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Special Report

Towards an SI-Traceable Reference Measurement System for Seven Serum Apolipoproteins Using Bottom-Up Quantitative Proteomics: Conceptual Approach Enabled by Cross-Disciplinary/Cross-Sector Collaboration

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ABSTRACT: Current dyslipidemia management in patients with atherosclerotic cardiovascular disease (ASCVD) is based on traditional serum lipids. Yet, there is some indication from basic research that serum apolipoproteins A-I, (a), B, C-I, C-II, C-III, and E may give better pathophysiological insight into the root causes of dyslipidemia. To facilitate the future adoption of clinical serum apolipoprotein (apo) profiling for precision medicine, strategies for accurate testing should be developed in advance.

Recent discoveries in basic science and translational medicine set the stage for the IFCC Working Group on Apolipoproteins by Mass Spectrometry. Main drivers were the convergence of unmet clinical needs in cardiovascular disease (CVD) patients with enabling technology and metrology. First, the residual cardiovascular risk after accounting for established risk factors demonstrates that the current lipid panel is too limited to capture the full complexity of lipid metabolism in patients. Second, there is a need for accurate test results in highly polymorphic and atherogenic apolipoproteins such as apo(a). Third, sufficient robustness of mass spectrometry technology allows reproducible protein quantification at the molecular level. Fourth, several calibration hierarchies in the revised ISO 17511:2020 guideline facilitate metrological traceability of test results, the highest achievable standard being traceability to SI.

This article outlines the conceptual approach aimed at achieving a novel, multiplexed Reference Measurement System (RMS) for seven apolipoproteins based on isotope dilution mass spectrometry and peptide-based calibration. This RMS should enable standardization of existing and emerging apolipoprotein assays to SI, within allowable limits of measurement uncertainty, through a sustainable network of Reference Laboratories.

Introduction

TRADITIONAL DIAGNOSIS OF DYSLIPIDEMIA IN CARDIOVASCULAR DISEASE

After many years, the evaluation of atherosclerotic cardiovascular disease (ASCVD) risk still centers around the procedurally defined classification based on density

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of lipoprotein particles [High Density Lipoproteins (HDL), Low Density Lipoproteins (LDL), Very Low Density Lipoproteins (VLDL), chylomicrons (CM)] and their quantification based on cholesterol and triglyceride content (1). The traditional lipid profile [HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), total cholesterol, and total triglycerides] allows effective identification of individuals who are at high risk for ASCVD due to familial hypercholesterolemia (2, 3). However, even in the age of intensive lipid lowering therapies, there is still a high proportion of patients who die prematurely or experience a major cardiovascular event while on lipid lowering therapy. In elderly populations with several comorbidities (i.e., obesity, diabetes, or chronic kidney disease), the traditional lipid profile is a weak predictor of ASCVD-related mortality (4). Meanwhile, basic research has greatly expanded our understanding of human lipid metabolism, which has been accelerated by the use of modern gene editing and omics technologies (5, 6). In clinical care, these advances in basic research and new drug developments will reach our patients only if our clinical laboratory measurements evolve accordingly. The traditional cholesterol-centered approach has placed a high bar for any newly emerging cardiovascular biomarker to overcome. This is due to the specificity, accuracy, precision, interlaboratory reproducibility, commutability, feasibility for automation, point of care use, and cost of analysis of the cholesterol assay. As a result, new assay technologies and tests will be adopted more swiftly if they both offer solutions for unmet clinical needs in existing care pathways and if tests results are anchored to higher order reference materials and methods in a sustainable way.

DIAGNOSTIC SIGNIFICANCE OF AN APOLIPOPROTEIN (APO) PANEL FOR ASCVD: A *COMING-OF-AGE* STORY

Apolipoproteins (apos) A-I and B provide scaffolding support for lipoproteins and are essential for the biogenesis, routing, and metabolism of HDL and non-HDL particles circulating in blood. Serum apoA-I and B measurements allow more direct quantification of HDL and LDL particle numbers, respectively (7). However, apoA-I and B themselves do not provide information on specific irregularities of HDL and non-HDL functions. Efforts to assess functional characteristics of lipoproteins based on size, density, charge of subfractions, or other functional considerations (e.g., cholesterol efflux capacity) have not achieved an acceptable level of interlaboratory comparability, due to lack of clear analytical definition of subclasses across different analytical platforms (8). A more feasible way to assess functional characteristics of lipoproteins (without subfractionation) is the measurement of other apolipoproteins along with apoA-I and B. Exchangeable apolipoproteins, such as apolipoproteins (apos) C-I, C-II, and C-III and E bind to apoA-I and B-containing lipoproteins and regulate the concerted action of lipase enzymes, transfer proteins, and receptors (9). From genetic studies, especially Mendelian randomization studies, there is now ample evidence that apos are risk markers in cardiovascular disease. Specifically, evidence has been gathered for apos AI, (a), B, C-I, C-II, CIII, and E (10). ApoC-III and E are now monitored in numerous drug trials and proof of concept intervention studies (11–15). However, the lack of standardized analytical measurements prevents the compilation of data from different laboratories into large scale epidemiologic studies, which will be necessary for these newer apolipoprotein biomarkers to be accepted as risk markers.

Currently there is heightened interest in Lp(a) research and its clinical application, as it is now considered a strong genetically determined risk factor for cardiovascular disease (CVD) (16). The lipoprotein(a) particle is composed of an LDL particle with an additional apolipoprotein associated with it, which is called apolipoprotein(a) (apo(a)) and shows a high structural homology with plasminogen. While Lp(a) is known to be synthesized in the liver, the site and mechanism of catabolism is more controversial. After more than 50 years of research, the physiological function of Lp(a) is still unclear (17). One astonishing characteristic of Lp(a) is the more than 1000-fold range of concentrations between individuals, from almost zero to more than 3000 mg/L (16). In addition, the distribution of Lp(a) is almost Gaussian in Blacks but skewed in Caucasians; roughly 50% of Caucasians have concentrations below 100 mg/L and about 25% have concentrations above 300 mg/L.

