardization activities. Differences and changes in analytical performance can be caused by a multitude of factors [24,63,64]. Addressing these factors requires programs that assess the analytical performance of these tests in a comprehensive, consistent, and scientifically appropriate manner. This is achieved most effectively by comparing test results to a reliable and continuous point of reference. The CDC CVD BSP has provided this point of reference and thorough analytical performance assessments for blood lipids for over 60 years [61,62,65,66].

Analytical performance of tests often varies at different biomarker concentrations, with tests typically aiming for optimal performance at clinical target concentrations. In the past, this was accomplished by the NCEP Expert Laboratory Panel, which developed recommendations for reliable measurements of blood lipids. These recommendations were adopted by the CDC CVD BSP, as well as manufacturers and laboratories. CDC CVD BSP had already standardized measurements conducted in the clinical trials on which the ATP guidelines were based. With advancements in each ATP guideline, the NCEP Expert Laboratory Panel added analytical performance specifications for lipid measurements, including TC [67], LDL-C [68], TG [69], and HDL-C [70]. With the discontinuation of the NCEP, the work performed by the NCEP Expert Laboratory Panel to set analytical performance specifications also stopped. As a result, recommendations on analytical performance requirements are no longer updated to reflect changes in current clinical guidelines and old performance requirements established in the 1990s are still being applied without appropriate modifications (Table 1). A recent study suggests that current NCEP standards may cause up to 10% misclassification of patients. This study also highlights the need for stakeholder collaborations to adjust analytical performance requirements [37].

As clinical practice guidelines evolved, the CDC CVD BSP engaged in research collaborations and generated new data on the analytical performance of CVD biomarker assays to broaden the scientific and clinical community's understanding of the strengths and weaknesses of traditional lipid biomarker tests used in patient care and research. For example, one key study reported increased measurement inaccuracies in patients with different comorbidities, such as diabetes and hypertriglyceridemia [41,71–74]. Findings from CDC CVD BSP collaborative studies, as well as studies conducted by other groups, are reflected in current guidelines, such as the recently suggested use of apoB or non-HDL-C in patients with high TG [75] to overcome the aforementioned measurement inaccuracies in these patients. Furthermore, based on these research findings, the CDC CVD BSP adjusted

its protocols to include samples from individuals with diseases known and/or suspected to affect lipid measurements. In addition, these new protocols [76] now focus on investigating LDL-C measurement accuracy at the low LDL-C concentrations targeted in current clinical practice guidelines [15,76]. CDC CVD BSP is continuing efforts in collaboration with NIH and other key stakeholders to adjust analytical performance criteria for both traditional blood lipids and new CVD biomarkers to facilitate the effective implementation of current guidelines, as proposed recently [15,16,21,22].

3 The CDC CVD Biomarkers Standardization Program

The CDC CVD BSP aims to ensure correct and consistent patient care and to enable effective implementation of research findings in health care and public health. It accomplishes this by creating measurement results in patient care, public health, and clinical research that are comparable across methods, locations, and over time and that are independent of analytical methodologies or technologies. CDC CVD BSP is doing this by assessing, improving, and maintaining the analytical performance of clinical laboratory tests. To conduct these activities appropriately and effectively, it established and maintains unique laboratory capacity and expertise within its clinical reference laboratories (CRL). It uses this capacity and expertise to assist assay manufacturers and clinical laboratories. Furthermore, this capacity is used to monitor the analytical performance of clinical tests as they are used in patient care and research, as detailed below.

3.1 CDC Reference Systems for CVD Biomarkers – Step 1

CDC CRL developed and maintains metrological reference methods (also called reference measurement procedures or RMPs) for TC, TG, HDL-C, and LDL-C (Supplemental Figures 1S–4S). The CDC CRL is actively collaborating with the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) to establish new RMPs for apolipoproteins such as Lp(a) (Supplemental Figure 5S), apoB, and apoA-I. These RMPs are not intended for use in patient care, as they are optimized to provide the highest level of accuracy and precision (analytical performance requirements for these RMPs are listed in Table 1). The high level of analytical performance is reflected in CDC CRL meeting the requirements of International Organization for Standardization (ISO) 15195:2018 for the competence of calibration laboratories using reference measurement procedures [77]. Compliance to this ISO standard includes use of ISO compliant RMPs and work conducted by CRL being compliant with ISO 17511:2020 for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples [78]. Using these RMPs, CRL is characterizing and assigning reference values to serum materials created by CDC CVD BSP programs and metrological organizations such as the U.S. National Institute for Standards and Technology (NIST). The combination of RMPs and reference materials are referred to as ‘reference systems’ and act as point of reference that is used for calibration and to assess the analytical performance of clinical tests. Establishing a point of reference, or reference system, is typically the first step in CDC’s clinical standardization activities.

To better address the global need for reference measurements and to ensure the continuity of those measurements, the CDC CVD BSP has established in 1989 the Cholesterol Reference Methods Laboratory Network (CRMLN) [65,79]. CRMLN has an average of 8 members in 8 countries covering different geographical regions. It provides reference measurements for TC, TG, HDL-C and LDL-C and collaborates with IVD manufacturers to calibrate clinical tests and to assess the analytical performance of these tests. The network conducts regular, well-defined interlaboratory comparisons studies where laboratories need to meet the analytical performance criteria outlined in Table 1. It is the only reference laboratory network available globally for TC, TG, HDL-C and LDL-C reference measurements.

The importance of having well-organized networks of reference laboratories recently became apparent with the loss of the reference systems for Lp(a), apoA-I, and apoB, which were operated and maintained in one highly specialized laboratory. For many years these systems consisted of specially prepared serum materials, made available through the World Health Organization (WHO), with values assigned by a well-characterized immunoassay operated in the aforementioned lab [80–83]. These WHO materials are now mostly exhausted. Furthermore, the well-characterized immunoassays and the specialized laboratory that operated these immunoassays no longer exist. The establishment of a network of reference laboratories, similar to CRMLN, may have ensured sustainability of this reference system over time; however, such a network was never organized. As a result, there is currently no reference system available for these biomarkers. This situation is especially concerning for Lp(a), which exhibits high inter-assay variability and is a biomarker of increasing clinical interest [84]. This impedes Lp(a) patient result comparisons across clinical assays, making comparisons across clinical trials and studies challenging. A lack of assay agreement may also hinder the appropriate assessment of pharmacologic efficacy in patients, which is important given that several national clinical trials (NCT) are underway for antisense oligonucleotide [NCT04023552] and small interfering RNA [NCT05581303; NCT05537571; NCT05565742] therapeutics that target and lower Lp(a) [85,86]. To avoid this situation in the future, a reference laboratory network for these biomarkers is being developed as outlined below.

3.2 CDC Procedures to Improve and Document Analytical Performance of CVD Tests – Step 2

Standardizing clinical laboratory tests is often incorrectly understood as solely re-calibration of tests to the highest available standard, as outlined in ISO 17511:2020 [78,87]. While correcting calibration is typically one main activity conducted in standardization efforts, it is often not sufficient for achieving accurate and reliable measurement results. Many times, improvements of other analytical performance parameters, such as analytical selectivity and sensitivity, are needed. However, identifying sources of inaccurate measurements, other than incorrect calibration, require thorough assessments using established protocols. These thorough assessments, including re-calibration activities, are conducted in the second step in CDC's CVD BSP.

