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Strategies to verify equimolar peptide release in mass spectrometry-based protein quantification exemplified for apolipoprotein(a)

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Abstract

Objectives: Quantitative protein mass spectrometry (MS) is ideally suited for precision diagnostics and for reference standardization of protein analytes. At the Leiden Apolipoprotein Reference Laboratory we apply MS strategies to obtain detailed insight into the protein-to-peptide conversion in order to verify that quantifier peptides are not partly concealed in miscleaved protein backbone.

Methods: Apolipoprotein(a) (apo(a)) was digested in a non-optimal manner to enhance the number of miscleaved peptides that were identified by high resolution liquid chromatography tandem-MS measurements. The protein-to-peptide conversion was carefully mapped with specific attention for miscleaved peptides that contain an apo(a) quantifier peptide. Four different isotopologues of each apo(a)-quantifier peptide were applied to evaluate linearity of internal peptide standards during measurement of specific real-life samples.

Results: Two apo(a) quantifier peptides that were concealed in two different miscleaved peptides were included into a multiple reaction monitoring list in our targeted MS-based apo(a) quantifications to alert for potential protein digestion discrepancies. The presence of miscleaved peptides could be ruled out when applying our candidate reference measurement procedure (RMP) for apo(a) quantification.

Conclusions: These data further corroborate the validity of our apo(a) candidate RMP as higher order method for certification of commercial Lp(a) tests that is endorsed by the International Federation of Clinical Chemistry and Laboratory Medicine. MS-based molecular detection and quantification of heterogeneous apo(a) proteoforms will allow manufacturers' transitioning from confounded lipoprotein(a) [Lp(a)] mass levels into accurate molar apo(a) levels.

Keywords: quantitative protein mass spectrometry; peptide quantification; equimolar proteolysis; apolipoprotein; lipoprotein(a)

Introduction

Quantitative protein mass spectrometry (MS) is an enabling technology that is ideally suited for precision diagnostics and for reference standardization of protein analytes [1–3]. In contrast to immunoassays that are based on indirect readouts MS also confirms the identity of the measurand [4]. Standardization of test results is essential for effective and safe patient treatment. To this end a calibration hierarchy according to the International Organization for Standardization (ISO) 17511:2020 facilitates anchoring of test results to reference materials that are value-assigned in a reference measurement procedure (RMP) [5]. The implementation of a higher order RMP demands high quality and confidence in the measurement accuracy within the metrological traceability chain. The framework for small molecules also applies for protein quantification [6]. Commonly, MS-based protein quantification relies on direct measurement of proteotypic (or surrogate) peptides that are generated after proteolytic digestion [7]. The selection of proteotypic peptides that are suitable for quantification occurs through a multistep process that combines *in-silico* digestion, peptide library information and empirical data obtained from purified protein standards. This selection process results in a precise definition of both quantifier and qualifier peptides used for quantification and identification purposes, respectively [8, 9]. For accurate protein quantification the

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proteolytic conversion of a protein into quantifier peptides should be either complete or it should be ascertained that the digestion efficiency is identical in all samples of interest [10, 11]. Upon raising the bar to reference standardization according to ISO 17511:2020 it must be assured that the metrological traceability concept is appropriately implemented. Since the protein measurand is changed into peptides equimolar conversion is key, especially for the quantifier peptides.

