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Quantifying apolipoprotein(a) in the era of proteoforms and precision medicine

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ABSTRACT

Lipoprotein(a) (Lp(a)) is an independent risk factor in the development of atherosclerotic cardiovascular diseases (ASCVD) and calcific aortic valve disease (CAVD). Lp(a) is an LDL-like particle to which apolipoprotein (a) (apo (a)) is covalently bound. Apo(a) contains a variable number of kringle IV repeats, a kringle V and a protease domain. Serum/plasma Lp(a) concentrations are traditionally expressed as total particle mass in mg/L. Concern has arisen lately as flawed Lp(a) mass tests have masked its clinical utility.

The determinants of variability in Lp(a) composition were investigated, including the apo(a) size polymorphism, post-translational modifications -N- and O-glycosylation- and the lipid:protein ratio. Depending on the number of kringle IV-2 repeats, the theoretical protein content of the Lp(a) particle varies between 30 and 46 (w/w) %, which inescapably confounds Lp(a) mass measurements.

The authors advocate that reporting of Lp(a) particle concentrations in mass units is metrologically inappropriate and should be abandoned, as it results in systematically biased Lp(a) results. Enabling technology, such as mass spectrometry, allows unequivocal molecular characterization of the apo(a) measurand(s) and accurate quantitation of apo(a) in molar units, unaffected by apo(a) size polymorphism. To guarantee that Lp(a)/apo(a) tests are *fit-for-clinical-purpose,* basic metrology principles should be implemented upfront during test development.

1. Introduction

Already in the 1970s lipoprotein (a) (Lp(a))[1] was reported to be a risk factor for the development of cardiovascular disease [1–3], which resulted in the development of Lp(a) mass tests. Biochemically, Lp(a) is an LDL-like particle, that contains a single copy of the apolipoprotein (a) (apo(a)) covalently linked by a disulfide bridge to apolipoprotein B (apoB) [4]. Importantly, Apo(a) carries a number of kringle IV repeats, a kringle V (KV) and a protease domain, and varies widely in size due to a size polymorphism in kringle IV-2 (KIV_2) [5]. In traditional tests Lp(a) mass concentrations are measured using polyclonal antibodies against apo(a). While these tests are relatively easy to develop, they overlook the potentially large variation in apo(a) size which lead to inaccurate Lp (a) mass results.

The position of Lp(a) as a risk factor for cardiovascular disease (CVD) has not always been clear. In the early 1990s several prospective studies evaluating Lp(a) levels as risk markers provided inconsistent results. Out of nine studies, summarized in 1995 by Ridker [6], five showed positive associations, but four did not. Specifically, of the large, well-designed studies, Positive associations between Lp(a) concentrations and coronary heart disease as well as risk of myocardial infarction were found in the Lipid Research Clinics Coronary Primary Prevention Trials (LRC-CPPT) [7] and Gottingen Risk Incidence and Prevalence Study (GRIPS) [8], respectively, while no association between Lp(a) and acute myocardial infarction was observed in the Physicians Health Study (PHS) [9]. These results invoked a major discussion on the

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[1] Lp(a) lipoprotein (a); apo apolipoprotein; KV kringle V; KIV_2 Kringle IV type2; CVD cardiovascular disease; LRC-SPPT Lipid Research Clinics Coronary Primary Prevention Trials; GRIPS Gottingen Risk Incidence and Prevalence Study; PHS Physicians Health Study; ASCVD atherosclerotic cardiovascular disease; CAVD calcific aortic valve disease; EFLM European Federation for Clinical Chemistry and Laboratory Medicine; APS analytical performance specifications; CV_{biol} biological variation; CV_a analytical variation; CV_g inter-individual biological variation; EQA external quality assessment; IFCC international federation for clinical chemistry and laboratory medicine; WHO world health organization; SI international system of quantities; IVDD EU IVD directive; IVDR EU IVD regulation; PL phospholipids; TG triglycerides; CE cholesteryl esters; FC free cholesterol; LC-MRM-MS liquid chromatography coupled to multiple reaction monitoring mass spectrometry; TE_a total allowable error; GWAS genome wide association studies; MRT mendelian randomized trials.

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clinical usefulness of Lp(a), and halted scientific interest in Lp(a) testing for more than a decade. Inadequate implementation of the metrological traceability concept as described in ISO 17511:2020 [10] and ignorance regarding the necessity of a unique, defined Lp(a) measurand contributed to poor analytical performance and incongruous reports on the clinical utility of Lp(a) as risk factor for atherosclerotic cardiovascular diseases (ASCVD) and calcific aortic valve disease (CAVD) [6,9,11].

Recent evidence at the genetic level from genome-wide association studies (GWAS) and mendelian randomization trials (MRT) clearly indicates that the *LPA* gene is causally associated with CVD [2], and that apo(a) is a major risk factor for CVD. These insights have triggered renewed interest in Lp(a) testing, and resulted in the recent development of Lp(a) lowering medication [12,13]. Therefore, next generation Lp(a) tests should enable correct identification of high-risk patients that would benefit from Lp(a) lowering therapies and should allow monitoring of the efficacy and safety of patients' treatment. To adequately address residual CVD risk beyond LDL-cholesterol with personalized therapies, Lp(a) test results should be accurate within allowable limits of uncertainty. Notably, this renewed interest has resulted in the establishment of an International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) working group on Apolipoprotein Quantitation, which is establishing a Reference Measurement System for standardization of Lp(a) tests in molar units [14]. Here, we discuss the relevance of proper implementation of the metrological traceability concept during Lp(a) test development and the absolute necessity to unequivocally define the molecular form intended to be measured in the calibration hierarchy. To fully unmask the clinical utility of Lp(a) and exploit the potential of Lp(a) testing, accurate Lp(a) or apo(a) test results are key because of the tight interdependency between analytical and clinical performance of a medical test.

2. Analytical performance of contemporary Lp(a) tests

Medical tests should be *fit-for-clinical-purpose*, to ensure that they contribute to intended patient management and patient outcome [15]. The Test Evaluation framework developed by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Test Evaluation working group considers five key elements that guide the development from promising biomarker to useful medical test [15]. There is a dynamic interrelation between unmet clinical needs in specific patient groups (such as ASCVD patients with residual risk due to hyperlipoproteinemia (a)), the clinical pathway, the analytical and clinical performance, the clinical and cost-effectiveness, and the broader impact of medical tests. Clinical needs and the intended use of the medical test in the clinical pathway should drive each of the components of test evaluation [16]. Ideally, analytical and clinical performance should be predefined so that a medical test will be developed that fulfils the performance requirements needed. This can be an iterative process because analytical and clinical performance are interrelated, as presented by the cog wheels in the EFLM Test Evaluation framework [15,16].

The EFLM has endorsed a hierarchy of three levels of desirable analytical performance specifications (APS) for medical tests [17,18]. Ideally, APS are based on clinical outcome studies, but in practice these are often not available [19]. Frequently, APS are deduced from biological variation data [20], and the least favourable level is based on the *state-of-the art* performance. Because outcome based data is not available for Lp(a) at this time, we here investigate the available data on biological variation.

According to Fraser, one of the founders of the biological variation concept, estimates of within-subject biological variation are independent of (*a*) the population examined, (*b*) the number and age of the study participants, (*c*) the setting where the study was conducted, (*d*) the health status of the subjects, (*e*) the sampling scheme /duration used, and (*f*) the analytical variability of the methodology used. However, as a result of the wide variation of published average biological variation estimates (CV_{biol}) for Lp(a), Fraser's points of departure do not seem to be universally valid across different Lp(a) studies and populations. Our group published biological variation data that (a) illustrate concentration dependency of analytical variation (CV_a) (from 1.3% to 8.4%) and CV_{biol} (from 5.7% to 29.4%) across the Lp(a) concentration range (from lower detection limit up to 2500 mg/L), implicating the inadequacy of using average CV_{biol} and CV_a values for Lp(a); (*b*) explain the controversy in the literature regarding intraindividual biological variability of Lp(a) and (*c*) underscore the fact that the intraindividual biological variability of Lp(a) is greater than previously believed, especially in the low concentration range [21].