Lp(a) concentrations are not significantly influenced by age, sex, fasting state, inflammation (18, 19), or lifestyle factors, such as diet and physical activity. However, the concentrations are under strict genetic control. Family studies have revealed a heritability estimate of Lp(a) concentrations of about 90%. The discovery of the size polymorphism of apo(a) in serum, which is based on a variable number of so-called kringle IV type 2 (K-IV_2) repeats in the *LPA* gene, resulted in the identification of the *LPA* gene as the major gene predicting Lp(a) concentrations. Each gene has a few to more than 40 repeats, with each repeat stretching 5.6 kB. This results in a highly polymorphic and informative copy number variation.

Interestingly, there is a pronounced inverse correlation between the number of K-IV_2 repeats and the serum/plasma concentration of Lp(a). Individuals expressing a low number of K-IV_2 repeats resulting in small apo(a) isoforms (up to 22 K-IV_2 repeats) have on average markedly higher serum Lp(a) concentrations, and thereby a higher CVD risk, compared with

individuals carrying only large apo(a) isoforms (more than 22 K-IV_2 repeats) (16). This K-IV_2 size polymorphism of apo(a) explains about 20%–80% of the variability of serum Lp(a) concentrations, depending on ethnicity. However, recent technological advancements have revealed a large number of genetic variants within the K-IV_2 repeats. Some of them can have a pronounced effect on Lp(a) concentrations resulting (e.g., in low Lp(a) concentrations even in a person carrying a small apo(a) isoform) (20, 21). Many Mendelian randomization studies provided strong support for an association between high Lp(a) concentrations and CVD risk (16).

The variable number of identical K-IV_2 repeats interferes with the accurate measurement of Lp(a) in serum and plasma (16), with lower Lp(a) concentrations being overestimated and higher concentrations being underestimated by K-IV_2 dependent tests, and masked its clinical utility for nearly 2 decades. Commercial IVD tests and technologies should be improved and properly standardized so that they are no longer prone to confounding by the apo(a) size polymorphism in native specimens, calibrators and reference materials.

ADOPTION OF SERUM/PLASMA APOLIPOPROTEINS IN CLINICAL PRACTICE

For primary prevention, the measurement of serum apoB and Lp(a) has been proposed to overcome the limitations of traditional lipid biomarkers and to better identify individuals at risk (22). In addition, proteomics using Multiple Reaction Monitoring (MRM)-MS platforms, which allows for simultaneous quantitation of multiple proteins, was selected by *Nature Methods* as the technology of the year 2012. Applied to epidemiological cohorts, the technique has demonstrated the association of serum apolipoprotein profiles with incident CVD in ongoing prospective community-based surveys such as the Bruneck or the EPIC-Norfolk study (23, 24). This parallels current efforts to obtain more detailed information from individual patient samples that could help us provide more customized diagnoses and treatments to our patients (*precision medicine*). Current technologies (next generation protein diagnostics with, for example, MRM-MS) and a more detailed understanding of the causes of dyslipidemia, allow for precision medicine to be applied, which was not possible a few years ago (10).

Consistent, effective, and sustainable diagnosis and treatment can only be achieved if lab tests measure well-defined measurands and have well-known trueness and uncertainty within allowable limits that match their clinical use. To that end, establishing metrological traceability is imperative (25). Metrological traceability requires the establishment of an advanced Reference Measurement System that accommodates evolution of our understanding of basic science as well as advances in the science of measurement, which will facilitate personalized patient management. It is also essential to make this Reference System robust, reproducible, and sustainable in a network of calibration labs across the globe.

PAVING THE WAY TO AN SI-TRACEABLE REFERENCE MEASUREMENT SYSTEM FOR MULTIPLE CLINICALLY RELEVANT APOLIPOPROTEINS

Metrological traceability of serum/plasma apolipoproteins: status update. It is well-known that metrological traceability of test results is essential for anchoring test results to internationally recognized, higher order materials, and methods in line with ISO 17511:2020 calibration hierarchies (see Supplemental Document for links to web resources). In addition, the measurement uncertainty (MU) on the reported test results should remain within acceptable limits to ensure that the test is *fit-for-clinical purpose*. To accomplish this, IFCC-WHO standardization efforts were initiated in the 1990s for serum/plasma apoA-I and B (26, 27) and Lp(a) (28). The contemporary metrological traceability chains for these 3 analytes are shown in Fig. 1 (left) and demonstrate traceability of test results to WHO-IFCC secondary reference materials, coded by WHO and IFCC as "SP1-01", "SP3-07" and "SRM2B".

For apoA-I, SP1-01 was prepared by lyophilizing a human serum pool to ensure long-term stability. The material was value assigned using a highly standardized radioimmunoassay, calibrated with purified apoA-I, for which the mass value had been determined by amino acid analysis. Using SP1-01, with an apoA-I concentration of 1.50 g/L as a common calibrator, inter-assay variation could be reduced from 9% to 5.4%, showing the success of this strategy (26).

For apoB, SP3-07 was prepared as a shock-frozen liquid preparation, because lyophilization was known to affect apoB structure (29). An apoB value of 1.22 g/L was assigned to SP3-07, using a nephelometric method that was calibrated with freshly isolated low-density lipoprotein for which the apoB mass value was determined by a standardized sodium dodecyl sulfate-Lowry procedure. Using SP3-07 as a common calibrator, the inter-assay variation of commercial tests could be reduced from 20% to 7% (27).

