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Development of an LC-MRM-MS-Based Candidate Reference Measurement Procedure for Standardization of Serum Apolipoprotein (a) Tests

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BACKGROUND: Medical results generated by European CE Marking for In Vitro Diagnostic or in-house tests should be traceable to higher order reference measurement systems (RMS), such as International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)-endorsed reference measurement procedures (RMPs) and reference materials. Currently, serum apolipoprotein (a) [apo(a)] is recognized as a novel risk factor for cardiovascular risk assessment and patient management. The former RMS for serum apo(a) is no longer available; consequently, an International System of Units (SI)-traceable, ideally multiplexed, and sustainable RMS for apo(a) is needed.

METHODS: A mass spectrometry (MS)-based candidate RMP (cRMP) for apo(a) was developed using quantitative bottom-up proteomics targeting 3 proteotypic peptides. The method was provisionally validated according to ISO 15193 using a single human serum based calibrator traceable to the former WHO-IFCC RMS.

RESULTS: The quantitation of serum apo(a) was by design independent of its size polymorphism, was linear from 3.8 to 456 nmol/L, and had a lower limit of quantitation for apo(a) of 3.8 nmol/L using peptide LFLEPTQADIALLK. Interpeptide agreement showed Pearson Rs of 0.987 and 0.984 for peptides GISSTVTGR and TPENYPNAGLTR, and method comparison indicated good correspondence (slopes 0.977, 1.033, and 1.085 for LFLEPTQADIALLK, GISSTVTGR, and TPENYPNAGLTR). Average within-laboratory imprecision of the cRMP was 8.9%, 11.9%, and 12.8% for the 3 peptides.

CONCLUSIONS: A robust, antibody-independent, MS-based cRMP was developed as higher order RMP and an essential part of the apo(a) traceability chain and future RMS. The cRMP fulfils predefined analytical performance specifications, making it a promising RMP candidate in an SI-traceable MS-based RMS for apo(a).

Introduction

There is currently an unmet need for a higher order, ideally multiplexed, and sustainable reference measurement system (RMS) for apolipoprotein (a) [apo(a)] that is globally accessible. Diagnostic laboratories require standardized tests and reference intervals to

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diagnose and treat patients effectively and safely, while RMS allow external quality assessment organizers to provide value assigned, commutable external quality assessment materials to monitor trueness of test results and aid in standardization of methods. As patients and clinicians expect equivalent results for similar tests among hospitals, metrological traceability of test results is essential. Implementation of innovations such as mass spectrometry (MS) technology for protein quantitation (1) and novel metrological insights, based on International Organization for Standardization (ISO) 17511:2020, are key to ensure accurate test results at a global level (2). It is therefore imperative that RMS for emerging measurands are developed and that already existing RMS are improved when needed.

The measurement of apo(a) at least once in an individual's lifetime has now been included in European and American clinical guidelines for risk assessment of cardiovascular disease (3). Moreover, drug therapies that target the apo(a) containing lipoprotein Lp(a) (4) have been developed and are currently in phase III clinical trials. These trials require apo(a) measurements to allow participants to meet inclusion criteria and for monitoring of therapy. Therefore, accurate measurement of apo(a) at a worldwide level, enabling the use of universal but ethnicity-dependent decision limits, cutoff points, and inclusion criteria, is desirable. However, in the current situation, large interlaboratory and intermanufacturer variation in the measurement of apo(a) is observed, where results of the same, commutable, sample in external quality assessment studies can vary by as much as 200% (5). Moreover, the units of measurement are not standardized, with both nmol/L and mg/dL being used. Given the complexity of the apo(a) molecule, apo(a) measurements should be performed in nmol/L, and ideally be Kringle 4 type 2 (KIV_2) independent, as emphasized in several publications recently [e.g., (5, 6)].

An individual WHO-International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) immunoassay-based RMS for apo(a) was developed more than 20 years ago in a single calibration laboratory (7). Technology has evolved, and direct protein measurement through proteolysis and the quantification of proteotypic peptides—peptides that uniquely identify a protein—has become achievable. Moreover, the stock of the existing reference material (RM) for apo(a) (SRM2B) has been depleted (https://www.ifcc.org/ifcc-scientific-division/sd-working-groups/wg-apo-ms/), and a recently developed candidate reference measurement procedure (RMP) for apo(a) is currently not operational (8); thus, the establishment of a highest-order, International System of Units (SI)-traceable RMS for apo(a) is timely. Here we describe the development and evaluation of a candidate RMP (cRMP) for apo(a), which is provisionally calibrated with a single serum-based human calibrator, traceable to the previous WHO-IFCC RMS (7). In subsequent steps, peptide-based apo(a) primary calibrators will be introduced that replace the native calibrator, and the method will be further developed into a multiplexed procedure for 7 apolipoproteins (Fig. 1). This paper describes our progress toward the cRMP while the selection and development of candidate reference materials is described in a companion manuscript (9).