This contrasts with a recent Lp(a) biological variation study which advocates for an average allowable CV_a of 3.8%, an average allowable bias of 34% and critical differences ranging from −22.6 to +29.2% [22]. Although compliant with the EFLM Biological Variation Data Critical Appraisal Checklist, this study has limitations due to 25% drop outs in the lowest concentration range (mainly specimens with Lp(a) concentrations below the lower detection limit of 7 nmol/L of the Roche Lp(a) test used) and due to absence of samples in the highest Lp(a) range (from > 300 up to 1000 nmol/L) [23]. However, facing the enormous interindividual concentration differences for Lp(a), Fraser's approach of calculating average indices for desirable imprecision and bias, and critical differences on the basis of average CV_a, CV_{biol}, and inter-individual biological variation (CV_g) values, does not seem to be ubiquitously valid for Lp(a), implying the need for more individualized indices [19].

Most medical laboratories measure Lp(a) mass using automated immunoassays with polyclonal antibodies that are directed against apo(a). When polyclonal apo(a) antibodies are used, they bind to the KIV_2 section of apo(a) as well, which may be larger or smaller, depending on the number of KIV_2 repeats. Apo(a) with more KIV_2 will then result in a higher signal than apo(a) with fewer KIV_2, thus introducing measurement bias. Accredited labs, compliant with e.g. ISO 15189:2012, regularly participate in EQA-surveys that give insight in interlaboratory variation. SKML, the Dutch External Quality Assessment (EQA) organization, carries out EQA-surveys with fresh frozen human samples for serum lipids, apoA-I, apo B and Lp(a) in the Netherlands. To evaluate Lp(a) test result variability among different laboratories and manufacturers, the Lp(a) EQA data of 2018 are presented. In total 17 laboratories participated with nearly complete data sets, which comprised 11 rounds of two blinded samples each. Each of the blinded samples was included twice in the EQA-survey: i.e., in the first respectively in the second half of the year. A scatterplot of the Lp(a) mass results produced by individual labs, stratified by in-vitro diagnostics (IVD) manufacturer, is shown in Fig. 1. Large inter-laboratory variations exist, which are partially, but certainly not entirely, attributed to differences between IVD-manufacturers. The overall interlab variation ranges from 16.4% to 32.1% at Lp(a) levels of ~ 150 to 450 mg/L, whereas the interlab variations were typically smaller within manufacturers. These data demonstrate that in identical EQA-specimens Lp(a) mass results scatter from 150 to 300 mg/L and from 250 to 450 mg/L across labs. Interestingly, Scharnagl et al. recently compared six commercial immunoassays, and also found substantial differences in mass results between the Lp(a) tests [11], despite the availability of WHO-IFCC reference material and a reference measurement system for apo(a) that should aid in standardization efforts.

3. Metrological traceability and standardization of Lp(a) anno 2020

In a global society, it is of utmost importance that medical test results and treatment decisions are based on standardized (or harmonized) reference intervals and/or decision limits. To make this happen, the total diagnostic testing process, encompassing the preanalytical, analytical and postanalytical phases, should be considered [24]. In addition, comparability of test results produced by end-users can only

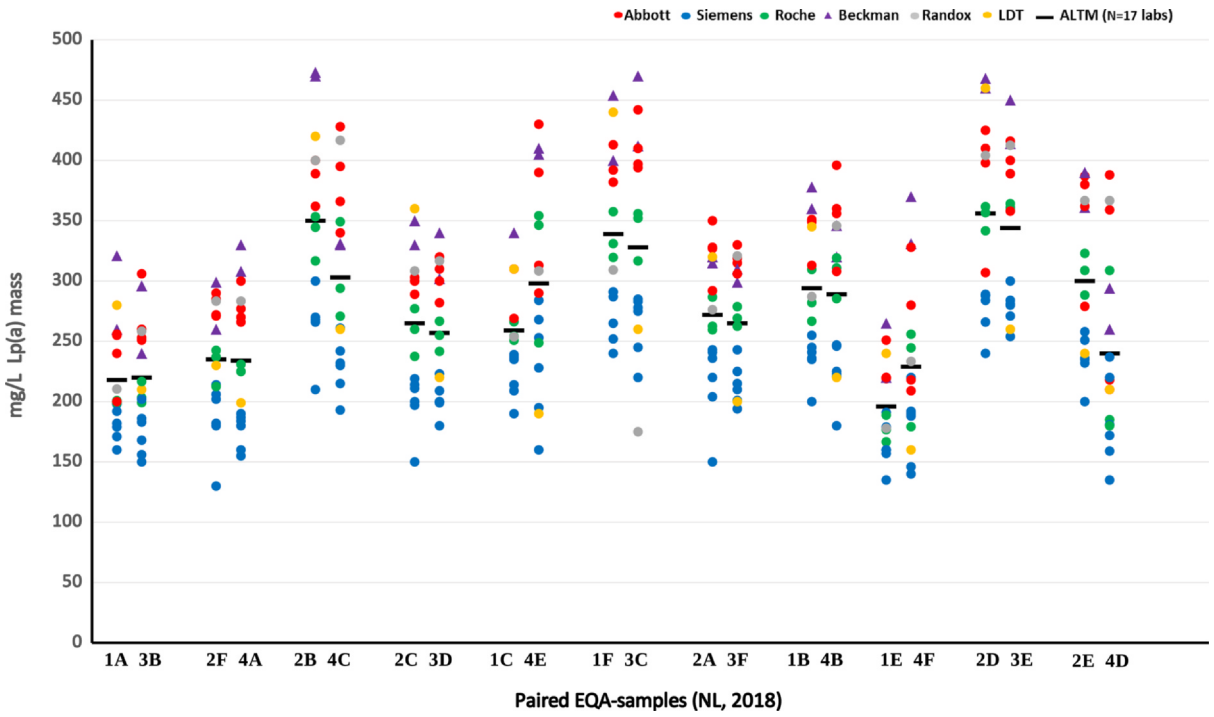


Fig. 1. National External Quality Assessment -results of Lp(a) surveys held in 2018 in the Netherlands. In total 17 accredited laboratories participated with nearly complete data sets, which comprised 11 rounds of two blinded samples each. EQA-samples were analyzed with 2-weekly intervals. Each of the blinded samples was included twice in the EQA-survey: i.e., in the first respectively in the second half of 2018, to evaluate independent duplicates within a year. A scatterplot of Lp(a) mass results (in mg/L) produced by individual labs and stratified by IVD-manufacturer is presented (manufacturers have different color codes). The All Lab Total Mean (ALTM) is presented with a black horizontal stripe. The overall interlab variation of Lp(a) mass tests ranges from 16.4% to 32.1% at Lp(a) levels of ~150 to 450 mg/L, Siemens demonstrating a negative bias and Abbott and Beckman a positive bias compared to the ALTM. The Roche Lp(a) test is closest to the ALTM.

be achieved through implementation of the concept of metrological traceability, which 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 the measurement uncertainty'* [10,25]. While major efforts have been initiated to implement the metrological traceability concept through harmonization or standardization efforts by e.g. the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and World Health Organization (WHO), adoption and implementation of test standardization by the IVD-manufacturers under the current EU IVD Directive 98/79/EC is slow and daunting. A system of self-declaration is used by manufacturers to comply with current the EU IVDD and bring their medical tests to the EU-market. The importance of metrological traceability of test results within allowable measurement uncertainty is not always recognized and acknowledged by the stakeholders involved [26]. Once the future IVD regulation 2017/746 enters into force per May 2022, extensive clinical evidence will be required, encompassing data on scientific validity, analytical and clinical performance, before tests can enter the EU-market.

The concept of metrological traceability is outlined in ISO guideline 17511:2020. An example of a traceability chain, as described in ISO 17511:2020, is shown in Fig. 2. Ideally, each analyte should have a full traceability chain in place that allows traceability to the units of the international system of quantities (SI). However, the development of complete reference measurement systems is challenging and demands extensive collaboration between reference institutes, metrology institutes, (candidate) reference laboratories, academia and IVD-industry. Also, to realize SI-traceability of test results, both a reference measurement procedure and primary reference materials have to be established and internationally endorsed. So far, for many tests, only incomplete reference measurement systems exist as no higher order reference materials and/or methods are in place.

More than 30 years ago, the IFCC-WHO acknowledged that a reference measurement system was required to reduce inter-lab variation of Lp(a) measurements and harmonize Lp(a) tests. A first IFCC working group on standardization of Lp(a) was established in the 1990s [27–29] in which a secondary reference material, SRM2B was developed. SRM2B is a lyophilized human serum pool with preservatives, that was selected out of four manufactured Lp(a) preparations as the material providing the best harmonization results in 27 Lp(a) tests. Value assignment of SRM2B was performed through KIV_2 independent ELISA measurements that were calibrated against two individually freshly isolated Lp(a) preparations from a donor exhibiting a single apo (a) isoform. The absolute mass of these isolates was determined using amino acid analysis [27]. However, when SRM2B was used to achieve traceability within the allowable measurement uncertainty, inter-laboratory CVs of up to 31% were still reported for the measurement of 30 fresh frozen serum samples with 22 assays [30]. It was already then acknowledged that these incongruent results are likely caused by variation of the measurand in patients' specimens as compared to calibrators, and the use of polyclonal antibodies, both contributing to kringle-dependent test results.