For Lp(a) standardization, SRM2B, a lyophilized human serum pool with preservatives, was selected from 4 manufactured Lp(a) materials as the material providing best harmonization results in 27 Lp(a) tests. Lp(a) isoforms consisted of 3 major apo(a) polymorphs of nearly equal gel intensity, containing 16, 17, and 18 K-IV_2 domains, and 3 minor polymorphs of 14, 20, and 32 K-IV_2 domains, respectively (28). Value assignment of SRM2B was performed through ELISA

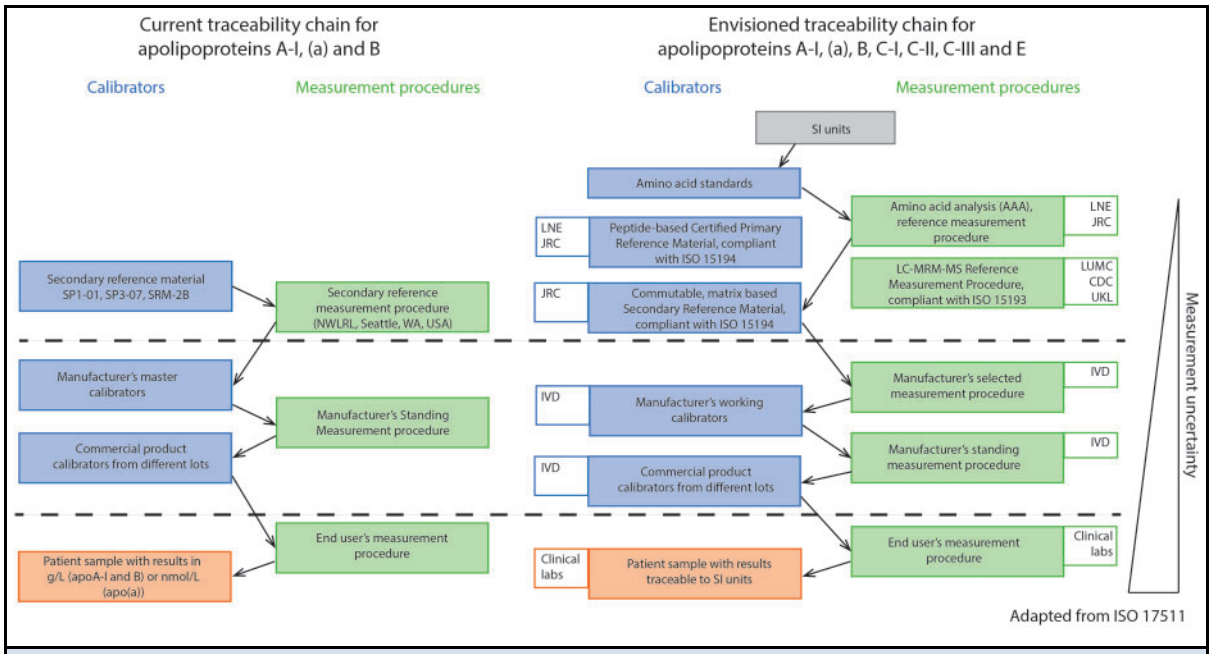

Fig. 1. Metrological traceability chains are presented for contemporary and emerging serum apolipoproteins consisting of an unbroken sequence of calibrators and measurement procedures that are used to relate a measurement result to a reference of higher order. Serum apos A-I, B, and (a)/Lp(a) test results are currently traceable to WHO-IFCC secondary reference materials (left), whereas envisioned traceability to SI for serum apos A-I, B, Cs, E, and (a) is presented on the right. JRC, Joint Research Centre, Geel, Belgium; LNE, Laboratoire National de Métrologie et d'Essais, Paris, France; IVD, In-Vitro Diagnostic; SI, Système Internationale.

measurements that were calibrated against 2 individually freshly isolated Lp(a) preparations from a donor exhibiting a single apo(a) isoform. The absolute mass of the isolates was determined using amino acid analysis, and 2 different K-IV$_2$ number-independent ELISAs were used for value assignment of SRM2B (30). However, when SRM2B was used to achieve uniformity of calibration, inter-laboratory CVs of up to 31% were still observed during the measurement of 30 fresh frozen serum samples with 22 assays (31). A significant portion of the inter-laboratory variance might lie in the variability of assay reactivity toward lipoprotein(a) particles with different numbers of apo(a) kringle domains, but another portion likely stems from assay nonlinearity and variable end-user adherence to the standard operating procedure (e.g., for predilutions to ensure that measurements are done in the optimal linear range). The need for next generation protein tests that measure apo(a) in a kringle-independent manner is obvious.

An important limitation of the WHO-IFCC materials discussed above is their unknown commutability. Also, some of these materials are no longer available or their general availability to the lab community is very limited. Existing stocks of material are running low, which creates the need for new materials. Further, new reference materials now need to meet current ISO requirements that were not in place at the time these other materials were created (coded. SP1-01, SP3-07, and SRM2B). For apolipoproteins C-I, C-II, C-III, and E standardization, no secondary WHO-IFCC reference materials exist at this moment.

Evolution in metrology: striving for SI-traceability of serum/plasma apolipoprotein test results. The science of measurement has evolved. To that end, ISO Technical Committee 212 working group 2 developed several international ISO standards, among them ISO 15193 (with requirements for reference measurement procedures), ISO 15194 (with requirements for reference materials), ISO 15195 and 17025 (describing competences of calibration laboratories), and ISO 17511:2020 (with requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples). All ISO standards contribute to a global infrastructure and guidance for standardization of medical tests. The primary goal is to accomplish metrological traceability of test results across the globe. Metrological traceability is defined as the property of a measurement result whereby the result can be related to a reference through a documented

unbroken chain of calibrations, each contributing to measurement uncertainty. Measurement traceability is important because it assures that medical test results are clinically effective and safe by agreeing with national or international standards within the statement of uncertainty in measurement. Without traceability, a laboratory can claim anything they want in a test or calibration report. To implement the metrological traceability concept, calibration hierarchies are essential. The most complete calibration hierarchy is the one that allows the community to trace test results back to a well-defined system of units, based on the International System of Quantities (SI), as adopted by the General Conference on Weights and Measures (CGPM) [VIM 3, JCGM 200:2012].