The technology for protein quantitation has evolved substantially over the past decade. Specifically, the introduction of targeted MS with isotope dilution internal standardization allows for multiplexed and direct quantitation of proteins at the molecular level (1, 5) independent of antibodies, which are known to be potentially problematic (10). Representative peptides obtained through proteolytic digestion of the target proteins are quantified, theoretically enabling peptide-based calibrators, provided the proteins are converted into their representative peptides in an equimolar manner. If successful, the use of synthetic peptides as primary reference materials enables the implementation of a sustainable, highest-order traceability chain as recently described by the ISO in ISO 17511:2020 (11, 12) and as we recently outlined (13). Indeed, 2 lab-developed LC-MS-based tests for apo(a) have already been reported (8, 14).

RMPs should provide highly accurate results and should ideally consume <50% of the total error budget of a specific measurand (15). For apo(a), only limited studies on biological variation have been reported, and, more importantly, the biological variation is not constant over the full concentration range (16). Therefore, the use of biological variation as outlined in the Milan consensus statement (17) does not seem valid. Instead, within the IFCC working group for standardization of apolipoproteins by mass spectrometry (WG APO-MS), we defined the total allowable error for the RMS for apo(a) at 12%, based on current state-of-the-art. ISO guidelines 15193:2009 and 15195:2018 have been developed to guide the development and validation of high-quality RMPs and their implementation in calibration laboratories, respectively. The requirements are stringent and require in-depth description of the developed methods in combination with laborious experiments to prove analytical reliability. To ensure the work can be described in sufficient detail, we here focus on the procedure for serum apo(a) Meanwhile, secondary reference materials are being developed at the European Commission's Joint Research Center (Fig. 1). An assessment of the sample requirements for a commutable secondary reference material for apo(a) is presented as a companion paper (9).

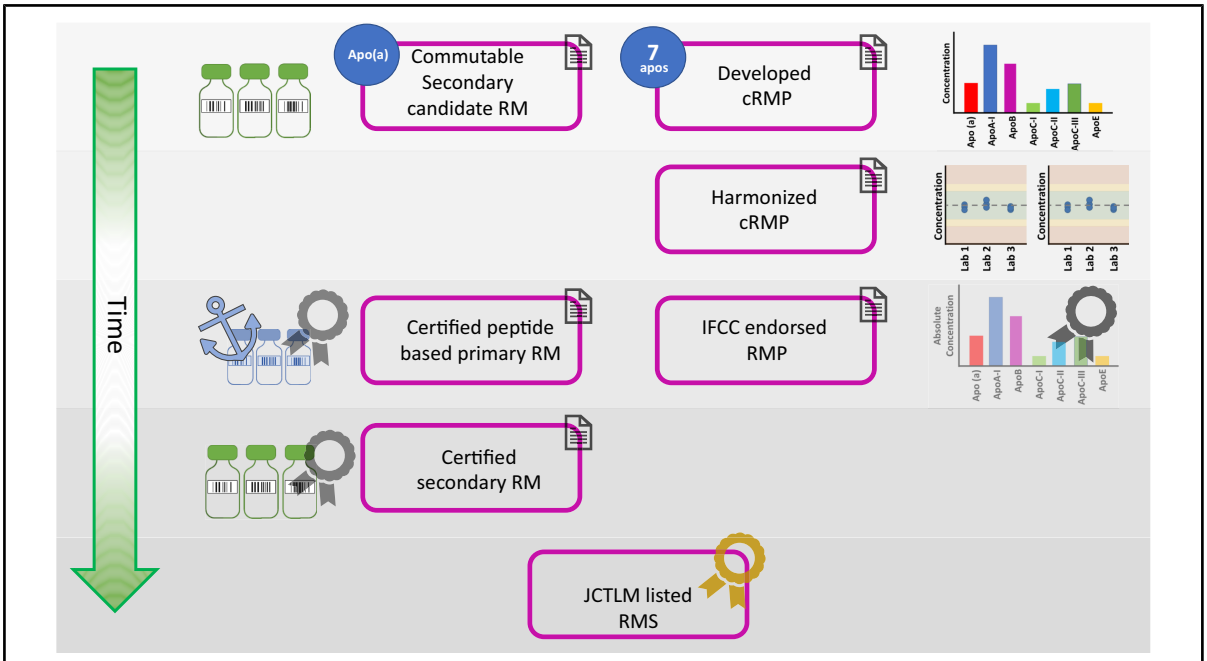

Fig. 1. Overview of the envisioned step-up approach toward development of a JCTLM-listed RMS for 7 apolipoproteins, including apo(a), in at least 5 steps. In a first step, the developed candidate RMP is presented in this publication. The performance of the other apolipoproteins will be published separately. Transferability of the cRMP together with the degree of harmonization among 3 calibration laboratories of the IFCC WG apolipoproteins-MS will then be described. Once certified primary RMs are available, the cRMP will be validated for IFCC endorsement. A similar strategy is envisioned for the RM production, where the characteristics of a commutable secondary RM for apo(a) are described in parallel to the cRMP. Peptide-based primary RMs will provide traceability to SI in the future by serving as calibrators for the RMP, provided equimolar digestion can be proven. Using the primary RMs and the IFCC-endorsed RMP, commutable, secondary RM will be value assigned and certified. At a later stage, the complete RMS will then be validated for JCTLM listing. Document icons indicate envisioned publications.