CDC CRL and CRMLN members collaborate with manufacturers of clinical tests by providing reference measurements and materials, as well as technical assistance to

investigate and improve the analytical performance of CVD biomarker tests. Once these activities are completed, the analytical performance is assessed using specific protocols [88]. These protocols use multiple single-donor, unmodified sera covering the clinically relevant concentration range for each analyte evaluated. The use of multiple samples across the analytical measurement range enables detailed assessments of the analytical performance at different concentrations. Unique to this CDC program is the use of unmodified single-donor sera. They closely mimic regular patient samples and thus provide information about the actual analytical performance occurring in patient care settings [89]. The measurement of these samples and evaluation of data are based on established protocols such as the Clinical Laboratory and Standards Institute (CLSI) protocol for Measurement Procedure Comparison and Bias Estimation Using Patient Samples [90].

Those laboratory tests meeting the analytical performance requirements outlined in Table 1 are considered sufficiently accurate and precise, and thus are commonly referred to as ‘standardized to CDC’. CDC and its CRMLN members have ensured consistent calibration of >800 assays and reagent kits over the past 11 years in the U.S. and globally. Current CDC-certified assays are listed on the CDC’s website at <https://www.cdc.gov/clinical-standardization-programs/php/cvd/list-of-cvd-certified-assays.html> [91].

3.3 Monitoring the Level of Agreement Across Clinical CVD tests – Step 3

Improving the analytical performance of commercial CVD biomarker tests at the manufacturer minimizes the burden on clinical laboratories as they do not need to conduct these activities. However, procedures need to be in place to monitor the analytical performance in clinical laboratories to ascertain that these improvements successfully impacted testing performed in patient care, public health and clinical research. This is typically referred to as the third step in CDC’s Standardization Program activities. These monitoring activities are conducted in collaboration with External Quality Assessment (EQA) program providers and through the CDC’s CVD BSP monitoring program.

Data obtained in EQA schemes provide cross-sectional information about the analytical performance of laboratory test used in patient care [92]. EQA assessments are typically conducted several times per year and each evaluation is performed independently. The laboratories participating in each challenge may vary. For over 30 years, CDC CVD BSP has provided reference measurements to EQA providers such as the U.S. College of American Pathologists (CAP) for their accuracy-based surveys for TC, TG, HDL-C, and LDL-C [93]. In 2023, over 100 laboratories participated in the CAP accuracy-based lipids survey. Data obtained in these CAP surveys have been used in scientific statements and other documents [94]. Discussions on more recent survey findings are available at <https://www.cap.org/member-resources/councils-committees/accuracy-based-testing-committee-participant-summary-report-discussions> [93]. Similar activities are conducted by CRMLN members in other countries, such as the Dutch EQA program provided by the Stichting Kwaliteitsbewaking Medische Laboratoriumdiagnostiek (SKML) [95].

The CDC Lipids Standardization Program (LSP) provides participants with samples that are analyzed weekly and measurement performance is assessed using data collected over

3 consecutive months [66,96]. This longitudinal study design facilitates the detection of changes in measurement accuracy over time and provides more detailed information about the analytical performance of an individual laboratory. In 2023, the CDC was monitoring the analytical performance of over 260 laboratories in this program. The average duration of participation in this program is seven years. The list of successful participants in this program can be found at <https://www.cdc.gov/clinical-standardization-programs/php/cvd/list-of-cvd-certified-assays.html> [91]. LSP ensured the accuracy of clinical trials and epidemiological studies since its inception over 40 years ago.

Data obtained with EQA and the LSP programs complement each other and, together, provide comprehensive information about the accuracy and reliability of CVD biomarker tests used in patient care settings. These programs use pooled sera and a small number of samples. Therefore, they typically provide an indication of potential concerning analytical performance but often cannot provide sufficient information to identify the cause of the concern. CDC CVD BSP is using the information obtained in these monitoring programs to guide test improvements performed in step two of the CDC standardization activities.

4. Standardizing traditional blood lipids.

CVD biomarkers currently used in patient care and public health range from chemically well-defined molecules, such as cholesterol, to highly complex particles consisting of a range of different small to highly complex molecules, such as LDL-C. The complexity of these particles and the concepts used to define them are not always fully appreciated. However, they determine how certain standardization activities are implemented and, most importantly, the level of agreement among assays that is technically achievable. Herein, we discuss the specific needs of various analytes used in clinical practice. Specifics with respect to the current status of CVD biomarker standardization and improvement efforts underway are discussed in the sections below.

4.1. Total Cholesterol

Cholesterol is a well-defined analyte with a specific, fully described molecular structure and molecular mass [97]. Total cholesterol measurements used in patient care are the sum of free and esterified cholesterol occurring in serum; thus, total cholesterol is measured after hydrolysis of esterified cholesterols. A reference system for total cholesterol has been established and consists of a certified, pure cholesterol RM [98] and well-described RMPs. The RMP used to standardize total cholesterol measurements in clinical research and patient care is based on a method initially described by Abell et al. [99] and has been updated later (Supplemental Figure S1) [100,101]. It is often referred to as the Abel-Kendall RMP. Current clinical decision points and treatment targets are based on measurements traceable to this RMP.

The Abel-Kendall RMP, operated by CDC and CRMLN members, has provided highly consistent reference measurements that are well within current performance requirements for decades (Figure 1) [79]. The median annual bias across all CRMLN members performing the Abel-Kendall RMP from 2013 – 2023 ranged between –0.4% and 0.2% (range of annual IQRs: 0.7% – 1.2%) and median annual precision across CRMLN members

ranged between 0.2% and 0.3% (range of annual IQRs: 0.2% – 0.4%) (Supplemental Tables 1S and 2S). The accuracy achieved by CRMLN members with assay manufacturers appears very similar to the accuracy observed in clinical laboratories participating in the CDC LSP program, with the yearly median bias across all LSP participant laboratories (average: 60 participants per year) ranging between –2.1% and 0.1% (annual range IQRs: 2.5% – 3.7%). The annual median precisions for LSP participants range between 0.4% and 0.6% CV (annual range of IQRs: 0.6% – 0.8%) (Supplemental Tables 1S and 2S). The high level of accuracy and precision observed at each level of the standardization process is achieved because total cholesterol is a well-defined compound that can be targeted by assays with high analytical specificity.

4.2. Triglycerides

TG, as measured in patient care, are less well-defined than cholesterol. The entity actually being measured is glycerol, which is obtained after hydrolyzing tri-, di- and monoglycerides. In typical patient samples, triglycerides constitute the majority of glycerides [97]. Therefore, measurement of glycerol is assumed to describe the amount of triglycerides present in blood. Measurements are further complicated by the presence of free glycerol in the blood. While free glycerol is typically a minor component, it can comprise up to 5% of the total glycerol measured in patients with conditions such as dyslipidemia, diabetes, pregnancy, or glycerol kinase deficiency [102–107]. Some clinical tests remove free glycerol prior to measuring esterified glycerol [106,108]. These tests are typically referred to as ‘glycerol-blanked’ assays. Higher-order reference materials, such as tripalmitin [109] and pure glycerol, and mass-spectrometry based RMPs [108,110–112] are available and make up the reference system for TG (Supplemental Figure 2S).

Performance criteria for clinical assays were published in 1995 [69] and reference systems were established and maintained by CDC and CRMLN members [108,110]. Criteria for TG RMPs and related certification activities were developed and implemented in 2016 (Table 1). The annual median biases observed in CRMLN between 2016 – 2023 range between –0.8% and 0.6% (range of annual IQRs: 1.1% – 2.6%) and the median precision ranged between 0.4 – 0.8 (IQR: 0.2 – 0.7) (Supplemental Tables 1S and 2S). Thus, the network has demonstrated consistent accuracy that is well below the required criteria.