In a recent study, the effects of KIV_2 number on the reported results were assessed using an immunoassay which was KIV_2 dependent respectively KIV_2 independent [31]. Tsimikas et al. clearly demonstrated tilting of the regression line at the calibration point, with lower Lp(a) concentrations being overestimated by kringle dependent tests, and higher concentrations being underestimated by kringle dependent tests [31].

To minimize the kringle-dependence of immunoassays and keep error within allowable limits of measurement uncertainty, a calibration strategy was developed by some IVD manufacturers in which five calibrators were selected over a range of apo(a) concentrations. The higher concentrations are expected to have a relatively low number of KIV_2 repeats, while the lower concentrations have a higher number of kringle IV-2 repeats. Each of the five calibrators would then be made

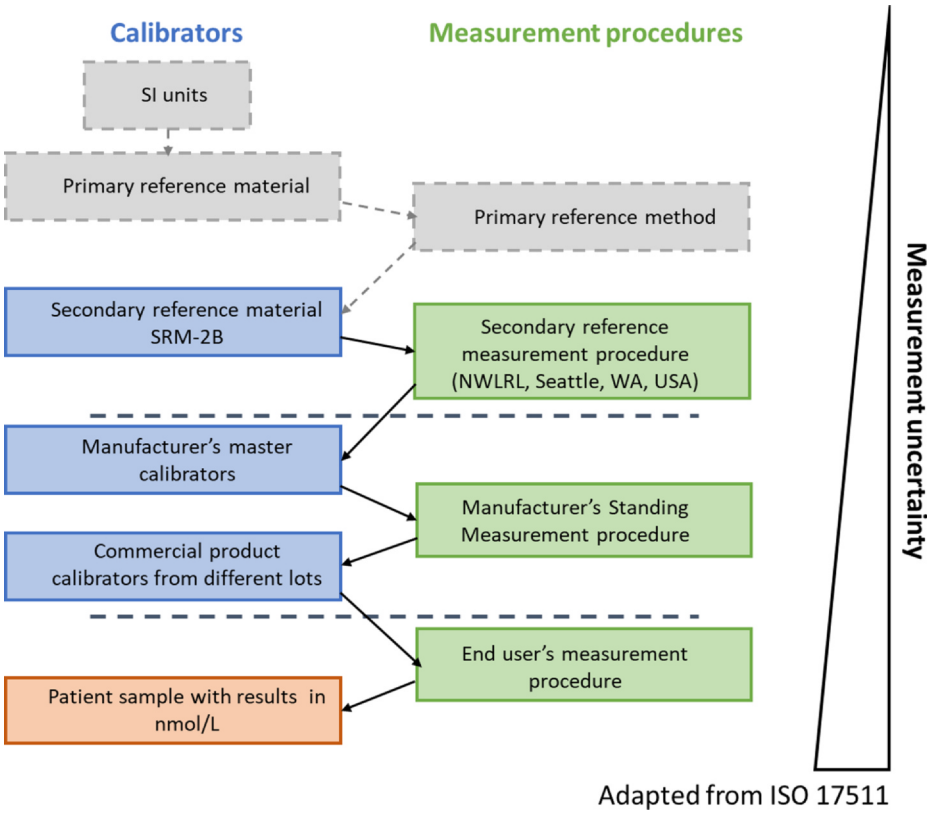


Fig. 2. Metrological traceability chain as outlined in ISO 17511:2020. Currently, tests for apo(a) can be traceable to WHO-IFCC secondary reference material SRM2B (blue), through an ELISA-based KIV_2 independent method (green). However, the top of the traceability chain is not in place (grey). To ensure SI-traceability primary reference materials and a higher order, KIV_2 independent reference measurement procedure are needed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

traceable to WHO-IFCC reference material SRM2B by ELISA using a KIV_2 independent reference measurement procedure. To confirm traceability, the correlation between the KIV_2 independent reference measurement procedure and the newly calibrated immunoassays was assessed using 80 well characterized samples with Lp(a) concentrations ranging from 8.7 to 276 nmol/L [27,32]. When 42 analytical systems were calibrated and evaluated using this procedure, an average inter-method CV of 5.5% could be achieved [33], demonstrating equivalence of results among manufacturers. However, this method of harmonization relies entirely on a reference measurement procedure run by a single laboratory, and as such is fragile and not sustainable enough for worldwide standardization.

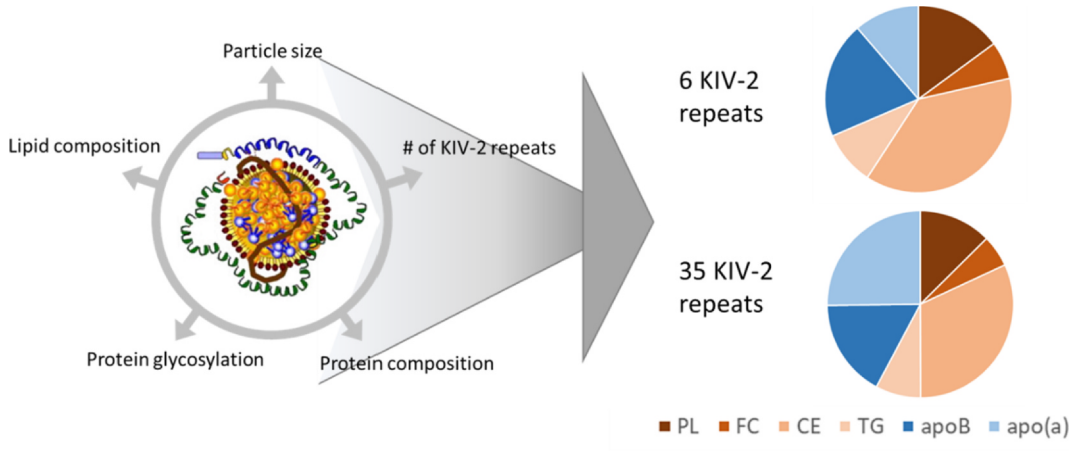
4. Determinants of variation in Lp(a) particle composition

Proper standardization of medical tests starts with defining the measurand, i.e. the starting point of the calibration hierarchy. According to the vocabulary international de metrology, the measurand is defined as *'a quantity intended to be measured'* [25], and should include both the analyte of interest as well as the matrix to be used. It might seem rather straightforward to define a protein measurand, but this was true in the era of the one-gene-one-protein paradigm [34]. Nowadays, it is known that a protein does not exist in a single molecular form, but may be present in many proteoforms [35]. Therefore, the definition of a measurand at the level of a particle (such as Lp(a)) or even a single protein (such as apo(a)) may not be sufficient. As stated in the introduction, Lp(a) is often measured in mass units, specifically mg/L, where Lp(a) mass reflects the mass of the entire Lp(a) particle. However, this is a poorly defined measurand, as Lp(a) does not have a single mass, but is rather a continuum of particles with variable masses. The Lp(a) particles hold a lipid core of cholesteryl esters (CE) and triglycerides (TG) surrounded by free cholesterol (FC), phospholipids (PL), apoB and apo(a) [36]. Variation in mass content may occur in each of these components.

As stated previously, a protein is likely to present itself in multiple proteoforms, and this holds true for both proteins associated with Lp(a). Specifically, it is well known that apo(a) is formed by an inactive C-terminal protease like domain and a kringle five (KV) domain, as well as a variable number of copies of the plasminogen-like kringle four (KIV) domain [5]. Each of ten KIV types is present once, with the exception of KIV_2, of which minimum 3 and maximum 40 copies may be present [2,33,37,38]. Moreover, several genetic variants with effects on the atherogenicity of Lp(a) have been reported [39,40].