Materials and Methods

The supplementary information provides a detailed description of the materials and methods. An overview of the essentials of the method and its validation is provided here.

PEPTIDE SELECTION AND TRANSITION DEVELOPMENT

Apolipoprotein (a) was digested in silico with trypsin and candidate peptides were selected that (*a*) are proteotypic; (*b*) do not contain amino acids tryptophan (W), methionine (M), or cysteine (C); (*c*) are not prone to endemic genetic mutations or post-translational modifications; and (*d*) have an AA length between 7 and 15. Transitions were developed for 3 peptides from apo(a) and in total 30 peptides for all 7 apolipoproteins (Supplemental Table 3): retention times, precursor ion m/z, and 3 fragment ion m/z as well as collision energies were selected per peptide.

ANALYSIS

Eight µL of 20-fold diluted serum was added to a 40 µL mixture of Na-Deoxycholate, Tris(2-carboxyethyl)phosphine, and ^{13}C, ^{15}N arginine (R) or lysine (K) labeled peptide analogues in a 96-well plate. Proteins in the samples were heat-denatured and cysteines were chemically reduced and alkylated. Proteolytic digestion was performed using 40 ng LysC at 37°C for 1 h, followed by 600 ng trypsin for 3 h. Digestion was quenched by addition of 106 µL 0.6% (v/v) formic acid, the sample plate was centrifuged, and supernatant was transferred for solid phase extraction using Oasis HLB stationary phase. The solid phase extraction plate was conditioned with methanol, equilibrated with water, and loaded with the digested samples. Upon washing with water, peptides were eluted using 80% methanol in water, brought to dryness, and reconstituted in 50 µL mobile phase A prior to analysis.

For analysis, 10 µL of sample were injected onto a C18 column and peptides were gradually eluted using

a 17 min gradient. Peptides were measured in dynamic multiple reaction monitoring (MRM) mode with a cycle time of 500 ms. To ensure that the LC-MS/MS instrumentation was performing accurately during the sample analysis, a system suitability testing procedure was performed with each analysis.

CALIBRATION AND INTERNAL QUALITY CONTROL

One deidentified native serum sample, prepared according to Clinical and Laboratory Standards Institute (CLSI) protocol C37A, was used as external calibrator during analytical validation of the cRMP. This calibrator is traceable to WHO/IFCC SRM2B for apo(a) (7) and contains 94.6 nmol/L apo(a), as determined by n = 20 measurements using the Roche LPA2 ITA method on a Roche Cobas C502 analyzer. A peptide-based calibration is in preparation and will replace the native calibrator once SI traceability can be assured. Two native serum samples were used for internal quality control.

DATA ANALYSIS AND ANALYTICAL EVALUATION

LC-MS/MS data were processed using Mass Hunter Workstation software (Agilent Technologies) and Skyline (18). A single-point linear calibration was used for apo(a), and concentrations were reported in nmol/L.

Intermediate analytical validation was performed in line with CLSI guideline C64, and, according to the respective CLSI protocols EP15 (precision), EP06 (linearity), EP17 (limit of quantification (LoQ)), and EP-10 (carryover) using deidentified native serum samples were prepared according to CLSI C37A. The predefined performance criteria are summarized in Table 1.

Results

METHOD DEVELOPMENT

The measurand was defined as "serum apolipoprotein (a)." A common accuracy base was developed, outlining the starting points of method development, including the use of a bottom-up proteomics strategy, reversed phase LC-MS/MS analysis, and already existing analytical infrastructure (19–21). The performance specifications were defined at 50% of the total allowable error; for apo(a) this was defined based on expert opinion to be 12%.

Peptide selection. In quantitative bottom-up proteomics, proteins are digested to corresponding peptides, which are quantified as the proteins' representative (Fig. 2, A). Because the moles of peptide should correctly reflect the moles of the protein of interest, peptide selection is of high importance. Ideally, proteotypic peptides are chosen, to be free of genetic or post-translational modifications and to be liberated easily and stably during proteolytic digestion. Three proteotypic peptides [GISSTTVTGR (Kringle 4 type 9 domain), LFLEPTQADIALLK (peptidase domain). and TPENYPNAGLTR (Kringle 4 type 5 domain)] were selected for Kringle IV-2 (KIV_2) independent quantitation at the molar level. Moreover, 2 KIV_2 peptides (GTYSTTVTGR and NPDAVAAPYCYTR) were selected to enable assessment of clinical relevance of the average number of KIV_2 repeats in future studies (Supplemental Table 3). The peptides were assessed for potential endemic genetic variants. Only for the selected peptide TPENYPNAGLTR was a genetic variant observed, in 6% of an African population (22). One peptide containing a cysteine was selected (NPDAVAAPYCYTR) but only for KIV_2 assessment. $^{13}C_6$, $^{15}N_2$ lysine, or $^{13}C_6$, $^{15}N_4$ arginine-labeled stable isotope labeled peptides were used as internal standards as this allows high analytical specificity, with collision induced dissociation fragmentation often yielding *y*-type ions, where the positively charged amino acid remains on at the C-terminal terminus side of the peptide.