With reference laboratory and manufacturer certifications starting after the TG analytical performance criteria were put in place in 2016, only a limited number of manufacturer certification data are currently available (Figure 2). The average annual bias observed with CRMLN participants ranged between –1.1% and 1.7% (annual IQR: 2.0% – 3.9%). Before formal certification was available, CRMLN members assisted manufacturers with calibration. The median annual bias observed with clinical laboratories participating in LSP between 2013 and 2023 ranging between –3.2% and 0.6% (IQR: 3.7% – 5.9%) and the median annual precision ranging between 0.5% to 0.8% (IQR: 0.7 – 1.2%) (Supplemental Tables 1S and 2S). The higher variability in TG measurements, as indicated by higher IQRs compared to TC measurements, could be explained by the heterogeneity of the analyte (i.e., blanked vs. non-blanked assays) and by the lack of certification in earlier years. Despite

these limitations, the overall analytical performance of TG measurements for certified tests is still well within performance limits.

4.3. LDL-C and HDL-C

Lipoproteins are complex particles comprised of hydrophobic lipids at their core and hydrophilic lipids at their surface, as well as one or more apolipoproteins [43]. The size and composition of these particles can differ among individuals and can be affected by diet and other factors [113,114].

LDL-C is defined as the amount of cholesterol in a serum fraction with a density between 1.02 and 1.06 g/mL, while HDL-C is the amount of cholesterol in a serum fraction with a density typically between 1.06 and 1.21 [21,24]. Thus, both parameters describe particles containing a diverse group of compounds with a common generic characteristic, namely density, that is not directly related to the atherogenic process. Because, HDL-C and LDL-C are the main vehicles used to transport cholesterol in blood, the total cholesterol determined in these fractions is assumed to reflect the amount of particles present in these fractions [115,116].

With LDL-C and HDL-C being defined based on density, the operational conditions used to obtain these density fractions, specifically the ultra-centrifugation conditions, need to be well-defined [115]. Therefore, LDL-C and HDL-C are commonly referred to as ‘procedure-defined analytes’, meaning the specific, generally agreed-upon operational conditions of the ultra-centrifugation step in the measurement procedure define what constitutes LDL and HDL. Complexity is furthered by the use of common, direct measurement assays in patient care for HDL-C and LDL-C measurements, which do not employ ultracentrifugation and use proprietary procedures that are assumed to provide LDL-C and HDL-C values equivalent to the corresponding ultracentrifugation fractions [63,73,117,118].

The specific quantitative composition of individual components in the LDL- and HDL-fractions is highly variable and not well-defined. As a direct result, unlike cholesterol and triglycerides, certified reference materials with pure HDL-C or LDL-C particles for assay calibration do not exist; however, serum-based reference materials are available [119] with reference values assigned by the CDC RMP [65,115,116]. Thus, the reference system for HDL-C and LDL-C is a RMP consisting of well-defined ultracentrifugation conditions (Supplemental Figure 3S) and serum-based reference materials.

Freeze-thaw can affect LDL-C measurements and can lead to incorrect assessments of measurement bias [73]. Therefore, the CDC CVD BSP requires the use of fresh, non-frozen materials for the evaluation and certification of analytical performance [88]. This requirement is in line with clinical practice, where LDL-C is typically measured in fresh, non-frozen sera; however, this requirement poses logistical challenges as samples need to be shared between manufactures and CRMLN members. These challenges are addressed by CRMLN members operating and covering measurement needs in different geographical regions. This freeze-thaw effect is also the reason LDL-C is not included in the CDC LSP program, which uses frozen sera.

Despite the described complexities and challenges, the CRMLN member laboratories were able to provide highly accurate and consistent reference measurements (Figure 3 and Figure 4). Across all CRMLN laboratories between 2013 and 2023, the median annual bias ranged between -0.6 mg/dL to -0.1 mg/dL (range of annual IQR: 0.7 mg/dL - 1.3 mg/dL) for HDL-C and -0.6% - 0.4% (range of annual IQRs: 1.3% - 2.7%) for LDL-C (Supplemental Table 1S). The median annual precision ranged between 0.5% - 0.8% (range of annual IQR: 0.4% - 1.0% , for HDL-C and 0.6% - 0.8% (range of annual IQR: 0.4% - 0.6%) for LDL-C (Supplemental Table 2S). The high level of accuracy and precision achieved across these RMPs has enabled CRMLN members to calibrate HDL-C and LDL-C appropriately. This is reflected in an annual median bias across manufacturers observed over the past 11 years of between -0.4% to 1.0% (range of annual IQRs: 4.4 to 7.7%) for HDL-C and between -2.4% to 2.3% (range of annual IQRs: 3.9% to 6.6%) for LDL-C. This is well within the performance requirements for both RMPs and manufactures (Table 1). However, the variability across assays, as reflected in the IQR, is higher than those observed for TG and TC. This could be explained by the analyte not being well-defined, which makes it difficult to develop analytical methods that recognize specific targets characteristic for LDL and HDL particles. Another potential source of variability is use of assays that apply testing principles other than ultra-centrifugation to separate HDL-C and LDL-C, which may lead to measurements results that differ from RMP measurements.

With clinical guidelines now aiming for much lower LDL-C concentrations, CDC CVD BSP conducted additional investigations about the analytical performance of blood lipid tests at current treatment goals using data available from CDC CVD BSP. The bias distributions observed across all samples and manufacturers participating in CDC's programs were normally distributed around zero bias and were consistent across analyte concentrations for TC and HDL-C. However, notable differences in bias distributions at different concentrations were observed for LDL-C and TG, with distributions exhibiting an increasing negative bias at decreasing LDL-C concentrations and increasing positive bias at decreasing TG concentrations (Figure 5). While the IQR of the bias distribution still falls within the TG bias criteria, that is not the case for LDL-C concentrations, where the IQR falls partially outside the performance criteria at LDL-C concentrations <70 mg/dL. Thus, there is a substantial portion of measurement results that may underestimate true LDL-C concentrations in the current LDL-C treatment target concentration range. Measurement results at the former NCEP clinical decision points remain accurate. These data support previous findings [37] that the current analytical performance criteria are not sufficient to support present day clinical practice guidelines. The level of accuracy of tests at the former NCEP targets suggest that the CDC's standardization procedures and activities are effective. The inaccuracy of LDL-C tests at new treatment goals suggest that the current analytical performance specifications are insufficient to maintain measurement accuracy at high as well as low LDL-C concentrations. Therefore, a review and revision of current analytical performance criteria is warranted and, as described, under way.

With the composition of LDL particles not being well-defined and affected by diet, certain diseases, and other factors, and with clinical test and RMPs using very different measurement principals, high sample-to-sample inconsistencies in measurement results between RMPs and clinical tests are anticipated. They have been confirmed in different

studies [73] and, as described, are observed in CDC CVD BSP, especially at low LDL-C concentrations. Though improvements in the analytical performance of LDL-C measurements seem possible, the heterogeneity of HDL-particles combined with the use of different measurement principles between RMP, and clinical tests indicate that these tests may not be able to reach the level of agreement and performance observed with TC.