Besides the variable amino acid length due to the KIV_2 polymorphism of apo(a), its mass also varies due to post-translational modifications, specifically N- and O-linked glycosylation. The asparagine (N) sites of N-glycosylation may be predicted based on the consensus sequence N-X-T/S, where X may be any amino acid except proline [41]. O-glycosylation occurs on serine (S) or threonine (T) amino acid residues, but the exact sites cannot be predicted. Apo(a) has 11 predicted N-glycosylation sites on the constant KIV and KV regions, as well as one N-glycosylation site on each of the KIV_2 domains. Variation in glycosylation can result in very high variation in mass, as each of the sites (both N- and O-glycosylation sites) may or may not be occupied, indicated by the glycosylation macroheterogeneity. Moreover, the exact glycan structures present at a particular site of glycosylation may vary largely between sites. This is coined glycosylation microheterogeneity. Because both macro- and microheterogeneity may vary between individuals, glycosylation may have a large impact on the mass of apo(a). The size of O-glycans may vary from approximately 200 Da (single GalNAc) to > 2000 Da, while N-glycans are often larger with masses ranging from approximately 1200 Da to > 3500 Da for tetra-antennary glycans. Similarly, the occupancy or inoccupancy of a singly glycosylation site may result in a mass variation of up to 3500 Da per glycosylation site for a large tetraantennary N-glycan [42]. Highly glycosylated proteins such as mucins may carry 50–80 of their weight in glycans, depending on the glycan heterogeneity.

A proteins' glycosylation can be studied using modern technology, specifically liquid chromatography (LC) and/or mass spectrometry (MS) [43,44]. Thus far, one study characterized apo(a) glycans [45]. In



Kringles IV-2	Lp(a) mass (kDa)	PL (%)	FC (%)	CE (%)	TG (%)	apoB (%)	apo(a) (kDa)	apo(a) (%)	lipids (%)	protein (%)
6	2821	14.9	6.5	37.8	9.4	20.1	319	11.3	68.6	31.4
10	2893	14.6	6.4	36.8	9.1	19.6	391	13.5	66.9	33.1
20	3074	13.7	6.0	34.7	8.6	18.5	572	18.6	62.9	37.1
30	3254	12.9	5.7	32.7	8.1	17.5	752	23.1	59.4	40.6
35	3344	12.6	5.5	31.8	7.9	17.0	842	25.2	57.8	42.2

Fig. 3. Theoretical model of Lp(a) mass and compositional variation depending on apo(a) KIV_2 size polymorphism based on literature. Lp(a) particle mass is dependent on the lipid: protein composition and amount, the apo(a) size polymorphism, and N- and O-glycosylation of apo(a) and apo B (upper left). Based on the variation in number of KIV_2 repeats in apo(a) alone, the distribution of lipid : protein in Lp(a) varies from 31 (w/w) % protein with 6 KIV_2 repeats to 42 (w/w) % protein with 35 KIV_2 repeats (upper right and bottom), leading to 19% difference in Lp(a) mass. PL = phospholipids, FC = free cholesterol, CE = cholesteryl esters, TG = triglycerides.

this study, Lp(a) particles were purified from human EDTA plasma samples, and apo(a) was isolated after reduction of the disulphide bridge linking apo(a) and apoB. The N-glycans as well as the O-glycans on apo(a) were individually chemically released and analysed using both LC and Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry. The stoichiometry of O-glycans to N-glycans was determined to be 5:1, indicating that for each N-glycan, five O-glycans are present on apo(a). The major N-glycans attached to apo(a) are non-fucosylated biantennary structures with one or two sialic acids, while the O-glycans present were of core 1 type, decorated with 0, 1 or 2 sialic acids [45]. These results indicate an 'average' glycosylation profile, but intra- and inter-individual variation of apo(a) glycosylation profiles have not yet been studied, and therefore the potential contribution of variable protein glycosylation cannot be estimated. Given that large variability in a protein's glycosylation has a large impact on its tertiary structure and is often detrimental, as indicated by the severe phenotypes observed in patients with congenital disorders of glycosylation, it seems unlikely that variability larger than ~15% in average weight occurs. This is further validated by the good concordance of test results between pulsed-field gel electrophoresis (DNA) and Western blot (protein) [46].

Post-translational modifications, specifically glycosylation, have also been reported for apoB. ApoB contains 19 potential N-glycosylation sites, of which 17 have previously been reported to be occupied by high-mannose, complex and hybrid type N-glycans [47,48]. Specifically, the glycosylation on apoB was determined to be approximately 4–10% of its total weight [49], with the most abundant glycans being the monosialylated biantennary glycans (29.2%), bisialylated biantennary glycans (23.6%), nonsialyated biantennary glycans (7.2%), and high mannose glycans (> 15.5%) [47]. Interestingly, glycosylation patterns of apoB were reported to be rather consistent between normolipidemic, hypertriglyceridemic and hypercholesterolemic individuals [47], but true biological variation data on apoB glycosylation are, similar to apo(a), lacking.

Besides variation in the Lp(a) associated proteins, the particles' lipid content should also be assessed. It is widely acknowledged that the lipid content of the Lp(a) particle is highly similar to that of LDL particles. Moreover, a high correlation was observed between Lp(a) and LDL density composition in individuals, indicating similar variation in lipid composition [50]. Shen et al. previously studied the lipid composition of LDL [51], and reported besides 21% protein, 22% phospholipids (PL), 11% triglycerides (TG), 37% cholesterol esters (CE) and 8% free cholesterol (FC). No biological variation or measurement uncertainty were reported, but rather similar results were obtained for the lipid composition of Lp(a) as determined by Kostner et al [36]. Further studies investigating the inter-individual variability of the lipid content of Lp(a) particles, as well as the variability in relation to apo(a) size are warranted to better assess its atherogenic properties, and to provide better estimates of the role of lipid variability in Lp(a) testing.

4.1. Effects of variation in Lp(a) mass on Lp(a) test results

To assess the extent of variation in Lp(a) composition that may be expected, we developed a model. In this model, we assumed an 'average' LDL particle core and simulated the potential effects of variation in apo(a) on Lp(a) mass. Based on the previously reported LDL composition, the lipid core of Lp(a) on average consists of 653 molecules PL, 475 molecules FC, 1310 molecules CE and 298 molecules TG [51], resulting in a lipid core of each Lp(a) particle with an approximate molecular mass of 1934 kDa. In addition, the amino acid sequence of apoB weighs 531 kDa and is decorated with on average ~37 kDa of glycans. Therefore, based on this average model, the 'LDL-core' of Lp(a) has a particle mass of ~2502 kDa. Assuming this mass is constant, independent of the apo(a) size, the total Lp(a) particle mass and composition can be calculated. Taking into account the average apo(a) glycosylation as described in Section 4, an Lp(a) particle containing only six kringle IV_2 repeats per apo(a) is calculated to have a particle mass of 2,821 kDa with a lipid portion of 69% (w/w), apoB content of 20% (w/w) and apo(a) content of 11% (w/w) (Fig. 3). However, for a particle containing 35 KIV_2 kringles, the particle mass is 3,344 kDa with