LC-MRM-MS analysis. For all peptides, 3 transitions were selected: 1 served as the quantifier and the others served as qualifying transitions. Congruent transitions were also selected for the stable isotope labeled peptides (Supplemental Table 3). The gradient was optimized to yield a minimum dwell time of 17 msec, and a typical chromatogram of the multiplexed method, including apo(a) peptides, is shown in Fig. 2, B. Supplemental Table 1 shows results of 10 runs of system suitability testing for apo(a). Stable retention times, relative responses, and ion ratios were obtained with average within-run CVs of 0.15%, 3.0%, and 3.2%, respectively. Average carryover was 0.31%, indicating suitable and expected LC-MRM-MS performance.

Sample preparation. Because the eventual objective is to achieve metrological traceability to SI units through the use of peptide-based calibrators, careful sample preparation and especially assessment of digestion is essential. The final sample preparation method is shown in Fig. 2, C and described in Supplemental Section 1.3 Sample Preparation. The method is based on our previously developed sample preparation strategy using trypsin digestion (20) with exchange to the more volatile ammonium bicarbonate buffer and comprises a combined LysC/trypsin digestion, which has previously been shown to yield more complete protein digestion of K-rich proteins (23). To initially assess completeness of digestion, a native serum sample with very low (1.8 nmol/L) apo(a) concentration as well as a serum sample with higher apo(a) concentration were spiked with 40 nmol/L of recombinant apo(a). Digestion time courses were prepared with trypsin digestion times of 0 min; 30 min; and 1, 3,

Table 1. Analytical performance characteristics of the candidate reference measurement procedure for serum apolipoprotein (a).

Analytical performance characteristic	Method	Desired performance criterion	Results
Precision	EP-15	<12% total imprecision	Average within laboratory imprecision of 8.9%, 11.9%, and 12.8% for peptides LFLEPTQADIALLK, GISSTVTGR, and TPENYPNAGLTR, respectively. (Supplemental Table 4)
Analytical selectivity	Interpeptide agreement	$R > 0.975$ and slope > 0.9 and < 1.1	R of 0.987 and 0.984 for comparisons of LFLEPTQADIALLK with GISSTVTGR and TPENYPNAGLTR, respectively. Slopes of 0.944 and 0.900 for comparisons of LFLEPTQADIALLK with GISSTVTGR and TPENYPNAGLTR, respectively. (Fig. 4 and Supplemental Fig. 1)
Limits of quantitation	EP-17, LoQ	LoQ for apo(a) <10 nmol/L	LoQ for apo(a) is 3.8 nmol/L, <0.63 nmol/L, and 10.9 nmol/L based on peptides LFLEPTQADIALLK, GISSTVTGR, and TPENYPNAGLTR, respectively (Fig. 4)
Stability	Yes	Within 3SD criteria as outlined through EP-15	Stable 2-year performance for all peptides (Supplemental Fig. 3)
Reference interval and medical cutpoints	To be defined once SI-traceable calibration is in place	—	—
Analytical interferences	Stability of ion ratios, CLSI C62A	Ion ratio should not deviate more than 20%	Difference in ion ratio between endogenous and SIL[a] peptides is <20%, except for qualifying transition 2 for peptide GISSTVTGR, which deviates 27%. (Supplemental Table 2)
Carryover	EP-10 and SST[b]	Carryover should not interfere at LoQ and should be <1%	Carryover does not affect apo(a) quantitation at LoQ and was below 1% for all 3 peptides in SST (Fig. 4)
Linearity	EP-06	R^2 over 0.98, deviation of linearity for apo(a) <12%	Quantitation of apo(a) is linear at least over a range of 3.8 to 456 nmol/L, 0.6 to 484 nmol/L and 10.9 to 484 nmol/L for LFLEPTQADIALLK, GISSTVTGR, and TPENYPNAGLTR, respectively (Fig. 4)
Method comparison	Yes	$R > 0.95$, bias below TEa[c]	R of 0.977, 0.969, and 0.960, slopes of 0.977, 1.033, and 1.085 and biases of -2.57%, 2.61%, and 7.61% for peptides LFLEPTQADIALLK, GISSTTVTGR, and TPENYPNAGLTR, respectively (Supplemental Fig. 2)

[a]stable isotope labeled.
[b]system suitability testing.
[c]total allowable error.