5. Standardization of Apolipoproteins

Initial standardization of traditional blood lipids, especially TC, was implemented at the beginning of major clinical trials conducted in the 1970s by the NIH Lipid Research Clinics [62]. Thus, clinical decision points for blood lipids stated in the ATP I guideline were generated using tests already standardized by CDCs. The findings from the NIH study stimulated the development of new clinical tests for blood lipids, which also underwent standardization before they were introduced commercially. This situation profoundly facilitated the implementation of the ATP guidelines in patient care and public health, and majorly contributed to the success and fast evolution of CVD risk assessment and treatment described above.

For apolipoproteins, standardization activities were introduced after clinical trials were conducted, guidelines were published, and commercial tests became available. This situation complicates the effective implementation of standardization activities as existing clinical tests need to be readjusted. Furthermore, it hampers the broad use of clinical decision points mentioned in clinical practice guidelines. This situation may explain in part the current limited use of apolipoprotein measurements in patient care and public health. With apolipoproteins being more recently recognized broadly to be beneficial in CVD risk assessment and treatment, standardization activities are less advanced compared to those available for blood lipids. As mentioned, the situation was further complicated with the loss of the initial reference systems for certain lipoproteins.

In general, Lp(a), apoB, and apoA-I are complex proteins with different isoforms and post-translational modifications [120]; however, they can be more easily quantified compared to HDL-C and LDL-C. Different methodologies are currently available to quantify these proteins by targeting well-defined segments/epitopes of the protein, and when appropriately designed with post-translational modifications and isoforms in mind, can accurately quantify the protein. For example, immunoassays currently utilize antibodies that recognize specific amino acid segments, or epitopes, that are specific to a certain apolipoprotein, while mass spectrometry (MS)-based methods currently measure peptides specific to a certain apolipoprotein protein after proteolytic digest [120–122]. Because the amino acid sequences and primary characteristics of these proteins are generally known, higher order reference materials for these proteins or relevant protein segments/epitopes can be produced. These reference materials can be used for calibration to represent the specific protein, including all known modifications and isoforms, when designed and produced accordingly.

5.1. Standardization of Lipoprotein(a)

Lp(a) is a complex protein that consists of an apoB-containing LDL-like particle that is covalently linked to a defining apolipoprotein(a) [apo(a)] protein [48]. The apo(a) protein

is comprised of a plasminogen-like domain, 1 kringle five (KV) domain, and 10 kringle four (KIV) domains known as KIV1 – KIV10. Analyte heterogeneity stems from variations in apo(a) isoform sizes, which are genetically determined by the number of KIV2 repeat sequences present (ranges from 1 to 40 KIV2). Most individuals in the population are heterozygotes and have two different apo(a) isoforms in circulation, of which the smaller isoform is typically present at higher abundances, as reviewed previously [123,124].

Current Lp(a) methods utilize assay calibrators that are traceable to the WHO/IFCC reference material, known as SRM-2B,[80,125,126] with reference values assigned in nmol/L by an ELISA-based, KIV2 independent reference method [81]. However, despite previous efforts to standardize Lp(a) clinical assays to SRM-2B, high inter-assay measurement variability persists [84] and the sources of this variability were recently investigated by the CDC CVD BSP (unpublished).

As described, the Lp(a) reference system no longer exists. A new, sustainable reference system is being developed by several stakeholders with efforts coordinated by the IFCC Apolipoproteins by Mass Spectrometry Working Group [127] [IFCC website and [122,128,129]]. The new RMP for Lp(a) utilizes a MS-based, KIV2-independent reference measurement procedure (RMP) (Supplemental Figure 5S) [122]. The RMP measures specific peptides from KIV2-independent regions of the apo(a) protein after proteolytic digest. SI-traceable primary reference materials, as well as serum-based reference materials, are being established by metrological organizations [130]. For example, a commutability assessment of the serum-based candidate reference materials for Lp(a) was conducted [128]. As an interim solution, until a new reference system is in place, serum materials with values assigned by the IFCC endorsed Lp(a) RMP are available from the CDC CVD BSP and the Laboratoire national de métrologie et d'essais (LNE) in France [127]. These serum materials consist of individual donor samples and pooled sera used in the CDC's monitoring program and LNE's EQA program.

In preparation for upcoming standardization activities, the CDC CVD BSP conducted an interlaboratory comparison study to establish the degree of inter-assay variability prior to standardization. As part of this study, the potential impacts of moving from the previous ELISA-based reference system to the new mass spectrometry reference system were evaluated. The high correlation of values observed (personal communication based on unpublished data) indicates that no major changes in calibration are expected for most methods assessed. The information obtained in this study will be used to guide standardization activities coordinated by the IFCC.

Lp(a) is currently reported by commercial assays in either SI units (i.e., nmol/L) or conventional units (i.e., mg/dL). While the conversion of units is a straight-forward process for analytes like cholesterol, which has a well-defined molecular mass, this is not the case for Lp(a). Apo(a) isoform molecular weights range from ~250 – 800 kDa, making the interpretation and conversion of Lp(a) concentrations reported in conventional mass units more difficult than those reported in molar units (nmol/L), which is a unit of measure that indicates the number of particles and is not dependent on molecular weight [52]. The typical presence of two different apo(a) isoform sizes in blood further complicates

the use of mass units. Furthermore, there is no unbiased conversion factor for mg/dL to nmol/L concentrations as a direct result of variations in Lp(a) isoform sizes [131]. Interestingly, the WHO/IFCC reference material called SRM-2B, which is used by assay manufacturers to calibrate assays has reference values assigned in nmol/L, while some clinical assays calibrated with this material report results in mg/dL. As a result of an improved understanding of apo(a) isoform diversity, several guidelines and statements have recommended reporting Lp(a) concentrations in molar units, expressed as nmol/L of apo(a) [123,124,131].

5.2 ApoB and ApoA-I

Current apoB assays are calibrated to the WHO/IFCC reference material SP3–8 with values assigned by the previous immunonephelometry-based RMP in g/L. [75]. Similarly, current apoA-I assays are calibrated using WHO-IFCC international reference material SP1–01, which was also value assigned in g/L using the previous immunonephelometric RMP. With the loss of the RMPs for apoB and apoA-I, and a declining inventory of WHO-IFCC RMs, new reference systems are also being developed by the IFCC Apolipoproteins by Mass Spectrometry Working Group using the same principles and approaches as described for Lp(a).

To prepare for this change and to gauge the potential impacts of changing from the immunonephelometric RMS to the mass spectrometry-based RMS, the CDC CVD BSP used its LSP monitoring program and three samples with value assignments made by both the immunonephelometric and mass-spectrometry RMPs. Preliminary data obtained with 17 different assays operated in approximately 40 laboratories annually indicate no major impacts of switching to the IFCC MS-based RMP for apoB and apoA-I (Figures 6 and 7). A more comprehensive study to compare the previous RMS to the MS-based RMS for additional LSP samples covering a broader concentration range is currently being conducted.

Analytical performance data are only available from clinical laboratories, such as laboratories participating in the CDC LSP monitoring program (Figure 6 and Figure 7). The observed measurement median bias to the immunoassay-based RMP across samples and assays observed between 2013 and 2021 ranged between –0.7% and 2.5% (IQR 5.2% – 6.5%) for apoA-I –6.7% and –0.3% (IQR: 6.4 – 8.6%) for apoB (Supplemental Table 1S). There is a small but notable difference in measurement accuracy between nephelometric and immunoturbidimetric assays (Figure 8), which will be investigated further. Comparing IQRs of these apolipoprotein measurements with those observed for HDL-C and LDL-C indicate that apoB and apoA-I measurements are less variable, suggesting that these biomarkers are potentially less susceptible to sample specific differences.