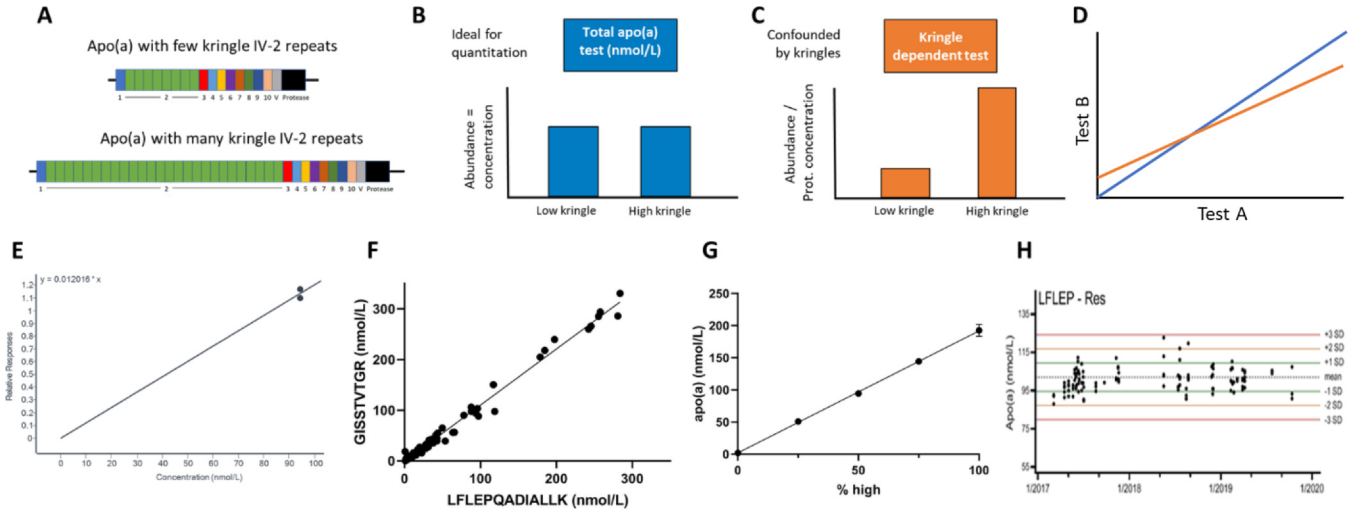


Fig. 4. Proteoform-independent, molar quantitation of serum apo(a) by mass spectrometry. Considering apo(a) with few (8) and many (26) KIV_2 repeats (A), quantitation of a serum sample with equal molar concentrations will result in the same molar apo(a) test result using a KIV_2 independent test (B), but not in a KIV_2 dependent test (C). Due to negative correlation between apo(a) KIV_2 repeats and apo(a) concentration, molar concentrations reported by kringle dependent tests are too high for concentrations below the calibrator level, and too low for concentrations above the calibrator level (D, blue = kringle independent tests, orange = kringle dependent test). Our in-house developed MS-based test for apo(a), currently has a single-point calibration that is traceable to SRM2B (E), and shows good interpeptide agreement (F). Linearity of the method was evaluated through admixing experiments (G). Robust and stable 2-year performance is illustrated in Levey-Jennings plot with internal quality control (H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a lipid portion of 58% (w/w), apoB portion of 17% (w/w) and apo(a) portion of 25% (w/w) (Fig. 3). These compositions largely agree with previous literature [31,36,52], but the apo(a) content varies, due to different size assumptions. Importantly, this model is based on average apo(a) glycosylation profiles in combination with an average LDL particle mass. Because substantial inter-individual variation in total plasma proteome glycosylation has been reported [53–55], inter-individual variation in apo(a) could further add to the effect of apo(a) size on Lp(a) mass. Based on our model, the Lp(a) particle containing 35 KIV_2 repeats would be 19% heavier in mass than the particle containing just 6 KIV_2 repeats. Given that this variation is prompted just from the apo(a) variation, and the other components of the Lp(a) particle will likely also contribute, it should be obvious that the measurement uncertainty in Lp(a) mass tests can be substantial.

5. Towards accurate Lp(a) tests in the era of precision Medicine

To guarantee accuracy of test results, knowledge on metrology and the science of measurement is key. In case of Lp(a) mass tests, one should realize that the protein : lipid composition of Lp(a) particles varies widely, even within individuals. Therefore, the measurand intended to be measured in Lp(a) mass tests cannot be defined unequivocally, causing both measurement and diagnostic uncertainty with respect to risk classification and treatment of ASCVD patients. Facing residual CVD needs beyond LDL-cholesterol in high risk ASCVD patients on one hand and the slow adoption and implementation of the metrological traceability concept under the current IVDD on the other hand, a wakeup call to laboratory specialists and IVD-manufacturers to take the metrological traceability concept seriously is warranted! The time has come to perform molar apo(a) testing, with apo(a) test results reflecting the number of Lp(a) particles present in an individuals' serum/plasma, independent of the number of KIV_2 repeats, the degree of N- and O-glycosylation of apo(a) and the lipid composition of Lp(a)! It is therefore imperative that tests are developed that truly measure Lp(a)/apo(a) in a kringle IV_2 independent manner, and not simply make use of an imperfect correction factor [33].

It should be noted here that previous standardization of commercial Lp(a) tests was based on the ELISA "gold standard" method of Dr Marcovina. IVD-manufacturers who received certification through the Lp(a) certification program of Dr Marcovina produced accurate Lp(a) results within allowable uncertainty. While the commercial Lp(a) tests were likely not kringle independent, the results were mostly good enough to provide useful diagnostic information on the relation between Lp(a) and patient outcome. Given the overestimation of lower apo(a) concentrations and underestimation of higher apo(a) concentrations by KIV_2-dependent test [31], it is even likely that the association of Lp(a) with CVD is underestimated by such tests. Previous evidence therefore remains valid. However, negative results should likely be questioned. As the science of measurement evolves with enabling technology such as LC-MS/MS, we can now unequivocally define the apo(a) measurand and make Lp(a) or apo(a) test results truly traceable to SI.

Targeted quantitative proteomics by mass spectrometry (MS) using liquid chromatography coupled to multiple reaction monitoring MS (LC-MRM-MS) is ideally suited for the characterization of (apolipo) proteins at the molecular level. The technique was selected as method of the year in 2012 [56], and relies on the so called 'bottom-up proteomics' strategy [57], in which proteins are enzymatically converted to their characteristic peptides, which can then be quantified. By carefully selecting peptides (or glycopeptides) of interest, specific proteoforms can be monitored [44,58]. This enables selective quantitation of the measurands of interest at the molecular level. However, as genetic variation due to for instance SNPs cannot always be predicted from databases, it is imperative to monitor at least two (proteotypic) peptides per protein, to ensure accurate quantitation [59]. Within our laboratory, we have developed an analytically validated MS-based test for immunoassay-independent quantitation of serum apolipoproteins A-I, B, C-I, C-II, C-III and E [60], and were able to show long-term robust performance of the extended variant MS-based test, including apo(a) [61] (Fig. 4H). Using a semi-automated approach in combination with a 21-minute gradient nine apolipoproteins may now be quantified in up to 350 samples per week on a single MS instrument. Such numbers may not be sufficient for high-volume laboratories with daily sample loads of thousands of samples, but the LC-MRM-MS technology is currently evaluated for its potential as a Reference Measurement Procedure (RMP), enabling SI-traceability. Commercial KIV_2 independent methods can then be standardized through the traceability chain encompassing

the RMP, as outlined in Fig. 2.

KVI_2 independent quantitation of apo(a) by mass spectrometry was achieved through the selection of peptides that are specific for apo(a) and not present in the KIV_2 domain (Fig. 4). These peptides should also not be affected by known genetic variations [39] and post-translational modifications, such as glycosylation, and ideally do not carry labile amino acids that can be chemically converted [62]. Thus far, the most ideal peptide for MS-based quantitation of apo(a) has been LFLEPTQADIALLK, which is present in the plasminogen-like peptidase S1 domain [63]. Using this peptide, an R^2 correlation coefficient of 0.98 was obtained between the LC-MS based method and a kringle-independent ELISA method [63].

Within our laboratory, we integrated the molar quantitation of apo (a) in our multiplexed apolipoprotein test panel (54) through the KIV_2 independent peptides LFLEPTQADIALLK (quantifying peptide) and GISSTVTGR (qualifying peptide). These peptides are quantified in the same LC gradient, with peptide GISSTVTGR eluting much earlier in the gradient than LFLEPTQADIALLK, relative to their stable isotope labelled (C^{13},N^{15} arginine or C^{13},N^{15} lysine) internal standard analog. A single-donor native human serum calibrator with unknown KIV_2 repeats is used in the lab-developed MS-test, tentatively value-assigned for apo(a)) using the Roche molar TinaQuant immunoassay with claimed traceability to SRM2B. For the MS-based test, a single point calibration strategy was selected to circumvent non-linearity issues caused by tweaked calibrators in the immunoassay (information from the manufacturer). To extend the measuring range for apo quantitation and allow for simultaneous measurement of both high abundant apolipoproteins such as ApoA-I (μmol/L range) and low abundant apo(a) (nmol/L range), a solid phase extraction using OASIS HLB material was included during sample preparation. Robust performance was achieved with TE_a values ranging from 7% to 15% in five specimens with widespread concentration levels measured five times each during 5 different days ($n = 25$) according to CLSI EP-15 (unpublished data). The apo(a) linear range was determined to be at least from 2 to 244 nmol/L (Fig. 4G). A method comparison with the molar Lp(a) Roche TinaQuant test demonstrated acceptable relative bias of −16% across the measuring range. Quantitative bottom-up proteomics has the potential to produce unconfounded apo(a) results, traceable to SRM2B, through direct measurement of unique, proteotypic apo(a) peptide fragments. Our results and data from Lassman et al. [63] show that MS-based quantitation enables molecular characterization of the apo(a) measurands and accurate quantitation of apo(a) in molar units, unaffected by apo(a) size polymorphism and glycosylation through the selection of KIV_2 independent peptides. So far, the extended multiplex MS-based apo method has run steadily for $>$ two years (Fig. 4).