The reference materials developed for apoA-I, apoB, and Lp(a), as well as the reference measurement procedures, were *state-of-the-art* in the 1990s. Today, it is clear that for some medical tests, methods such as liquid chromatography coupled to liquid chromatography (LC)-MRM-MS, with its higher selectivity, have become promising alternatives for radioimmunoassays and ELISAs. As the current Reference Measurement Systems for apoA-I, apoB, and Lp(a) only allow traceability to secondary WHO-IFCC reference materials, there is a need to reconsider the existing Reference Measurement Systems (RMS) and to achieve SI-traceability in a sustainable manner. By evolving into Next Generation Protein measurement technology, RMS should improve and allow molecular characterization of the measurands (apos) of interest.

An IFCC working group on quantitating Apolipoproteins by Mass Spectrometry was formed in 2017 to establish such a mass spectrometry-based RMS. The objectives are 2-fold: To develop an RMS for a panel of clinically relevant serum apos A-I, (a), B, C-I, C-II, C-III, and E (including qualitative phenotyping where needed). Measurement results should be traceable to SI as outlined in ISO 17511:2020, and the envisioned calibration hierarchy in Fig. 1 (right). Other traceability chains will be used in cases where traceability to SI cannot be achieved. A second objective will be to evaluate clinical performance and clinical utility of serum apolipoprotein panel(s) for CVD risk stratification and treatment, in comparison to or together with contemporary blood lipids once the RMS is in place. The working group is cross-disciplinary and includes representation from metrology institutes and the Joint Research Centre (JRC, former IRMM, Geel, Belgium), candidate reference laboratories that developed LC-MRM-MS-based tests for absolute quantification of serum apolipoproteins, experts in apolipoprotein biochemistry and genetics, and IVD-representatives. The first step in the development of a reference measurement system was the establishment of a common accuracy base, with pre-agreed starting points. An overview of the common accuracy base is presented in Table 1, and the proposed strategy to achieve apolipoprotein standardization is outlined in Fig. 2.

It was agreed that an affordable and robust reference measurement system should be developed in line with technological advances and the latest ISO standards on metrological traceability and standardization. LC-MRM-MS, using isotope dilution, is currently a widely accepted methodology for standardization of protein tests in serum/plasma (Fig. 3). The technology is attractive, as it allows quantification of proteins in an antibody-independent manner, minimizes by design batch-to-batch variations (e.g., proteolytic enzymes used for digestion, LC-columns used for separation), is independent of specific suppliers, and can be reproduced by other laboratories using that technology. Moreover, isotope dilution mass spectrometry (IDMS) allows specific (molecular) characterization of the measurand(s), which currently cannot be achieved using immunoassay-based techniques. Third, the technique allows multiplexing. Particularly because the technology easily enables multiplexed quantification of apolipoproteins, IDMS was the first method of choice for establishing an SI-traceable RMS for multiple apolipoproteins.

A major challenge of quantitative proteomics using LC-MRM-MS is that proteins are enzymatically digested into their peptides, which has been termed the bottom-up proteomics strategy (Fig. 3). As the measurands are altered during the measurement process, demonstrating completeness of digestion is an essential prerequisite for accurate quantitation. From a metrological viewpoint, this methodology may be advantageous too: if the digestion of intact apolipoproteins into proteotypic peptides is sufficiently optimized to enable equimolar conversion with consistent reproducibility, a peptide-based calibration strategy is possible to accomplish SI-traceability. Main advantages of peptide-based primary reference materials as compared to recombinant protein-based reference materials are the fact that peptide-based reference materials can be obtained more readily, and their purity assessment is much easier as compared to that of recombinant apolipoproteins. Peptide-based calibration will be achievable if peptide-based primary reference materials can be developed for each apolipoprotein (32). It was therefore decided, as a starting point, that primary reference materials should ideally be synthetic peptides of high purity, allowing SI-traceability for the setup of a sustainable and global RMS for apolipoproteins. See Fig. 1.

IDMS-based protein quantification through bottom-up proteomics relies on several assumptions that should be met to take full advantage of this powerful technology and correctly implement the metrological traceability concept. First, the apolipoproteins in

Table 1. Predefined starting points for the establishment of an SI-traceable IDMS-based Reference Measurement System for multiple serum apolipoproteins[a].