6. Discussion and Conclusions

Over the past decades, CVD risk assessment and ASCVD treatments have changed profoundly, as reflected in an evolution of clinical guidelines and new recommendations for CVD biomarker testing. While blood lipids, namely TC, HDL-C, LDL-C, and TG remain central to CVD risk assessment and treatment, apolipoproteins such Lp(a), apoB, and apoA-I

are now included as part of the CVD testing panel to provide additional information about residual CVD risk and/or to overcome limitations of traditional blood lipid measurements.

With treatment targets now focusing on LDL-C concentrations that are profoundly lower than those in earlier guidelines, the assessment of analytical performance of LDL-C tests is now focused on those lower concentrations. While these assessments revealed that the analytical performance of LDL-C measurements at the old treatment targets are appropriate, the analytical performance at the new treatment targets needs to be improved. Furthermore, LDL-C measurements require further improvements to provide more accurate measurement results in patients with certain conditions, such as hypertriglyceridemia.

The data collected in CDC's CVD BSP indicate that the achievable level of improvement depends, in part, on how well a biomarker can be defined and on the consistency of the measurement principles used by clinical assays and RMPs. The highest level of agreement among different assays was achieved for total cholesterol, a well-defined analyte, while the same level of agreement may not be technically feasible with less well-defined biomarkers, such as LDL-C, especially since clinical tests for LDL-C use different, proprietary techniques to emulate ultra-centrifugation. Traditionally, cholesterol in LDL particles is assumed to reflect the number of LDL particles. With a better understanding of the composition of lipoprotein particles and their role in the atherosclerotic process, measurements of apoB concentrations are now considered a more accurate parameter for the number of atherogenic particles present in blood. One of technical advantage of apoB assays is that they appear less affected by triglycerides and other comorbidities than LDL-C. This may explain why the described IQR for apoB are smaller than for LDL-C. Despite these limitations, LDL-C remains a primary biomarker used for CVD risk assessment; thus, further standardization activities for LDL-C are focused on improving analytical performance at LDL-C levels targeted in current clinical treatment guidelines, while maintaining the level of accuracy achieved at the higher levels.

Lp(a) is a biomarker that captures residual CVD risk not captured by traditional blood lipids measurements. While guideline recommendations differ with respect to Lp(a) clinical decision cut-off concentrations, units and frequency of measurement, and patient populations for which measurements are recommended, the overarching consensus is that Lp(a) should be measured. This is important given that recent studies estimate that over 1.4 billion people worldwide have elevated levels of Lp(a), which represents ~20% of the world's population [50]. Indeed, a recent multicenter, cross-sectional, epidemiological study demonstrated that >25% of established ASCVD patients from 48 countries had Lp(a) levels exceeding the aforementioned clinical decision thresholds for increased CVD risk [132,133]. With clinical decision threshold being defined, the assays need to be accurate and comparable to consistently use these decision thresholds. Despite previous standardization efforts, Lp(a) assays are still not sufficiently accurate and comparable. The previous reference system, which is no longer available, is being replaced by a new, more sustainable SI-traceable reference system. Standardization activities for Lp(a) will continue as a collaborative effort among stakeholders.

Fundamental to the efficient and reliable use of traditional and new CVD biomarker measurements in patient care, clinical research, and public health is the consistent accuracy and comparability of biomarker test results across measurement systems, time, and location. The CDC CVD BSP has successfully performed these activities for traditional CVD biomarkers for decades. As a result, total cholesterol measurements, standardized since the early 1960s, are highly accurate globally. The current lack of reference systems for apolipoproteins that initially were maintained by a single laboratory highlight the importance of maintaining networks of reference laboratories to ensure sustainability of reference systems once established. Thus, the success of standardization of traditional blood lipids over decades, which is mainly attributed to the work conducted by CRMLN, will provide a template for maintaining a sustainable system for apolipoproteins. As a first step towards this goal, CDC CVD BSP is collaborating with stakeholders to establish a new, sustainable mass spectrometry reference system for apoB, apoA-I, and Lp(a). With formal, more sustainable standardization programs for apolipoproteins being available soon, more data will be generated to further solidify their role in CVD risk assessment.

The success of previous clinical practice guidelines can, in part, be attributed to successful coordination of guideline implementation and related laboratory standardization activities. Building on these experience, CDC CVD BSP will collaborate with stakeholders to continue facilitating successful implementation of new guidelines.

7.0 Outlook

Mass spectrometry will become the preferred technology for RMPs. This technology can provide highly selective measurements and is easily sustainable as it does not depend on proprietary antibodies. This is reflected in new RMPs for apolipoproteins being mass spectrometry-based. Also, activities are in preparation to change from the traditional Abel-Kendall RMP for cholesterol to mass spectrometry-based RMPs. CDC CVD BSP is supporting these activities by including these mass spectrometry-based RMPs in CRMLN. Both RMPs for TC have different analytical selectivity and therefore measurement results obtained with both methods on the same samples are not interchangeable [134]. Given that all clinical decision points and patient data are traceable to the Abel-Kendall RMP, additional assessments are warranted to assess the impact of such a change on clinical decision making and public health investigations such as assessments of prevalence of high TC or calculated LDL-C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations:

ACC	American College of Cardiology
AHA	American Heart Association
apo(a)	apolipoprotein (a)
apoA-I	apolipoprotein A-I
apoB	apolipoprotein B
ASCVD	atherosclerotic cardiovascular disease
ATP	Adult Treatment Panel
CDC	Centers for Disease Control and Prevention
CAD	coronary artery disease
CRMLN	Cholesterol Reference Method Laboratory Network
CSP	Clinical Standardization Programs
CVD	cardiovascular disease
EAS	European Atherosclerosis Society
EQ/PT	external quality assurance/proficiency testing
ESC	European Society of Cardiology
HDL-C	high-density lipoprotein cholesterol
hsCRP	high sensitivity C-reactive protein
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IQR	interquartile range
LDL-C	low-density lipoprotein cholesterol
Lp(a)	lipoprotein (a)
LSP	Lipid Standardization Program
NCEP	National Cholesterol Education Program
NIH	National Institutes of Health
NIST	National Institute of Standards and Technology
RM	reference material
RMP	reference measurement procedure

SCORE	Systematic Coronary Risk Evaluation
TC	total cholesterol
TG	triglycerides

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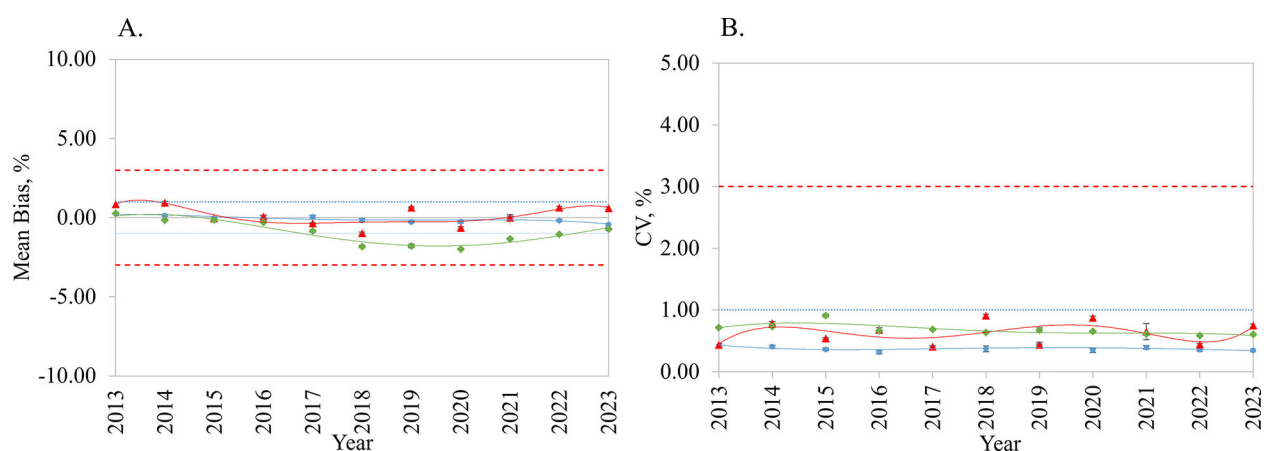