Global standardization requires a sustainable infrastructure that guarantees longitudinal traceability and accuracy in time and space. Therefore, a full metrological traceability chain should be established, encompassing well-characterized primary reference materials that are preferentially traceable to SI and a robust reference measurement procedure for selective protein/peptide measurement. Lately, mass spectrometry is considered an attractive technique for the development of reference measurement procedures. The technique allows protein characterization at the molecular level and combined with peptide-based primary reference materials, mass spectrometry could enable SI-traceability of test results. Importantly, preparation, characterization and accurate quantitation with traceability to SI is likely more feasible with peptide-based calibration than protein-based calibration [64]. To ensure the establishment of a global Reference Measurement System, the Scientific Division of the International Federation for Clinical Chemistry appointed a working group on Apolipoprotein quantitation by Mass Spectrometry (https://www.ifcc.org/ifcc-scientific-division/sd-working-groups/wg-apo-ms/), that should also enable formation of a network of Reference Laboratories [14].

6. Conclusions and future perspectives

Just over a decade ago, it was debated whether Lp(a), a highly atherogenic and heritable lipoprotein, was an independent ASCVD risk factor. The discordant results were attributable to both biological variation and analytical flaws. Clinical outcome studies by Ridker et al. in the nineties [6,9] brought confusion, reporting lack of clinical utility of Lp(a). Retrospectively it is now speculated that these studies might have been flawed by the apo(a) size polymorphism [11]. The recent, wider availability of improved Lp(a) tests coupled with data from observational population studies, GWAS and MRT presented evidence that Lp(a) is an independent risk factor for ASCVD, including stroke, coronary heart disease, peripheral arterial disease and CAVD [65].

The currently unharmonized Lp(a) mass results lead to variable detection rates of high risk ASCVD patients with Lp(a) excess and unequal treatment of patients requiring Lp(a)-lowering therapies. We here outline the absolute necessity for adequate implementation of the metrological traceability concept, especially in heterogeneous protein analytes such as apo(a). This requires a clear definition of the measurand intended to be measured and an internationally endorsed, well established calibration hierarchy that ensures anchoring of test results, within allowable limits of measurement uncertainty, to standards of higher order. Thus far, Lp(a) mass testing has been oversimplified, which resulted in poor test development since its discovery in 1963. Faults in Lp(a) mass tests include expressing Lp(a) concentration in mass units and ignorance of a potential 19% mass difference between an Lp(a) particle with 6 KIV_2 in apo(a) as compared to Lp(a) with 35 KIV_2 in apo(a), beyond neglecting variation caused by differences in apo(a) and apo B glycosylation as well as Lp(a) lipid content. This has resulted in defective calibration strategies, improperly correcting for the type of apo(a) isoforms present in used single or multi-calibrators and the immunoassay technology with its inherent shortcomings and its “blindness” for molecular forms.

The variability in protein mass outlined here should be a major concern for laboratory specialists and clinicians at a much larger scale, as it is now obvious that nearly all proteins exist in multiple molecular forms, the so-called proteoforms, each varying in mass. Therefore, laboratory specialists and IVD-industry should rethink their calibration strategy for accurate measurement of molecular forms of proteins, taking into account the interrelation between analytical and clinical performance of medical tests. Proteins should be quantified in such a way that the measurands are well defined, as a first step to enable intended metrological traceability to SI units. Mass spectrometry using LC-MRM-MS is ideally suited for the characterization of proteins at the molecular level, and should be considered for quantitation of specific proteoforms, especially in reference laboratories and eventually in medical laboratories.

We conclude that Lp(a) should be evaluated in terms of its apo(a) component and no longer in terms of Lp(a) mass. Lp(a) tests should unequivocally and accurately measure apo(a) in molar units. MS-based quantitation of the highly polymorphic apo(a) has potential as a higher order measurement procedure and a useful alternative to flawed immunoassay-based Lp(a) mass tests. Accurate, molar-based tests for apo (a) will certainly lead to increased trust and recommendation by clinicians [66], while also improving downstream patient management and patient outcome [65].

CRediT authorship contribution statement

L.R. Ruhaak: Conceptualization, Investigation, Writing - review & editing. **C.M. Cobbaert:** Conceptualization, Investigation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] G.M. Kostner, P. Avogaro, G. Cazzolato, E. Marth, G. Bittolo-Bon, G.B. Qunici, Lipoprotein Lp(a) and the risk for myocardial infarction, Atherosclerosis 38 (1–2) (1981) 51–61.

[2] B.G. Nordestgaard, A. Langsted, Lipoprotein (a) as a cause of cardiovascular disease: insights from epidemiology, genetics, and biology, J. Lipid Res. 57 (11) (2016) 1953–1975.

[3] G. Dahlen, K. Berg, T. Gillnas, C. Ericson, Lp(a) lipoprotein/pre-beta1-lipoprotein in Swedish middle-aged males and in patients with coronary heart disease, Clin. Genet. 7 (4) (1975) 334–341.

[4] J.J. Albers, H. Kennedy, S.M. Marcovina, Evidence that Lp[a] contains one molecule of apo[a] and one molecule of apoB: Evaluation of amino acid analysis data, J. Lipid Res. 37 (1) (1996) 192–196.

[5] J.W. McLean, J.E. Tomlinson, W.J. Kuang, D.L. Eaton, E.Y. Chen, G.M. Fless, A.M. Scanu, R.M. Lawn, cDNA sequence of human apolipoprotein(a) is homologous to plasminogen, Nature 330 (6144) (1987) 132–137.

[6] P.M. Ridker, An epidemiologic reassessment of lipoprotein(a) and atherothrombotic risk, Trends Cardiovasc. Med. 5 (6) (1995) 225–229.

[7] E.J. Schaefer, S. Lamon-Fava, J.L. Jenner, J.R. McNamara, J.M. Ordovas, C.E. Davis, J.M. Abolafia, K. Lippel, R.I. Levy, Lipoprotein(a) levels and risk of coronary heart disease in men, lipid Res. Clin. Coronary Primary Prevent. Trial, JAMA 271 (13) (1994) 999–1003.

[8] P. Cremer, D. Nagel, B. Labrot, H. Mann, R. Muche, H. Elster, D. Seidel, Lipoprotein Lp(a) as predictor of myocardial infarction in comparison to fibrinogen, LDL cholesterol and other risk factors: results from the prospective Gottingen Risk Incidence and Prevalence Study (GRIPS), Eur. J. Clin. Invest. 24 (7) (1994) 444–453.

[9] P.M. Ridker, C.H. Hennekens, M.J. Stampfer, A prospective study of lipoprotein(a) and the risk of myocardial infarction, JAMA 270 (18) (1993) 2195–2199.

[10] International, Organization, for, Standardization, ISO 17511:2020, In vitro diagnostic medical devices — Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples, ISO, Geneva, 2020.

[11] H. Scharnagl, T. Stojakovic, B. Dieplinger, H. Dieplinger, G. Erhart, G.M. Kostner, M. Herrmann, W. Marz, T.B. Grammer, Comparison of lipoprotein (a) serum concentrations measured by six commercially available immunoassays, Atherosclerosis 289 (2019) 206–213.

[12] N.J. Viney, C. Yeang, X. Yang, S. Xia, J.L. Witztum, S. Tsimikas, Relationship between "LDL-C", estimated true LDL-C, apolipoprotein B-100, and PCSK9 levels following lipoprotein(a) lowering with an antisense oligonucleotide, J. Clin. Lipidol. 12 (3) (2018) 702–710.

[13] M.L. O'Donoghue, S. Fazio, R.P. Giugliano, E.S.G. Stroes, E. Kanevsky, I. Gouni-Berthold, K. Im, A. Lira Pineda, S.M. Wasserman, R. Ceska, M.V. Ezhov, J.W. Jukema, H.K. Jensen, S.L. Tokgozoglu, F. Mach, K. Huber, P.S. Sever, A.C. Keech, T.R. Pedersen, M.S. Sabatine, Lipoprotein(a), PCSK9 inhibition, and cardiovascular risk, Circulation 139 (12) (2019) 1483–1492.

[14] C.M. Cobbaert, H. Althaus, I. Begcevic Brkovic, U. Ceglarek, S. Coassin, V. Delatour, L. Deprez, I. Dikaios, J. Dittrich, A.N. Hoofnagle, G.M. Kostner, F. Kronenberg, Z. Kuklenyik, U. Prinzing, H.W. Vesper, I. Zegers, L.R. Ruhaak, 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, Clin Chem. in press.

[15] A.R. Horvath, S.J. Lord, A. StJohn, S. Sandberg, C.M. Cobbaert, S. Lorenz, P.J. Monaghan, W.D. Verhagen-Kamerbeek, C. Ebert, P.M. Bossuyt, M. Test evaluation working group of the European federation of clinical chemistry laboratory, from biomarkers to medical tests: the changing landscape of test evaluation, Clin. Chim. Acta 427 (2014) 49–57.