Principle n	Starting points	Rational choices agreed by the IFCC Working Group members for establishing a Mass-Spectrometry (MS)-based Reference Measurement System (RMS). All definitions are explained in VIM3, JCGM 200:2012.
1	General	The RMS for serum apo standardization by MS has to be feasible and sustainable and should be set up preferentially in at least 3 calibration laboratories.
2		Metrological traceability to the highest calibration hierarchy in ISO 17511:2020 should be the aim (i.e., apolipoprotein test results should preferentially be traceable to SI). The apolipoprotein measurands have to be defined and quantitated unequivocally, notwithstanding the heterogenous nature of proteins.
3		Beyond the standardization of the analytical RMP/RMS, standardization of the Total Testing Process is needed, including the preanalytical conditions, the type of matrix (serum), the intended measurands, the units (molar), and the reference values and decision limits.
4		Terminology should be compliant with relevant ISO documents, such as ISO 17511; 15193; 15194; 15195; 17025, VIM3, and the IFCC C-NPU terminology and silver book.[b]
5	Reference Materials	The goal is to prepare primary reference materials for each individual apolipoprotein. These primary reference materials will be prepared and stored individually, to ensure stability. However, for use in the RMP, these materials will be mixed to form reference standards in serum for constructing calibration curves.
6		Peptide standards are the primary reference materials of choice, however, if that is not feasible, recombinant proteins will be considered.
7		Commutable and human serum-based reference materials are developed as secondary reference materials.
8	Reference Method	The goal is to establish one harmonized bottom-up proteomics procedure for 7 apolipoproteins by mass spectrometry across the calibration labs involved.
9		We aim to develop a multiplexed primary Reference Measurement Procedure (RMP) for detecting and/or quantifying serum apos (a), A-I, B, C-I, C-II, C-III, E, and the apoE phenotype. The analytical selectivity should be guaranteed through use of appropriate quantifying peptides.
10		The RMP should enable metrological traceability of serum apo test results to SI, within allowable measurement uncertainty. (i.e., enough uncertainty budget should be left for the manufacturers and end-users of routine apo tests). As a rule of thumb and in case that biological variation data are available, less than 50% of the allowable uncertainty budget derived from biological variation should be consumed by the candidate RMP.

[a]The starting points in this table were defined after thorough preparational discussions with the working group members and corporate members, taking into consideration the pre-agreed objectives.
[b]Web-links to the respective regulatory documents are provided in the Supplemental Information.

serum-based secondary reference materials should be present in their intact and native form to ensure accurate quantification. Second, the quantifying peptides should be specific for the endogenous apolipoproteins and selected carefully to reflect the intended measurand(s) accurately. Third, equimolar digestion from apolipoprotein into quantifying peptide should be achieved using standardized sample preparations and independent of individual patients' matrices. These aspects should all be thoroughly investigated during the development of the RMS.

STEPWISE APPROACH TO ESTABLISHING AN MS-BASED REFERENCE SYSTEM

Defining the measurands (quantities intended to be measured). The first step in the development of a measurement system is the accurate definition of the