Figure 1:

Analytical accuracy and precision of total cholesterol measurements by years. Figure A: mean percent bias (Standard Error) between the CDC lab and all CRMLN members (circles), CRMLN members and all manufacturers (triangles) and CDC Labs and all LSP participants (diamonds). Dotted line represents analytical performance limits for CRMLN members, dashed line represents analytical performance limits for clinical assays. The mean bias provides information about calibration accuracy. Figure B: mean percent coefficient of variation (CV) observed across CRMLN members (circles), manufacturers (triangles) and LSP participants (diamonds). The mean CV provides information about the overall precision of the analytical methods. Each symbol in both figures represents on average 250, 2500, and 5500 data points for CRMLN, manufacturers and LSP participants, respectively.

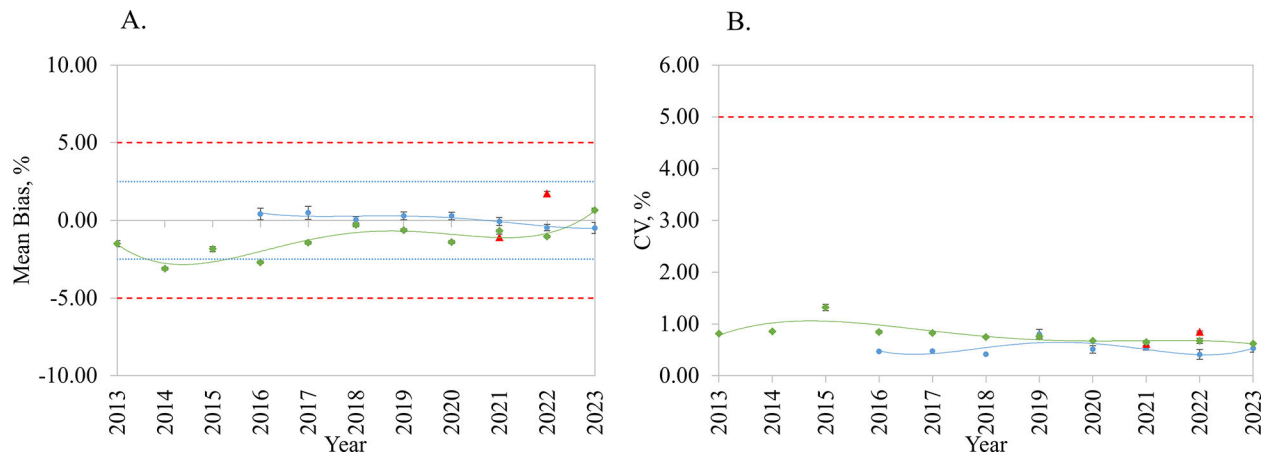
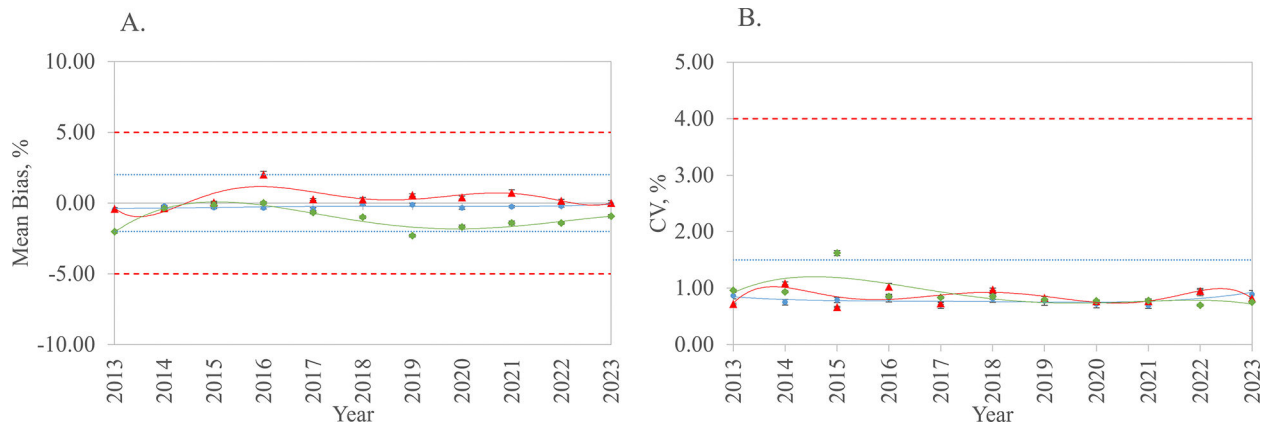


Figure 2:

Analytical accuracy and precision of total glycerides measurements by years. Figure A: mean percent bias (Standard Error) between the CDC lab and all CRMLN members (circles), CRMLN members and all manufacturers (triangles) and CDC Labs and all LSP participants (diamonds). Dotted line represents analytical performance limits for CRMLN members, dashed line represents analytical performance limits for clinical assays. The mean bias provides information about calibration accuracy. Figure B: mean percent coefficient of variation (CV) observed across CRMLN members (circles), manufacturers (triangles) and LSP participants (diamonds). The mean CV provides information about the overall precision of the analytical methods. Each symbol in both figures represents on average 80, 1300, and 5500 data points for CRMLN, manufacturers and LSP participants, respectively.

**Figure 3:**

Analytical accuracy and precision of HDL-C measurements by years. Figure A: mean percent bias (Standard Error) between the CDC lab and all CRMLN members (circles), CRMLN members and all manufacturers (triangles) and CDC Labs and all LSP participants (diamonds). Dotted line represents analytical performance limits for CRMLN members, dashed line represents analytical performance limits for clinical assays. The mean bias provides information about calibration accuracy. Figure B: mean percent coefficient of variation (CV) observed across CRMLN members (circles), manufacturers (triangles) and LSP participants (diamonds). The mean CV provides information about the overall precision of the analytical methods. Each symbol in both figures represents in average 590, 4000, and 5600 data points for CRMLN, manufacturers and LSP participants, respectively.