[16] P.J. Monaghan, S.J. Lord, A. St John, S. Sandberg, C.M. Cobbaert, L. Lennartz, W.D. Verhagen-Kamerbeek, C. Ebert, P.M. Bossuyt, A.R. Horvath, C. Test evaluation working group of the European federation of clinical, M. Laboratory, biomarker development targeting unmet clinical needs, Clin. Chim. Acta 460 (2016) 211–219.

[17] S. Sandberg, C.G. Fraser, A.R. Horvath, R. Jansen, G. Jones, W. Oosterhuis, P.H. Petersen, H. Schimmel, K. Sikaris, M. Panteghini, Defining analytical performance specifications: consensus statement from the 1st strategic conference of the European federation of clinical chemistry and laboratory medicine, Clin. Chem. Lab. Med. 53 (6) (2015) 833–835.

[18] M. Panteghini, F. Ceriotti, G. Jones, W. Oosterhuis, M. Plebani, S. Sandberg, C. Task force on performance specifications in laboratory medicine of the European federation of clinical, M. Laboratory, strategies to define performance specifications in laboratory medicine: 3 years on from the Milan strategic conference, Clin. Chem. Lab. Med. 55 (12) (2017) 1849–1856.

[19] A.R. Horvath, P.M. Bossuyt, S. Sandberg, A.S. John, P.J. Monaghan, W.D. Verhagen-Kamerbeek, L. Lennartz, C.M. Cobbaert, C. Ebert, S.J. Lord, C. Test evaluation working group of the European federation of clinical, M. Laboratory, Setting analytical performance specifications based on outcome studies - is it possible? Clin. Chem. Lab. Med. 53 (6) (2015) 841–848.

[20] C. Ricos, V. Alvarez, C. Perich, P. Fernandez-Calle, J. Minchinela, F. Cava, C. Biosca, B. Boned, M. Domenech, J.V. Garcia-Lario, M. Simon, P.F. Fernandez, J. Diaz-Garzon, E. Gonzalez-Lao, Rationale for using data on biological variation, Clin. Chem. Lab. Med. 53 (6) (2015) 863–870.

[21] C. Cobbaert, J.C. Arentsen, P. Mulder, N. Hoogerbrugge, J. Lindemans, Significance of various parameters derived from biological variability of lipoprotein(a), homocysteine, cysteine, and total antioxidant status, Clin. Chem. 43 (10) (1997) 1958–1964.

[22] N. Clouet-Foraison, S.M. Marcovina, E. Guerra, A.K. Aarsand, A. Coskun, J. Diaz-Garzon, P. Fernandez-Calle, S. Sandberg, F. Ceriotti, A. Carobene, Analytical performance specifications for lipoprotein(A), apolipoprotein B-100, and apolipoprotein A-I using the biological variation model in the EuBIVAS population, Clin. Chem. 66 (5) (2020) 727–736.

[23] A.K. Aarsand, T. Roraas, P. Fernandez-Calle, C. Ricos, J. Diaz-Garzon, N. Jonker, C. Perich, E. Gonzalez-Lao, A. Carobene, J. Minchinela, A. Coskun, M. Simon, V. Alvarez, W.A. Bartlett, P. Fernandez-Fernandez, B. Boned, F. Braga, Z. Corte, B. Aslan, S. Sandberg, C. European federation of clinical, V. Laboratory medicine working group on biological, Task, D. Finish group for the biological variation, the biological variation data critical appraisal checklist: a standard for evaluating studies on biological variation, Clin. Chem. 64 (3) (2018) 501–514.

[24] M. Plebani, Harmonization in laboratory medicine: the complete picture, Clin. Chem. Lab. Med. 51 (4) (2013) 741–751.

[25] JCGM200:2012 International vocabulary of metrology – Basic and general concepts and associated terms.

[26] C. Cobbaert, N. Smit, P. Gillery, Metrological traceability and harmonization of medical tests: a quantum leap forward is needed to keep pace with globalization and stringent IVD-regulations in the 21st century!, Clin. Chem. Lab. Med. 56 (10) (2018) 1598–1602.

[27] F. Dati, J.R. Tate, S.M. Marcovina, A. Steinmetz, C. International Federation of Clinical, M. Laboratory, I.W.G.f.L.A. standardization, First WHO/IFCC international reference reagent for lipoprotein(a) for Immunoassay–Lp(a) SRM 2B, Clin. Chem. Lab. Med. 42 (6) (2004) 670–676.

[28] J.R. Tate, K. Berg, R. Couderc, F. Dati, G.M. Kostner, S.M. Marcovina, N. Rifai, I. Sakurabayashi, A. Steinmetz, International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) standardization project for the measurement of lipoprotein(a). Phase 2: selection and properties of a proposed secondary reference material for lipoprotein(a), Clin. Chem. Lab. Med. 37 (10) (1999) 949–958.

[29] J.R. Tate, N. Rifai, K. Berg, R. Couderc, F. Dati, G.M. Kostner, I. Sakurabayashi, A. Steinmetz, International Federation of Clinical Chemistry standardization project for the measurement of lipoprotein(a). Phase I. Evaluation of the analytical performance of lipoprotein(a) assay systems and commercial calibrators, Clin. Chem. 44 (8 Pt 1) (1998) 1629–1640.

[30] S.M. Marcovina, J.J. Albers, A.M. Scanu, H. Kennedy, F. Giaculli, K. Berg, R. Couderc, F. Dati, N. Rifai, I. Sakurabayashi, J.R. Tate, A. Steinmetz, Use of a reference material proposed by the international federation of clinical chemistry and laboratory medicine to evaluate analytical methods for the determination of plasma lipoprotein(a), Clin. Chem. 46 (12) (2000) 1956–1967.

[31] S. Tsimikas, S. Fazio, N.J. Viney, S. Xia, J.L. Witztum, S.M. Marcovina, Relationship of lipoprotein(a) molar concentrations and mass according to lipoprotein(a) thresholds and apolipoprotein(a) isoform size, J. Clin. Lipidol.. 12 (5) (2018) 1313–1323.

[32] S.M. Marcovina, M.L. Koschinsky, J.J. Albers, S. Skarlatos, Report of the national heart, lung, and blood institute workshop on lipoprotein(a) and cardiovascular disease: recent advances and future directions, Clin. Chem. 49 (11) (2003) 1785–1796.

[33] S.M. Marcovina, J.J. Albers, Lipoprotein (a) measurements for clinical application, J. Lipid Res. 57 (4) (2016) 526–537.

[34] G.W. Beadle, Biochemical genetics, Chem. Rev. 37 (1) (1945) 15–96.

[35] R. Aebersold, J.N. Agar, I.J. Amster, M.S. Baker, C.R. Bertozzi, E.S. Boja, C.E. Costello, B.F. Cravatt, C. Fenselau, B.A. Garcia, Y. Ge, J. Gunawardena, R.C. Hendrickson, P.J. Hergenrother, C.G. Huber, A.R. Ivanov, O.N. Jensen, M.C. Jewett, N.L. Kelleher, L.L. Kiessling, N.J. Krogan, M.R. Larsen, J.A. Loo, R.R. Ogorzalek Loo, E. Lundberg, M.J. MacCoss, P. Mallick, V.K. Mootha, M. Mrksich, T.W. Muir, S.M. Patrie, J.J. Pesavento, S.J. Pitteri, H. Rodriguez, A. Saghatelian, W. Sandoval, H. Schluter, S. Sechi, S.A. Slavoff, L.M. Smith, M.P. Snyder, P.M. Thomas, M. Uhlen, J.E. Van Eyk, M. Vidal, D.R. Walt, F.M. White, E.R. Williams, T. Wohlschlager, V.H. Wysocki, N.A. Yates, N.L. Young, B. Zhang, How many human proteoforms are there? Nat. Chem. Biol. 14 (3) (2018) 206–214.

[36] G.M. Kostner, A. Ibovnik, H. Holzer, H. Grillhofer, Preparation of a stable fresh frozen primary lipoprotein[a] (Lp[a]) standard, J. Lipid Res. 40 (12) (1999) 2255–2263.

[37] Y.Y. van der Hoek, M.E. Wittekoek, U. Beisiegel, J.J. Kastelein, M.L. Koschinsky, The apolipoprotein(a) kringle IV repeats which differ from the major repeat kringle are present in variably-sized isoforms, Hum. Mol. Genet. 2 (4) (1993) 361–366.