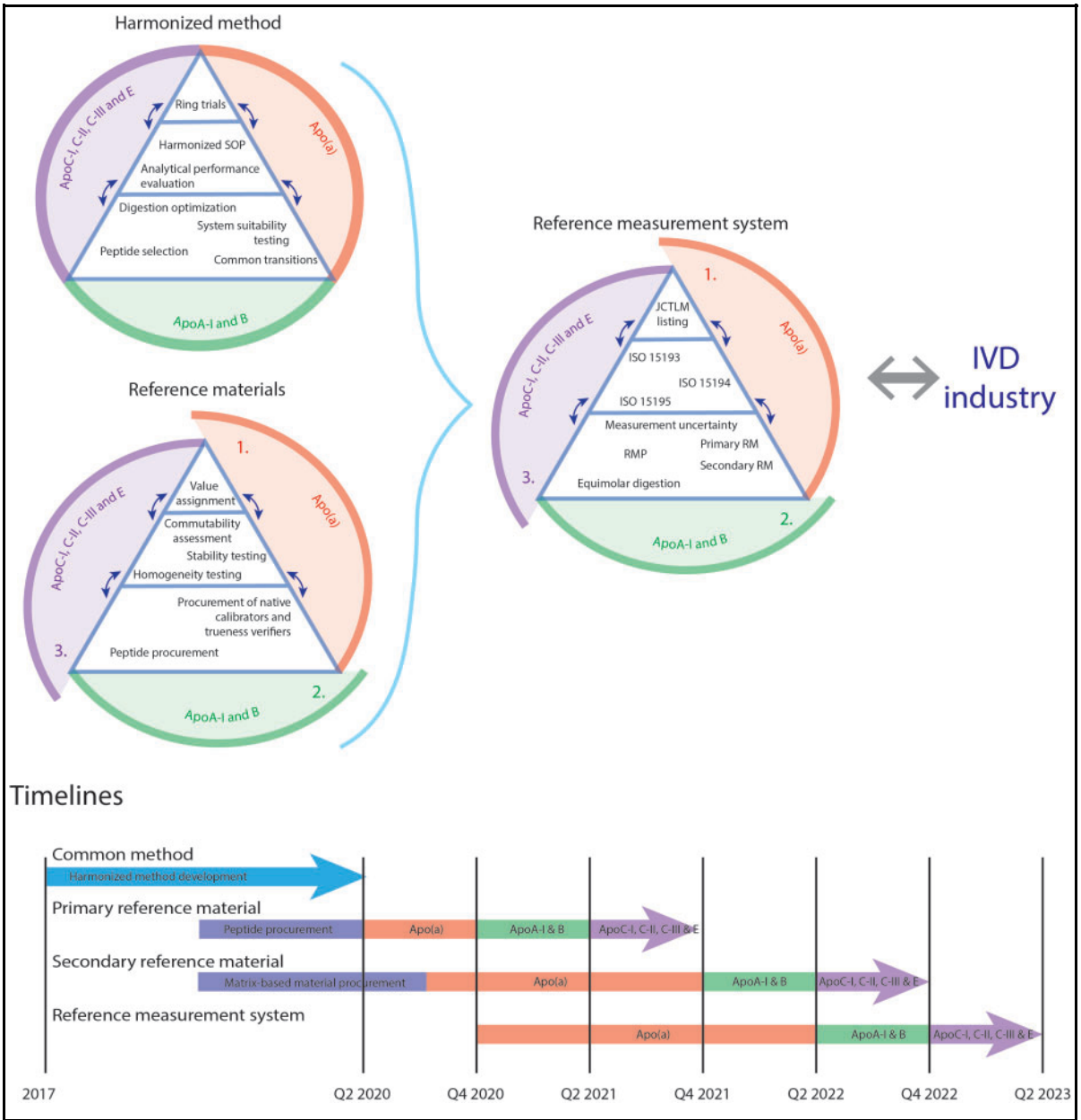

Fig. 2. Establishment of a multiplex Reference Measurement System (RMS) for 7 serum apolipoproteins, including timelines for its deliverables. The Apolipoprotein Reference Measurement System is being developed along 2 lines: the establishment of a harmonized multiplex Reference Measurement Procedure (RMP) and the development of primary respectively secondary, serum-based Reference Materials. The development of the candidate RMP is being done by the reference laboratories in University Hospital Leipzig, Leipzig, Germany; Leiden University Medical Centre, Leiden, the Netherlands and CDC, Atlanta, Georgia, USA. The development of the Reference Materials is the responsibility of the JRC from the European Commission, Geel, Belgium, and by the French metrology institute LNE, Paris, France. Both the common RMP and the uni-or multiplex RMs are needed for establishing the complete RMS. The timelines are presented below: the harmonized (note: not yet standardized!) multiplex Reference Measurement Procedure will be available at the end of 2020, whereas the development of the primary and secondary Reference Materials is sequential and ongoing. The development of apo(a) RMs has been prioritized, followed by RMs for apos A-I and B, and finally by apos C and E RMs. Primary RMs are expected to be available by the end of 2021, whereas secondary RMs are estimated to be available by the end of 2022.

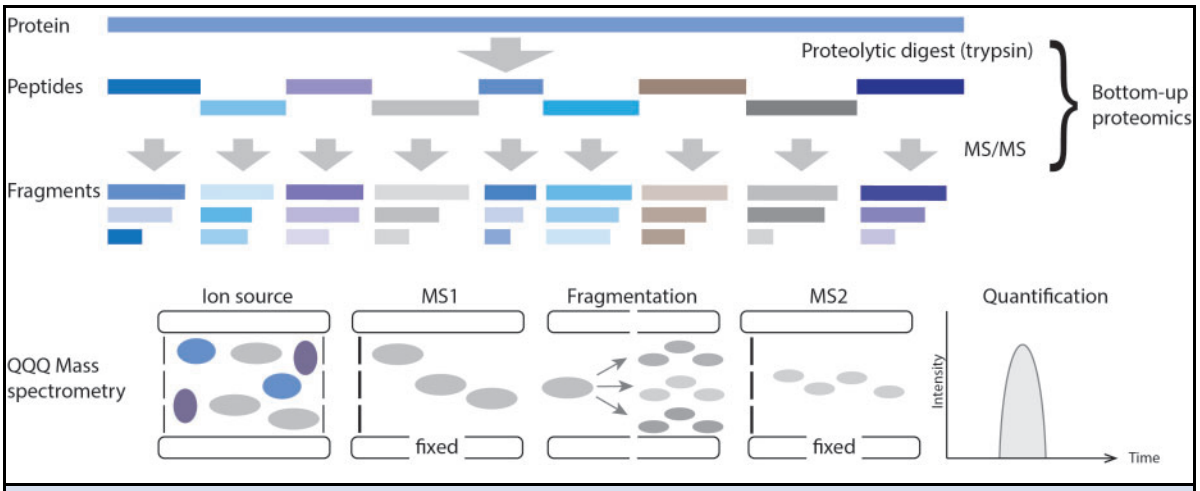

Fig. 3. Principles of Mass Spectrometry-based bottom-up proteomics for absolute quantification of serum apolipoproteins. Apolipoproteins are enzymatically digested into proteotypic peptides, which are then quantified by LC-MRM-MS. Analytical specificity is achieved through the LC separation in combination with the selection of specific peptide fragments in MS2.

measurand. Because proteins exist in multiple proteoforms, which each have a different mass, it was decided to quantify each of the proteins in molar units. Specifically for apo(a), which has a size polymorphism affecting its quantification, the peptides should be from a unique KIV_2-independent domain. For apoE, 2 clinically relevant amino acid polymorphisms are known, resulting in the genetic variants ε2, ε3, and ε4, represented by phenotypes E2, E3, and E4. Therefore, apoE phenotype identification was also deemed important.

Peptides will be selected for each measurand. However, the selection of the representative peptides is not a trivial task, as was outlined previously (33). Importantly, 2 of the 3 major assumptions for bottom-up protein quantification should be met: the proteotypic peptide should reflect the intended measurand, and digestion from protein to peptide should be equimolar. The first criterion can be met theoretically, while the second should be evaluated empirically. To ensure peptides will be selected that accurately reflect the intended measurand, the following criteria were agreed upon: the selected peptides should be unique for the target protein (proteotypic peptides) and at least 2 proteotypic peptides should be quantified per protein to ensure analytical specificity. Moreover, to avoid misrepresentation, the peptides should ideally not contain cysteine, methionine, or tryptophan residues, not be affected by (common or rare) mutations, and not carry any post-translational modifications. Exceptions to these criteria should only be allowed if no other peptides are available. For apoE phenotyping, the genetically variable peptides CLAVYQAGAR (E2), LAVYQAGAR (E3 and E4), LGADMEDVR (E4), and LGADMEDVCGR (E2 and E3) should be measured (34).

Development of primary and secondary reference materials, including purity and stability testing, evaluation of commutability and value-assignment, according to relevant ISO guidelines. A major requirement for establishing, implementing, and maintaining a reference system relying on an MS-based reference measurement procedure (RMP) is to have primary reference materials (RMs) of well-characterized purity [17511:2020] (Fig. 2). Primary calibrators consist of purified peptides (or proteins) that are used to calibrate the reference measurement procedure. Different approaches are available for determination of the concentration of calibration solutions: mass balance, amino acid analysis, quantitative nuclear magnetic resonance, and elemental analysis (32). While technically challenging, rigorous purity assessment of primary calibrators is an essential requisite to establish metrological traceability of results to SI units (32) and to obtain consistent results over prolonged periods of time during which different batches of the primary calibrators will be used.