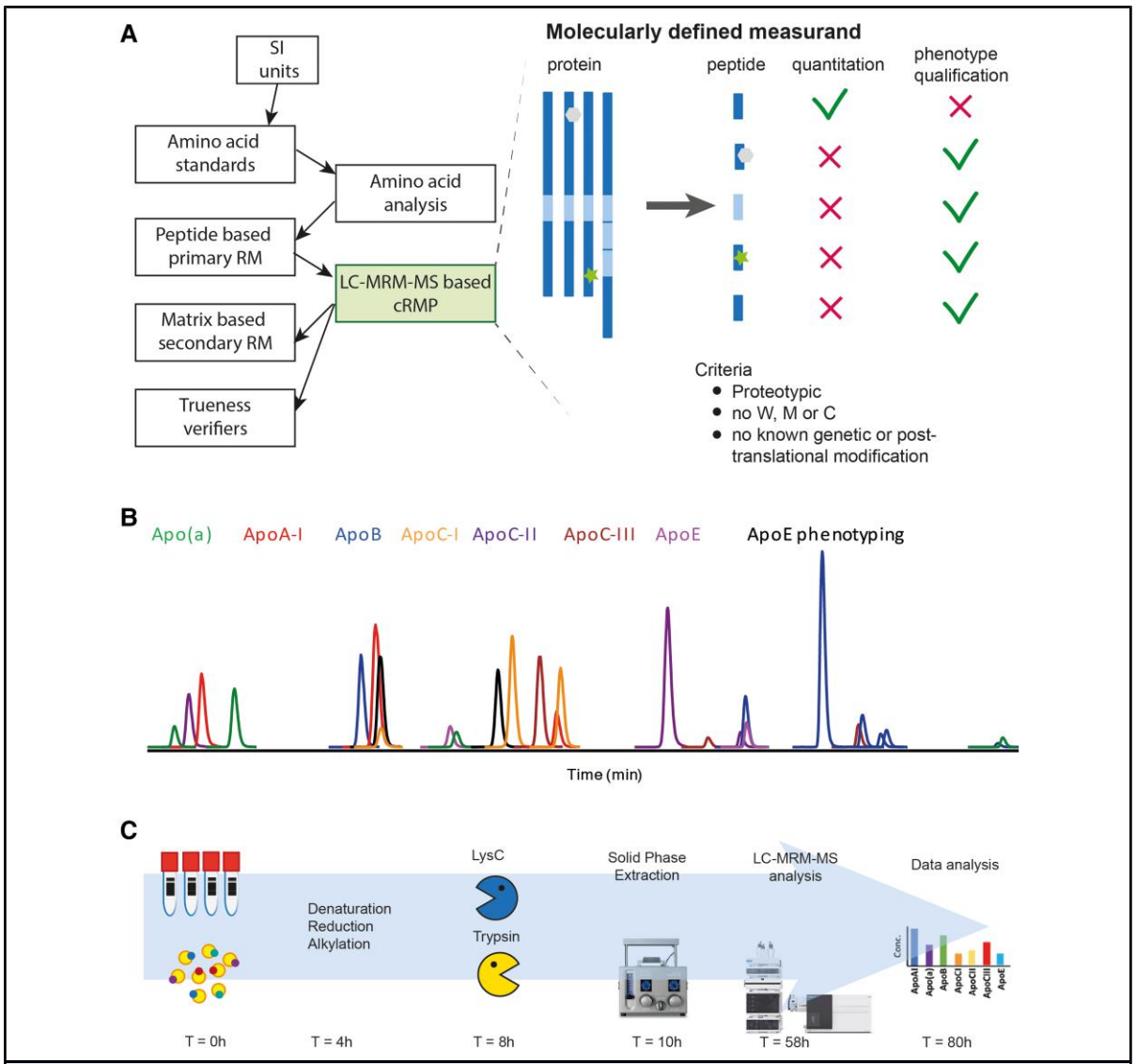

Fig. 2. Overview of the LC-MRM-MS cRMP for quantitation of apo(a). LC-MRM-MS allows definition of the measurand at the molecular level through the quantitation of proteotypic peptides and qualitative measurement of specific peptides representing proteoforms (A). The cRMP provides a chromatogram comprising peptide signals from 32 endogenous peptides as well as their synthetic stable isotope labeled analogues (B) and uses a bottom-up proteomics strategy, with a combined LysC—trypsin digestion. The total analysis time of the procedure, including data evaluation, takes approximately 80 hours (C). Abbreviations: W, tryptophan; M, methionine; C, cysteine.

and 18 h, and plateaus were reached for all 3 peptides within 1 h trypsin digestion time for both the native serum sample as well as the serum samples spiked with recombinant apo(a) (Fig. 3). Moreover, the plateaus reached target levels in concordance with their expected concentrations. While not yet conclusive, these results hint toward a well-optimized digestion process and likely completeness of digestion. Notably, further evidence will have to be generated to prove equimolar digestion as well as the requirements for preparation, storage, and handling of the envisioned peptide-based calibration curve, which is beyond the scope of the current publication.

INTERMEDIATE ANALYTICAL VALIDATION

The analytical validation of the cRMP was performed using a single external native serum calibrator, which is provisionally value assigned to the current WHO-IFCC

RMS. The cRMP was evaluated for analytical selectivity, carryover, matrix effects, linearity, method comparison, stability, and imprecision (Table 1).

Analytical selectivity at the peptide level was achieved through the monitoring of ion ratios, calculated as the ratio of the chromatographic peak area of the qualifying transition to that of the quantifying transition for each specific peptide. Average ion ratios for the measurement of 39 clinical samples (n = 3) yielded deviations of <20% for all transitions from the 3 peptides, except for 1 qualifying transition for GISSTVTGR, which deviates 27% (Supplemental Table 2), compared to the target ion ratios observed for pure peptides in system suitability samples. This specific transition yields low ion counts, resulting in higher imprecision, particularly for clinical samples with lower concentrations. Overall, no analytical interferences from other compounds are observed.