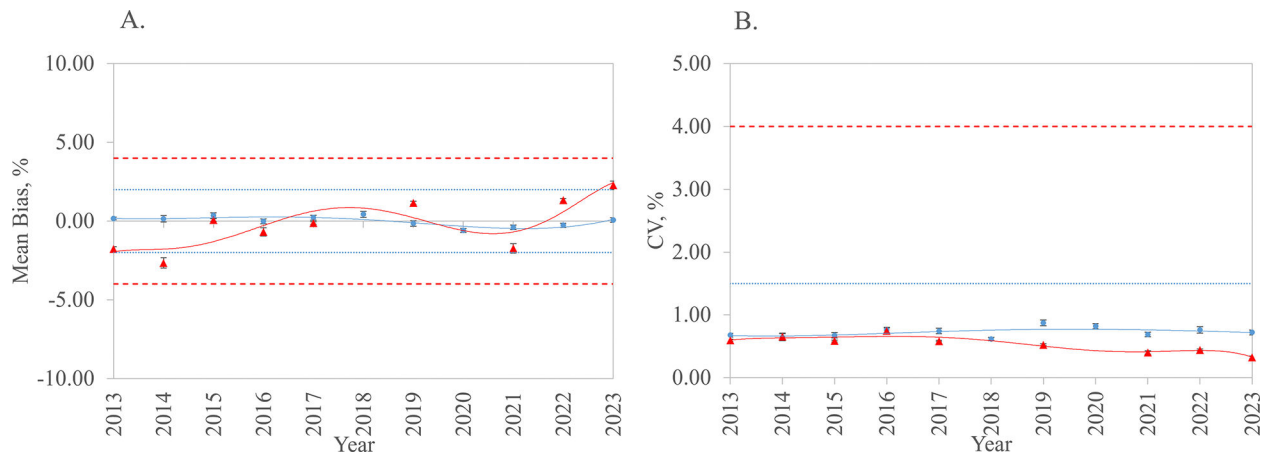


Figure 4:

Analytical accuracy and precision of LDL-C measurements by years. Figure A: mean percent bias (Standard Error) between the CDC lab and all CRMLN members (circles), CRMLN members and all manufacturers (triangles) and CDC Labs. Dotted line represents analytical performance limits for CRMLN members, dashed line represents analytical performance limits for clinical assays. The mean bias provides information about calibration accuracy. Figure B: mean percent coefficient of variation (CV) observed across CRMLN members (circles) and manufacturers (triangles). The mean CV provides information about the overall precision of the analytical methods. Each symbol in both figures represents on average 700 and 1600 data points for CRMLN and manufacturers, respectively.

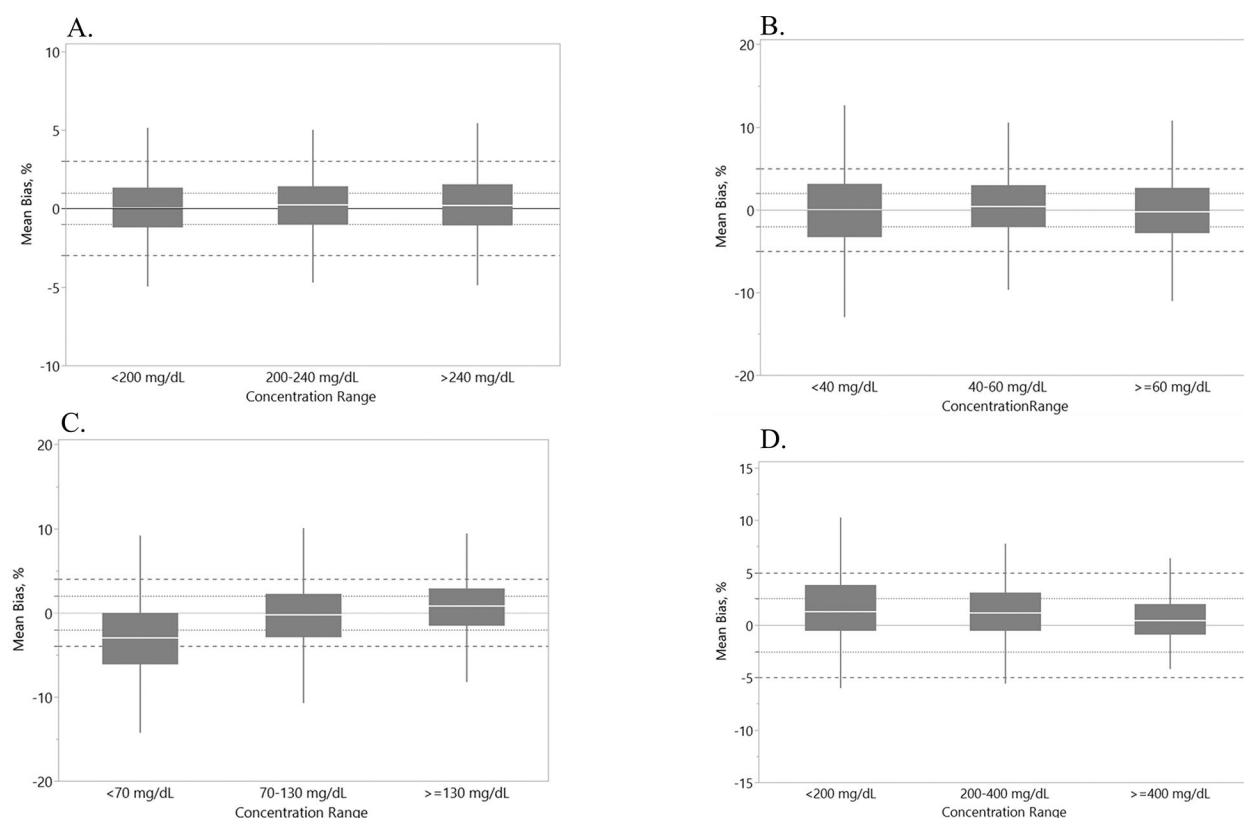


Figure 5:

The bias distributions observed across all samples and manufacturers participating in CDC's CRMLN between 2013 and 2023. Figure A: The bias distributions for total cholesterol (TC) manufacturers in 2013 to 2023; Figure B: The bias distributions for HDL-C manufacturers in 2013 to 2023; Figure C: The bias distributions for LDL-C manufacturers in 2012 to 2023; Figure 5D: The bias distributions for TG manufacturers in 2021 to 2023. Dashed line represents performance criteria for manufacturers, dotted line represents performance for corresponding RMPs.

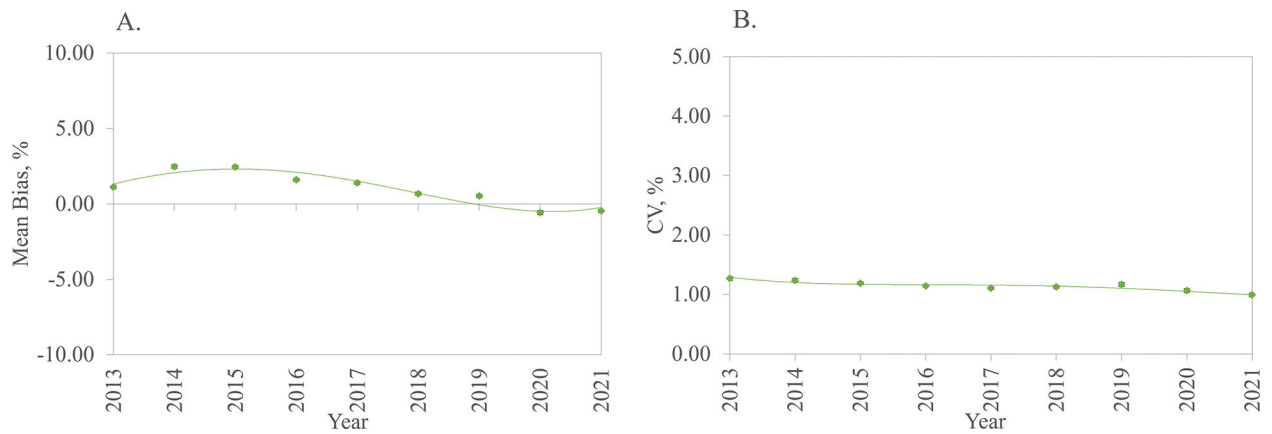


Figure 6:

Analytical accuracy and precision of apoA-I measurements by years. Figure A: mean percent bias (standard error) between RMP and all LSP participants. The mean bias provides information about calibration accuracy. Figure B: mean percent coefficient of variation (CV) observed across LSP participants. The mean CV provides information about the overall precision of the analytical methods. Each symbol in both figures represents on average 4000 data points for LSP participants.