Provided that the RMP spends less than 50% of the total allowable uncertainty derived from biological variation data, the RMP can then be used to assign target values to 2 types of secondary RMs (serum-based matrix materials): secondary calibrators used to calibrate IVD-tests and trueness verifiers used to verify accuracy in external quality assessment (EQA) programs or as part of the post-market vigilance of IVD tests. Both secondary calibrators and trueness verifiers should have

appropriate stability, homogeneity, and commutability properties, in compliance with ISO 15194.

Commutability relates to the closeness of agreement between results for the candidate RMs and results for clinical samples (CSs) when measured with ≥2 measurement procedures. Assessment of commutability requires a dedicated study in which the RMs and a sufficient number of CSs (and matrices) are measured with the RMP and all relevant IVD tests. The study design and the criteria applied for assessing the commutability should be consistent with the required analytical performance specifications (APS) of the IVD tests (35).

The availability of well-characterized and commutable secondary calibrators is no guarantee for successful standardization of commercial IVD tests. Variations in calibration procedure, including the number and level of calibration points or the type of diluent, may still cause relevant differences in measurement results. A harmonized calibration protocol might be needed to further improve inter-method comparability. The calibrated IVD tests should also have an adequate selectivity for measurand. Selectivity differences between an IVD test and the RMP will persistently lead to biased results on individual CSs, even after calibration. These sample-specific effects can become visible in a split-sample comparison of the RMP and IVD test(s) on a large group of fresh, unprocessed CSs. If the sample-specific effects are at a clinically undesirable level, a modification of the medical test improving its selectivity is required.

Development of a common IDMS-based candidate reference method in a network of calibration laboratories. To facilitate protein standardization, a full RMS should be in place, consisting of both primary and secondary RMs and a RMP (Fig. 2). We aim to develop an IDMS-based candidate RMP according to the highest ISO 17511:2020 calibration hierarchy and C62A quality requirements. Moreover, the RMP should be compliant with ISO 15193 for reference measurement procedures and ISO 15195 for reference laboratories.

To ensure a sustainable RMP, the procedure is developed in a network of 3 independent candidate reference laboratories: 2 in Europe (Leiden University Medical Centre (LUMC) in Leiden, The Netherlands, and University Hospital Leipzig (UKL) in Leipzig, Germany) and 1 in the United States (Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA). The first step was agreement on the starting points for a common MS-based RMP, taking into account the Terms of Reference of the IFCC WG. During method development, common target peptides will be selected, and common transitions will be developed. The use of stable isotope labelled peptides as internal standards is essential, not only for reliable and accurate qualification and quantification, but also to account for variations in ionization efficiency. Initial method comparisons will be performed using mixtures of synthetic peptides. These mixtures may also be used to monitor instrument performance in the respective labs according to system suitability testing procedures. When sample preparation procedures are harmonized, the enzymatic digestion will be optimized to reach equimolar apoprotein-to-peptide conversion. Given that the WG aims for peptide-based primary calibration, transfer of concentrations from quantifying peptide to intact protein through the candidate RMP assumes identical behavior between the peptide-calibrators and the endogenous peptides (representing the proteins) produced through enzymatic digestion. This is the most crucial step of the candidate RMP development (36) that has to be proven with dedicated experiments for each of the apolipoproteins. Behavior of peptides as calibrators, protein digestion kinetics, and recovery of the surrogate proteotypic peptides will be examined to provide evidence that the sample preparation procedure guarantees complete protein digestion.

Analytical performance validation of a common candidate RMP. Similar to any medical test, the candidate RMP should be *fit-for-purpose*, considering its higher order role in the calibration hierarchy. Based on our objectives and starting points for a common accuracy base, an envisioned traceability chain is drafted (Fig. 1). Due to error propagation, each step in the chain introduces additional MU, hence the error budget for the candidate RMP should, as a rule of thumb, be maximally 25%–50% of the total allowable error budget per measurand. We hypothesize that a limited MU contribution of the candidate RMP is achievable, as such uncertainties were achieved in previous evaluations of MS-based apolipoprotein tests already (37–39), and demonstrate long-term stable performance of such a method (39).

Once a common, multiplexed LC-MRM-MS-based candidate reference method is developed, a detailed Standard Operating Procedure will be written, according to ISO 15193, 15195, and 17025 requirements. To evaluate the analytical performance of the common candidate RMP, each laboratory will assess analytical sensitivity, linearity, limit of detection and quantification, repeatability, and uncertainty. Ring trials are periodically organized to monitor accuracy of test results produced by candidate reference laboratories running the candidate RMP. All 3 reference labs should fulfil the predefined APS before the candidate RMP can be submitted to the IFCC Scientific Division and the protein

review team of the Joint Commission on Traceability in Laboratory Medicine (JCTLM).

An approach to improve the accuracy and reliability of routine IVD-tests by applying the Reference Measurement System for calibration and monitoring: future perspective. Adequate implementation of the RMS and the Metrological Traceability concept demands a 3-step process. In the first step, the measurands are defined and the reference measurement system, consisting of reference methods and materials, is established. In the second step, the reference measurement system is used to calibrate and verify the analytical performance of assays operated by IVD manufacturers and laboratories providing in-house developed tests. In the third step, the accuracy and analytical performance of testing in patient care and research settings is monitored on its fitness with clinical needs. This 3-step process, which is called standardization, has been successfully applied to improve global reliability and accuracy of cholesterol, HbA1c, and other clinical analytes, and can be applied to apolipoproteins. It needs to be noted that in any measurement process, the instrumentation, reagents, and laboratory operations can change over time, which can introduce a change in analytical performance. Therefore, this standardization process needs to be continuously applied.