Analytical selectivity at the protein level was achieved through the use of at least 2 peptides per protein. Interpeptide comparisons were performed on measurements of 157 individual clinical serum samples (Fig. 4, A and B and Supplementary Fig. 1). Pearson's R for both interpeptide comparisons were 0.987 and 0.984 for peptides GISSTVTGR and TPENYPN AGLTR relative to LFLEPTQADIALLK, while slopes of 0.944 and 0.900 were obtained for these same comparisons, indicating generally good correspondence between peptides and therefore good analytical selectivity.

The LoQ was determined for apo(a) according to CLSI EP-17 to be 3.8 nmol/L, <0.63 nmol/L, and 10.9 nmol/L for the peptides LFLEPTQADIALLK, GISSTVTGR, and TPENYPNAGLTR, respectively (Fig. 4, C). The linearity of the method was assessed for apo(a) through a procedure similar to the one described in CLSI protocol EP-06. Using a maximum degree of nonlinearity of 12%, or half the total allowable error for apo(a), the quantitation of apo(a) was determined to be linear over a range of 3.8 to 450 nmol/L for peptide LFLEPTQADIALLK, 0.6 to 484 nmol/L for peptide GISSTVTGR, and 10.9 to 484 nmol/L for peptide TPENYPNAGLTR. These extended linear ranges will enable quantitation of apo(a) without dilution for nearly all clinical samples. (Fig. 4, D).

The carryover was assessed for apo(a), since this apolipoprotein has a large biological variation, and an up to 1000-fold variation in protein concentration may be expected among individuals. A CLSI-EP10 protocol was used, in which a sample with a high apo(a) concentration (468 nmol/L), was measured concurrently with a sample with low apo(a) concentration (1.8 nmol/L). The concentrations of the low sample measured within the sequence either immediately following the low concentration or the high concentration sample were compared, but no significant carryover was observed (Fig. 4, E).

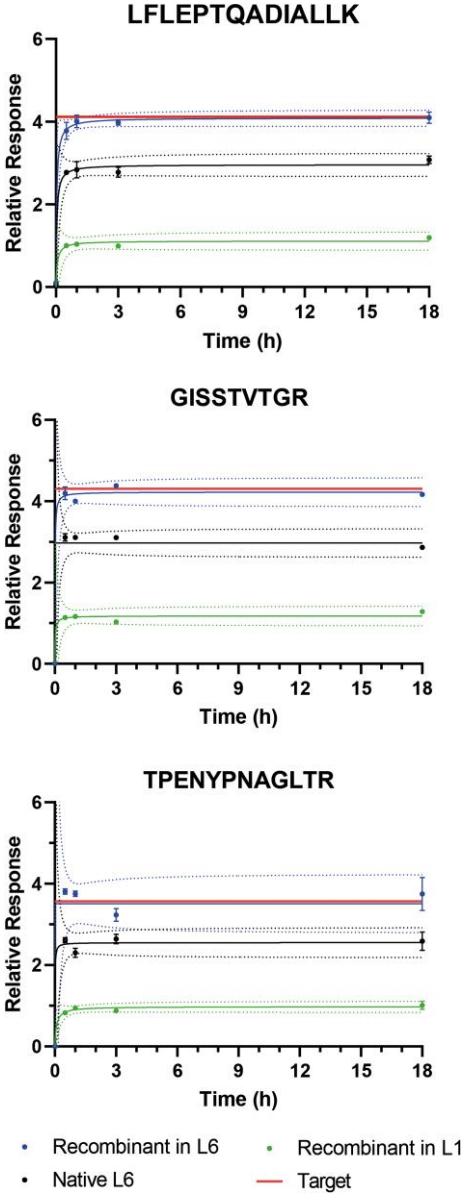

Fig. 3. Evaluation of digestion efficiency of apo(a). Digestion kinetics curves of known concentrations of recombinant apo(a) (40 nmol/L) spiked into serum containing 1.8 nmol/L (L1) and 94.6 nmol/L (L6) apo(a), indicating stable and similar digestion kinetics with recoveries as expected for both the spiked recombinant and native serum samples. Relative responses were obtained relative to stable isotope labeled internal standards (Supplemental Table 3), which were added prior to proteolysis. Error bars represent standard deviation (often not visible), and dotted lines indicate the 95% CIs around the digestion kinetics curves.