[38] C. Lackner, J.C. Cohen, H.H. Hobbs, Molecular definition of the extreme size polymorphism in apolipoprotein(a), Hum. Mol. Genet. 2 (7) (1993) 933–940.

[39] S. Coassin, G. Erhart, H. Weissensteiner, M. Eca Guimaraes de Araujo, C. Lamina, S. Schonherr, L. Forer, M. Haun, J.L. Losso, A. Kottgen, K. Schmidt, G. Utermann, A. Peters, C. Gieger, K. Strauch, A. Finkenstedt, R. Bale, H. Zoller, B. Paulweber, K.U. Eckardt, A. Huttenhofer, L.A. Huber, F. Kronenberg, A novel but frequent variant in LPA KIV-2 is associated with a pronounced Lp(a) and cardiovascular risk reduction, Eur. Heart J. 38 (23) (2017) 1823–1831.

[40] S. Coassin, S. Schonherr, H. Weissensteiner, G. Erhart, L. Forer, J.L. Losso, C. Lamina, M. Haun, G. Utermann, B. Paulweber, G. Specht, F. Kronenberg, A comprehensive map of single-base polymorphisms in the hypervariable LPA kringle IV type 2 copy number variation region, J. Lipid Res. 60 (1) (2019) 186–199.

[41] R.D. Marshall, The nature and metabolism of the carbohydrate-peptide linkages of

glycoproteins, Biochem. Soc. Symp. 40 (1974) 17–26.
[42] A. Varki, R.D. Cummings, J.D. Esko, P. Stanley, G.W. Hart, M. Aebi, A.G. Darvill, T. Kinoshita, N.H. Packer, J.H. Prestegaard, R.L. Schnaar, P. Seeberger, Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY), 2015–2017.
[43] L.R. Ruhaak, G. Xu, Q. Li, E. Goonatilleke, C.B. Lebrilla, Mass spectrometry approaches to glycomic and glycoproteomic analyses, Chem. Rev. 118 (17) (2018) 7886–7930.
[44] L.R. Ruhaak, The use of multiple reaction monitoring on QQQ-MS for the analysis of protein- and site-specific glycosylation patterns in serum, Methods Mol. Biol. 1503 (2017) 63–82.
[45] B. Garner, A.H. Merry, L. Royle, D.J. Harvey, P.M. Rudd, J. Thillet, Structural elucidation of the N- and O-glycans of human apolipoprotein(a): role of o-glycans in conferring protease resistance, J. Biol. Chem. 276 (25) (2001) 22200–22208.
[46] H.G. Kraft, S. Kochl, H.J. Menzel, C. Sandholzer, G. Utermann, The apolipoprotein (a) gene: a transcribed hypervariable locus controlling plasma lipoprotein (a) concentration, Hum. Genet. 90 (3) (1992) 220–230.
[47] B. Garner, D.J. Harvey, L. Royle, M. Frischmann, F. Nigon, M.J. Chapman, P.M. Rudd, Characterization of human apolipoprotein B100 oligosaccharides in LDL subfractions derived from normal and hyperlipidemic plasma: deficiency of alpha-N-acetylneuraminyllactosyl-ceramide in light and small dense LDL particles, Glycobiology 11 (10) (2001) 791–802.
[48] A. Harazono, N. Kawasaki, T. Kawanishi, T. Hayakawa, Site-specific glycosylation analysis of human apolipoprotein B100 using LC/ESI MS/MS, Glycobiology 15 (5) (2005) 447–462.
[49] C.Y. Yang, F.S. Lee, L. Chan, D.A. Sparrow, J.T. Sparrow, A.M. Gotto Jr., Determination of the molecular mass of apolipoprotein B-100. A chemical approach, Biochem. J. 239 (3) (1986) 777–780.
[50] D.L. Rainwater, M.J. Ludwig, S.M. Haffner, J.L. VandeBerg, Lipid and lipoprotein factors associated with variation in Lp(a) density, Arterioscler. Thromb. Vasc. Biol. 15 (3) (1995) 313–319.
[51] B.W. Shen, A.M. Scanu, F.J. Kezdy, Structure of human serum lipoproteins inferred from compositional analysis, Proc. Natl. Acad.. Sci. USA 74 (3) (1977) 837–841.
[52] J.P. McConnell, P.A. Guadagno, T.D. Dayspring, D.M. Hoefner, D.L. Thiselton, G.R. Warnick, W.S. Harris, Lipoprotein(a) mass: a massively misunderstood metric, J. Clin. Lipidol. 8 (6) (2014) 550–553.
[53] A.G. Gebrehiwot, D.S. Melka, Y.M. Kassaye, I.F. Rehan, S. Rangappa, H. Hinou, T. Kamiyama, S.I. Nishimura, Healthy human serum N-glycan profiling reveals the influence of ethnic variation on the identified cancer-relevant glycan biomarkers, PLoS ONE 13 (12) (2018) e0209515.
[54] A. Knezevic, O. Polasek, O. Gornik, I. Rudan, H. Campbell, C. Hayward, A. Wright, I. Kolcic, N. O'Donoghue, J. Bones, P.M. Rudd, G. Lauc, Variability, heritability and environmental determinants of human plasma N-glycome, J. Proteome Res. 8 (2) (2009) 694–701.
[55] L.R. Ruhaak, H.W. Uh, M. Beekman, C.H. Hokke, R.G. Westendorp, J. Houwing-Duistermaat, M. Wuhrer, A.M. Deelder, P.E. Slagboom, Plasma protein N-glycan profiles are associated with calendar age, familial longevity and health, J. Proteome Res. 10 (4) (2011) 1667–1674.
[56] Method of the Year 2012, Nature methods 10(1) (2013) 1.
[57] M.L. Fournier, J.M. Gilmore, S.A. Martin-Brown, M.P. Washburn, Multidimensional separations-based shotgun proteomics, Chem. Rev. 107 (8) (2007) 3654–3686.
[58] Y.E.M. van der Burgt, C.M. Cobbaert, Proteoform analysis to fulfill unmet clinical needs and reach global standardization of protein measurands in clinical chemistry proteomics, Clin. Lab. Med. 38 (3) (2018) 487–497.
[59] Quality requirements for quantitative clinical chemistry proteomics, Translational Proteomics 2 (2014) 1–13.
[60] I. van den Broek, F.P. Romijn, J. Nouta, A. van der Laarse, J.W. Drijfhout, N.P. Smit, Y.E. van der Burgt, C.M. Cobbaert, Automated multiplex LC-MS/MS assay for quantifying serum apolipoproteins A-I, B, C-I, C-II, C-III, and E with qualitative apolipoprotein E phenotyping, Clin. Chem. 62 (1) (2016) 188–197.
[61] L.R. Ruhaak, N.P.M. Smit, F. Romijn, M.M. Pieterse, A. van der Laarse, Y.E.M. van der Burgt, C.M. Cobbaert, Robust and accurate 2-year performance of a quantitative mass spectrometry-based apolipoprotein test in a clinical chemistry laboratory, Clin. Chem. 64 (4) (2018) 747–749.
[62] V. Lange, P. Picotti, B. Domon, R. Aebersold, Selected reaction monitoring for quantitative proteomics: a tutorial, Mol. Syst. Biol. 4 (2008) 222.
[63] M.E. Lassman, T.M. McLaughlin, H. Zhou, Y. Pan, S.M. Marcovina, O. Laterza, T.P. Roddy, Simultaneous quantitation and size characterization of apolipoprotein (a) by ultra-performance liquid chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 28 (10) (2014) 1101–1106.
[64] R.D. Josephs, G. Martos, M. Li, L. Wu, J.E. Melanson, M. Quaglia, P.J. Beltrão, D. Prevoo-Franzsen, A. Boeuf, V. Delatour, M. Öztug, A. Henrion, J.-S. Jeong, S.-R. Park, Establishment of measurement traceability for peptide and protein quantification through rigorous purity assessment—a review, Metrologia 56 (4) (2019).
[65] L. Renee Ruhaak, A. van der Laarse, C.M. Cobbaert, Apolipoprotein profiling as a personalized approach to the diagnosis and treatment of dyslipidaemia, Ann. Clin. Biochem. 56 (3) (2019) 338–356.
[66] K.L. Ellis, A. Chakraborty, E.K. Moses, G.F. Watts, To test, or not to test: that is the question for the future of lipoprotein(a), Expert Rev. Cardiovasc. Ther. 17 (4) (2019) 241–250.