This implies that the RMS needs to be maintained and operational, which can best be achieved through a network of reference laboratories that ensures consistency of analytical performance of reference measurements over time. This network performs regular interlaboratory comparison studies where network members need to demonstrate appropriate analytical performance and metrological traceability in line with relevant ISO standards. Examples of successfully operated reference laboratory networks are the IFCC Network for Standardization of HbA1c, the network of the National Glycohemoglobin Standardization Program, and the CDC Cholesterol Reference Method Laboratory Network. The reference laboratories also assign reference values to commutable serum materials for the purpose of (re)calibration or trueness verification.

Recalibrated IVD manufacturers need to ensure that the level of accuracy and reliability established at the manufacturer in the second step of the standardization process is properly transferred to the tests deployed by end-users in patient care, public health, and research. This can be assessed by monitoring the analytical performance of end-users. Programs such as the CDC Lipids Standardization Program (CDC LSP), Accuracy-based Monitoring Program (CDC AMP), or Accuracy-based External Quality Assessment (EQA) programs successfully monitor the analytical performance of individual laboratories. While CDC LSP and CDC AMP assess the analytical performance using multiple time points, EQA programs typically assess laboratories using a single time point. The combination of longitudinal monitoring programs and cross-sectional EQA programs provides comprehensive information about measurement accuracy and reliability. Once the entire reference system for apolipoproteins is established (Fig. 2) a formal monitoring program will be introduced. Lp(a) is already part of several accuracy-based EQA programs, and it is being included in the CDC LSP program.

Conclusion

In parallel with technological innovations and our enhanced understanding of the role of blood apolipoproteins in the pathophysiology of ASCVD, the science of measurement of these potential risk markers also is evolving. Therefore, existing WHO-IFCC based Reference Measurement Systems need to be improved and redefined. Hence, the IFCC working group on Apolipoproteins by Mass Spectrometry has established a scientific and technical basis for SI-standardization of 7 relevant serum apolipoproteins. Currently, a complete Apolipoprotein Reference Measurement System is under development, consisting of a harmonized multiplex Reference Measurement Procedure, uniplex primary Reference Materials, and secondary serum-based Reference Materials. The analytical selectivity of the apolipoprotein measurements is guaranteed with quantitative LC-MRM-MS, based on bottom-up proteomics and peptide calibrators. The metrological traceability of contemporary and emerging apolipoprotein tests will be accomplished according to the latest standardization insights as laid down in ISO 17025:2018 and 17511:2020. A harmonized, multiplex Reference Measurement Procedure will be available by the end of 2020, whereas the development of the primary and secondary RMs has been postponed due to the COVID pandemic and is still ongoing. The development of RMs for apo(a) has been prioritized, followed by RMs for apos A-I and B, and finally for the apos C and E. Primary RMs are expected to be available by the end of 2021, whereas secondary RMs are estimated to be available by the end of 2022. The IFCC WG on Apolipoprotein standardization by mass spectrometry strives to have the SI-traceable Reference Measurement System for apo(a) standardization of medical tests in place in 2021, whereas the complete Reference Measurement System for apo A-I, B, C-I, C-II, C-III, and E test standardization will be ready for use by the end of 2022/early 2023 (Fig. 2). Meanwhile, the network of calibration labs running the Reference Measurement System will further improve its analytical

performance, periodically run ring trials, and establish Apolipoprotein Standardization Programs. The convergence of RMS development and implementation is essential for global apolipoprotein standardization in the near future. As the analytical performance and clinical performance of medical tests are interdependent and interrelated key features that have to be considered thoroughly during biomarker-to-test development, concomitant apolipoprotein standardization according to the latest metrology insights is a natural "evolutionary" step in the science of measurement and a prerequisite for improving patient management in this era of precision cardiology.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: Apo, apolipoprotein; Apo(a), apolipoprotein(a); Apos, apolipoproteins; APS, analytical performance specifications; ASCVD, atherosclerotic cardiovascular disease; CDC, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; CDC LSP, CDC lipid standardization program; CDC AMP, CDC accuracy-based monitoring program; CGPM, General Conference on Weights and Measures; CLSI, Clinical and Laboratory Standards Institute; CM, chylomicrons; CS, clinical sample; CVD, cardiovascular disease; DGKL, German Society for Clinical Chemistry and Laboratory Medicine; ELISA, enzyme-linked immunosorbent assay; EQA, External Quality Assessment; HbA1c, Hemoglobin A1c; HDL, High Density Lipoprotein; HDL-C, High Density Lipoprotein cholesterol; IDMS, isotope dilution mass spectrometry; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; ISO, International Organization for Standardization; IVD, In Vitro Diagnostic; JCGM, Joint Committee for Guides in Metrology; JCTLM, Joint Commission on Traceability in Laboratory Medicine; JRC, Joint Research Centre, Geel, Belgium; K-IV$_2$, kringle IV type 2; LC, liquid chromatography; LDL, Low Density Lipoprotein; LDL-C, Low Density Lipoprotein Cholesterol; LNE, Laboratoire National de Métrologie et d' Essais; *LPA*, lipoprotein(a) gene; Lp(a), lipoprotein(a) particle; LUMC, Leiden University Medical Centre; MRM, multiple reaction monitoring; MS, mass spectrometry; MU, measurement uncertainty; Non-HDL, Non-High Density Lipoprotein; SI, international system of quantities (*Systeme Internationale*); RM(s), Reference Material(s); RMP, Reference Measurement Procedure; RMS, Reference Measurement System; SRM, Standard Reference Material; UKL, University Hospital Leipzig; VLDL, Very Low Density Lipoprotein; VLDL-C, Very Low Density Lipoprotein Cholesterol; WG, Working Group; WHO, World Health Organization.

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