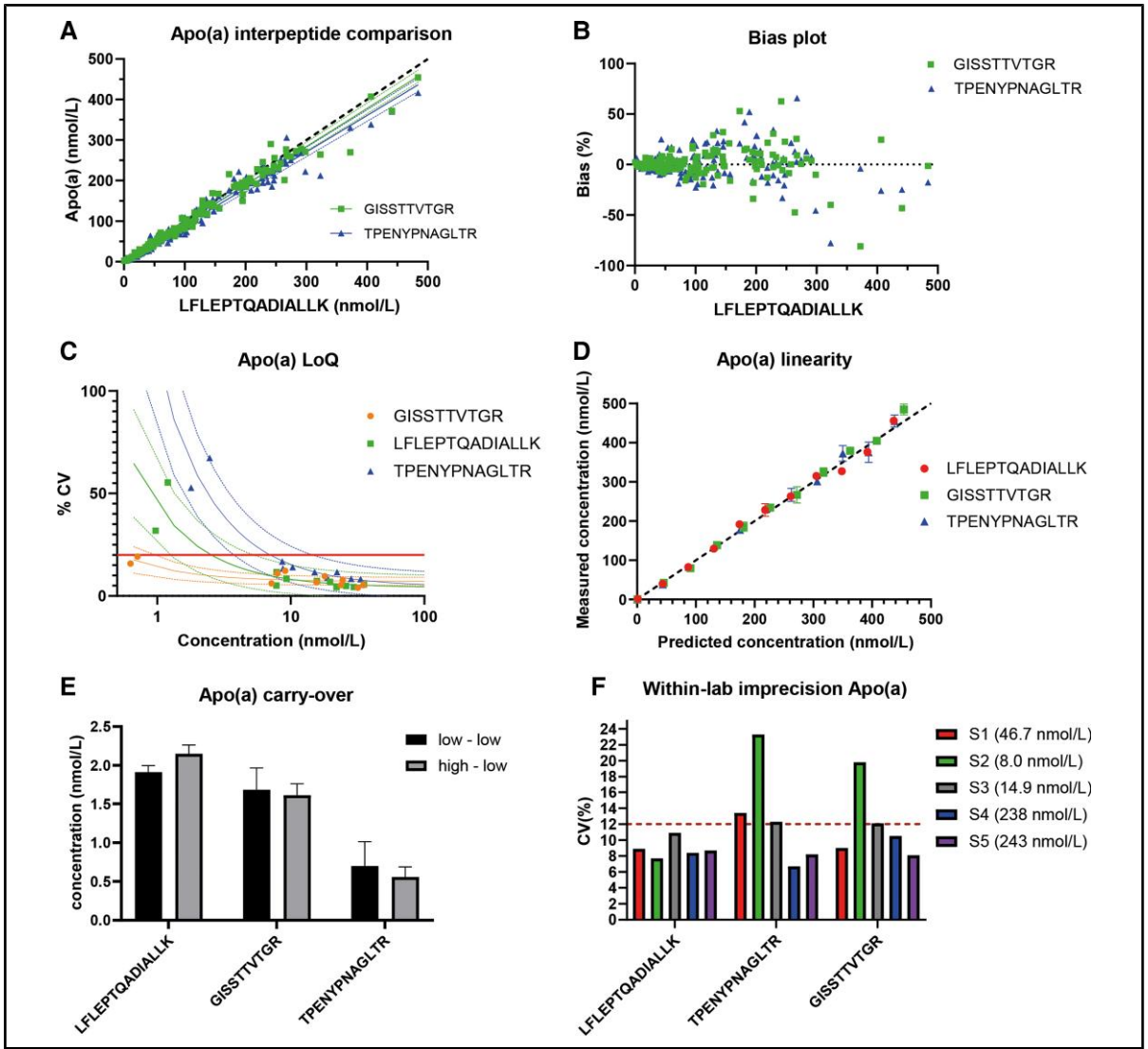

Fig. 4. Analytical validation of the cRMP for apo(a). Three KIV_2 independent peptides were monitored for apo(a). An interpeptide comparison was made in results originating from individual measurements of 157 native human serum samples, and Deming regression was performed, resulting in equations of 0.944 (± 0.022) *x + 0.45 (± 1.63) and 0.900 (± 0.020) *x + 0.48 (± 1.52) for LFLEPTQADIALLK vs GISSTTVTGR and TPENYPNAGLTR, respectively (A). Black dotted line represents line of identity, while colored dotted lines represent 95% CIs of the regression lines. Bias of the interpeptide comparison was also calculated to be on average −5.17% and −9.56% for LFLEPTQADIALLK vs GISSTTVTGR and TPENYPNAGLTR, respectively (B). Precision of quantitation of apo(a) in relation to concentration of apo(a) to assess LoQ through the replicate (n = 9) quantitation of native human serum samples with low apo(a) concentration (< 33 nmol/L) (C) and linearity of quantitation of apo(a) through admixing of a high (468 nmol/L) with a low (1.8 nmol/L) human serum sample (D). Carryover of apo(a) assessed through a sequence of low (1.8 nmol/L) and high (468 nmol/L) apo(a) concentration samples (E) and within-laboratory imprecision of quantitation of apo(a) by 3 proteotypic peptides through the repeated quantitation of 5 native human serum samples (samples 1–5) (F).

An EP-15 protocol comprising quantitation of 5 samples in quintuplicate on 5 different days was conducted to assess imprecision. Average total imprecision of the method was 8.9%, 11.9%, and 12.8% for peptides LFLEPTQADIALLK, GISSTVTGR, and TPENYPNAGLTR, respectively. (Fig. 4, F).