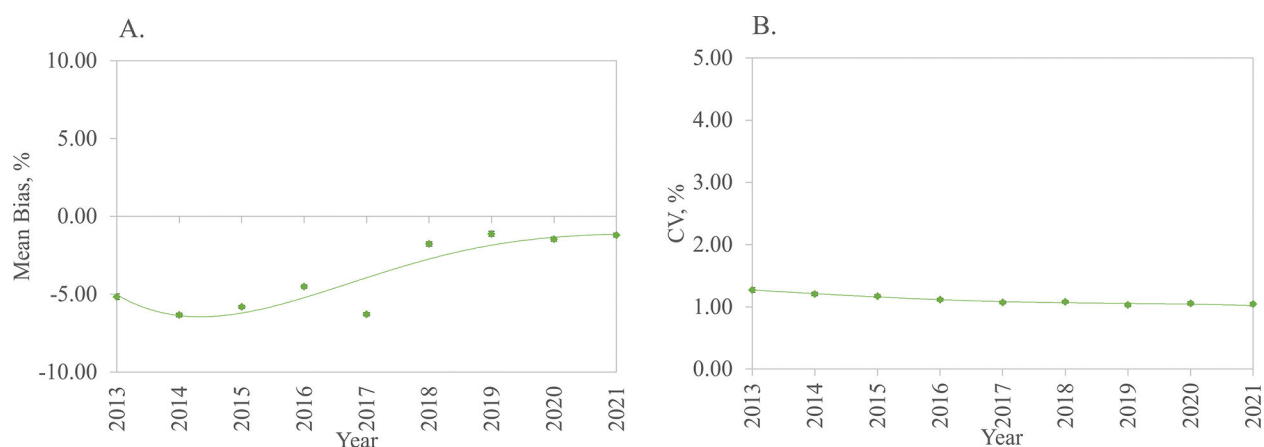


Figure 7: Analytical accuracy and precision of apoB measurements by years. Figure A: mean percent bias (Standard Error) between RMP and all LSP participants. The mean bias provides information about calibration accuracy. Figure B: mean percent coefficient of variation (CV) observed across LSP participants. The mean CV provides information about the overall precision of the analytical methods. Each symbol in both figures represents on average 4000 data points for LSP participants.

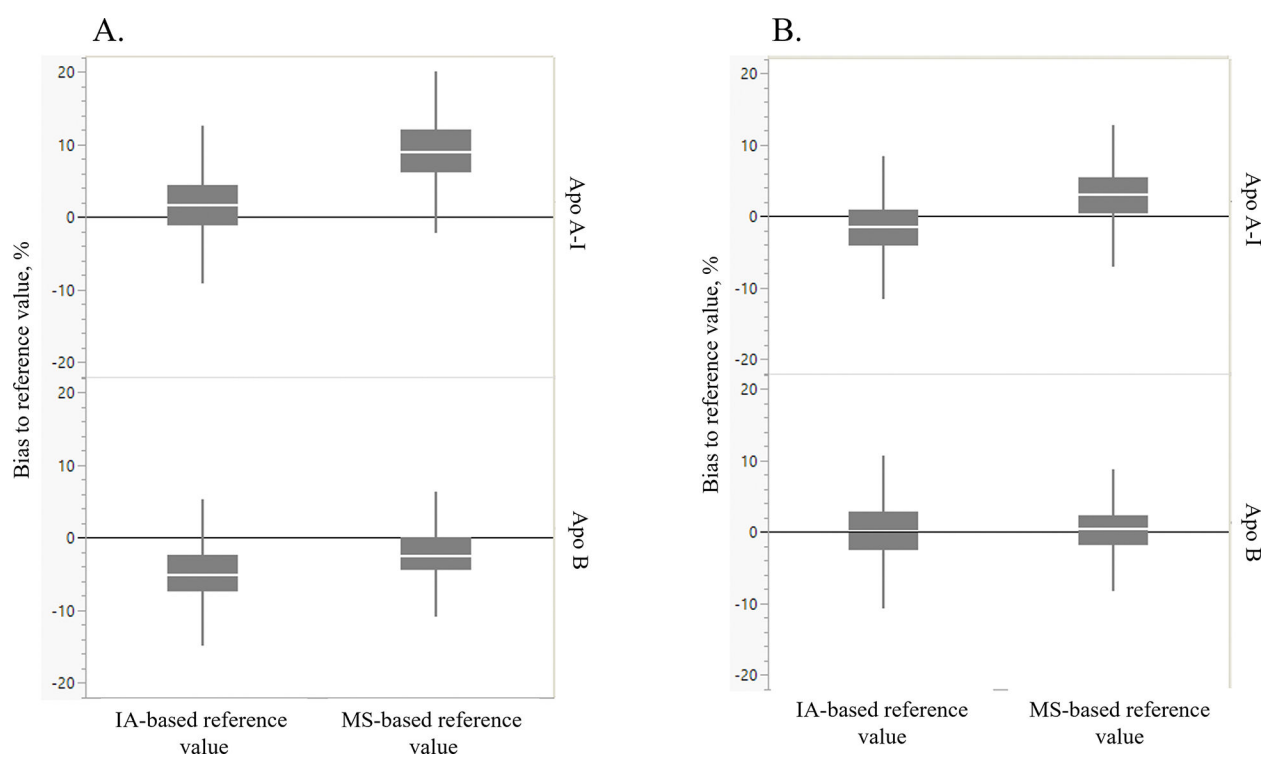


Figure 8:
Bias distribution of apoA-I and apo B assays enrolled in LSP between 2020–2023 compared to IA-based and MS-based reference values. A: Nephelometric assays. B: Immunoturbidimetric assays. Note: LSP used IA-based reference values before Q2 2022 and MS-based reference value starting in Q2 2022 were used.

Table 1:

Analytical performance requirements for RMPs and clinical assays for total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) used in the CDC CVD Biomarkers Standardization Program. Accuracy is expressed as difference to the CDC RMP in percent (bias) or absolute value. Precision is expressed as percent coefficient of variation or standard deviation. Total error (TE) calculated using the bias of a single measurement to the CDC RMP and the precision of the measurement expressed as percent coefficient of variation using a formula described previously [135].

Analyte	Level	Accuracy (bias)	Precision (%CV/SD)	Additional Requirements
TC	RMP	±1%	≤1%	
	Clinical Assay	±3%	≤3%	
TG	RMP	± 2.55 % per sample ± 2.00% per survey	- -	Range of sample bias ≤ 3.95 % Range of individual bias ≤ 6.96 %
	Clinical Assay	±5%	≤5%	TE ±13.3% per measurement
HDL-C	RMP	±1 mg/dL	≤1 SD	
	Clinical Assay	±5%	≤4%	
LDL-C	RMP	±2%	≤1.5%	
	Clinical Assay	±4%	≤4%	