Longer term stability was assessed through the follow-up of measurements of 2 internal quality control samples over a 2-year period, in which >10 experiments were performed. Levey-Jennings plots are shown for LFLEPTQADIALLK, GISSTVTGR, and TPENYPNAGLTR in Supplemental Fig. 3. No deviation between the first results obtained 2 years prior to the last results is observed, indicating longer term stability of the method as well as the internal quality control samples.

A method comparison using 157 native human serum samples was performed to assess the relationship between the KIV_2 independent LC-MRM-MS based cRMP for apo(a) and an immunoassay (Roche TinaQuant LPA2) expressing results in nmol/L traceable to the previous secondary reference material SRM2B and the previous ELISA reference method. Results of the method comparison for all 3 apo(a) peptides, including Deming regression, are shown in Supplemental Fig. 2. Slopes of 0.977, 1.033, and 1.085 and biases of −2.57%, 2.61%, and 7.61% are reported for peptides LFLEPTQADIALLK, GISSTTVTGR, and TPENYPNAGLTR, respectively, indicating good concordance between the 2 methods.

Discussion

We here report a candidate reference measurement procedure for apo(a) as a first step to fulfill the need for a globally available reference measurement system for apolipoproteins. The method is analytically validated using a provisional single point native human serum calibrator that is traceable to the current WHO-IFCC RMS. While a peptide-based calibration that enables future traceability to SI units is envisioned and required (Fig. 2, A), currently a native serum calibrator traceable to the previous WHO-IFCC RMS (7) was deliberately chosen to provide the clinical chemistry community and in vitro diagnostics manufacturers with a step-up approach and transition phase, in which the differences due to the measurement procedure and the new value assignment can be assessed without interference. As a consequence, this report does not contain data on combined measurement uncertainty at this time.

Mass spectrometry is ideally suited for detection and quantification of proteins at the molecular level, contrary to immunoassays, which rely on binding specificity of the antibodies used in the procedure. In LC-MS/MS, the combination of specific liquid chromatography retention times with characteristic precursor and fragment ions provides very high analytical specificity. In the new cRMP, it was further enhanced through the selection of at least 2 characteristic proteotypic peptides in combination with 3 transitions per peptide and evaluated through ion ratio monitoring and inter-peptide comparison. Overall, this provides high confidence in the identification of the specific protein of interest, without interferences.

The development of the highest-order candidate RMP for sustainable standardization of commercial Lp(a) or apo(a) tests is of utmost importance, as Lp(a) mass or apo(a) test results are significantly confounded for multiple reasons (5). Lp(a) is still a greatly misunderstood biomarker, and current commercial immunoassays still reveal large intermethod variation (5) as well as sample-specific scatter across methods (24). At equal numbers of KIV_2 repeats, a 200-fold variation in apo(a) concentration is observed (25). In contrast, the peptides we selected for apo(a) quantitation are outside the KIV_2 region; therefore the LC-MRM-MS based cRMP presented here is by design a KIV_2-independent method. The range of apo(a) concentrations varies 1000-fold, and its linearity ranges from 3.8 to 450 nmol/L, which is larger than the linear measuring range of current commercial immunoassays. The lower LoQ is respectively 3.8 nmol/L, <0.63 nmol/L, and 10.9 nmol/L for the apo(a) peptides LFLEPTQADIALLK, GISSTVTGR, and TPENYPNAGLTR. No significant carryover for apo(a) quantitation was observed, and overall imprecision for apo(a) is within the predefined analytical performance specifications (total allowable error of 12%).

In conclusion, the IFCC WG APO MS here presents a candidate reference measurement procedure for apo(a) that is to be extended for 6 other clinically relevant apolipoproteins. The method was developed at the LUMC in Leiden, the Netherlands, in collaboration with calibration labs at University Hospital Leipzig and the Centers for Disease Control and Prevention. Transferability is essential as it enables global and sustained availability of the cRMP during its life cycle and pilot experiments indicate transferability of the cRMP. The envisioned apolipoprotein reference measurement system comprises both a reference measurement procedure as well as reference materials. In parallel, to the cRMP, primary and secondary reference materials for apolipoproteins are being developed. Results of a first assessment of commutability of matrix-based reference materials for apo(a) is published concurrently to this report. A transition plan from the former WHO-IFCC RMS to a new envisioned SI-traceable reference measurement system is underway as the IFCC SRM2B reference material for apo(a) is depleted and the ELISA-based KIV_2 independent method is no longer available (https://www.ifcc.org/ifcc-scientific-division/sd-working-groups/wg-apo-ms/).

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: RMS, reference measurement system; apo(a), apolipoprotein (a); ISO, International Organization for

Standardization; KIV_2, Kringle 4 type 2; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; RMP, reference measurement procedure; RM, reference material; SI, International System of Units; cRMP, candidate RMP; WG APO-MS, IFCC working group for standardization of apolipoproteins by mass spectrometry; MRM, multiple reaction monitoring; CLSI, Clinical and Laboratory Standards Institute; LoQ, limit of quantification.

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