Earlier, the MS-based proteomics community proposed a three tier system using a fit-for-purpose approach for the discovery and quantification of protein biomarkers and anticipated translation into a medical test [12]. This tier system is a suitable starting point for analytical validation of proteolysis-aided assays and introduces tier 1 assays that enable the translation to a medical test. In line with this pursuit the Clinical and Laboratory Standard Institute (CLSI) recently reported a guidance document with a systematic approach for development and validation of "quantitative measurement of proteins and peptides by mass spectrometry" [8]. Here it is stated that peptide quantities reflect protein concentrations, provided that digestion conditions allow equimolar conversions. Hereby it is assumed that the protein of interest is present in the intact form in the matrix and that no *in vivo* biotransformations have occurred [13]. It is noted that *in vitro* protein cleavages near the proteolytic cleavage sites may affect the formation of surrogate peptides. Moreover, the digestion outcome can be changed due to changes in protein conformation or hampered by the presence of post-translational modifications [14–16]. For the establishment of an RMP the aggregated protein quantity (compiling all different proteoforms) derived from peptide readouts is an attractive and robust parameter [17]. Protein digestion is often thought to proceed to completion, however for reliable quantifications over various samples the "end-point" of proteolysis needs to be carefully mapped [18]. The extent of proteolysis is commonly inferred via agreement between multiple proteotypic peptides and can furthermore be monitored through digestion time courses and the detection of miscleaved peptides. Earlier studies on digestion kinetics of a protease focused on the specificity of the enzyme by identifying peptides that resulted from unexpected cleavages [19–21]. Such peptides are referred to as ragged peptides (or in case trypsin is used as non-tryptic), although these may alternatively originate from earlier mentioned *in vivo* biotransformations [8]. In the current study the identification of ragged peptides is complemented by detailed mapping of miscleaved peptides in MS-based quantification of apolipoprotein(a) (apo(a)). MS-based molecular detection and quantification of apo(a) will allow transition of confounded mass units of lipoprotein(a) (Lp(a)) into molar units [22–25]. The approach for establishing a state-of-the-art reference measurement system for apo(a) is endorsed by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), in which MS-based measurement of apo(a) will function as a cornerstone in quantification of Lp(a) in a next-generation RMP to replace the previous one that is based on an immunoassay [26–28].

Materials and methods

Lipoprotein(a) enrichment from serum samples

All solvents were liquid chromatography (LC) MS grade and the reagents used were of the highest available purity. Biotinylation of anti-apo(a) antibodies (Merck or Abcam) was performed using 100 µL of antibody solution (60 µg/100 µL). Streptavidin-coated magnetic beads were used for immobilization of anti-apo(a) antibody (Cat. Nr. 65801, DynaBeads™, Streptavidin Trial Kit, Invitrogen™, ThermoFisher, New York, NY). Two different serum samples (200 µL each) were used for enrichment, namely sample L8 ([apo(a)] =263 nmol/L, 17 kringles) and sample L3 ([apo(a)]=17 nmol/L, 29 kringles) (see Supplemental Table 1).

Protein digestion and N-glycan release

Captured Lp(a) was denatured and disulfide bonds were reduced with 5 mM tris(2-carboxyethyl) phosphine in ammoniumbicarbonate buffer at 56 °C for 30 min. The reduced cysteine residues were alkylated with 5 mM iodoacetamide at room temperature in the dark for 30 min. For optimal digestion, samples were incubated with 0.4 µg of Lys-C for 1 h followed by with 0.4 µg trypsin at 37 °C, pH=8 for 3 h in a total volume of 35 µL [29–31]. For non-optimal digestion, samples were incubated with 0.4 µg of trypsin at 20 °C, pH=8 for 15 min. In both cases proteolysis was quenched with 5 µL 40 % (v/v) methanol/4 % (v/v) formic acid in milliQ water to obtain pH=3. Enzymatic N-glycan release was performed by adding 0.4 mU PNGase F (Roche) and overnight incubation at 37 °C. Prior to LC-MS/MS analysis each protein digest was purified using Oasis HLB 1cc Vac Cartridge (Waters, 30 mg Sorbent per Cartridge, 30 µm). The solid-phase extraction was performed with two washes (0.1 % formic acid (FA) aqueous solution) and an elution with water/acetonitrile/FA 70/30/0.1 [v/v/v]). After lyophilization the digest was redissolved in 30 µL of 0.1 % FA aqueous solution.

LC–MS/MS analysis

Protein digests were analyzed (after SPE) by online C18 nanoHPLC MS/MS with a system consisting of an Easy-nanoLC 1,200 gradient HPLC system (Thermo Fisher Scientific) and an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer (Thermo). Samples (2 µL) were injected onto a homemade precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 1.9 µm; Dr Maisch) and eluted on a homemade analytical nano-HPLC column (30 cm × 75 µm; Reprosil-Pur C18-AQ 1.9 µm). The gradient was run from 2 % to 40 % solvent B (water/acetonitrile/FA 20/80/0.1 [v/v/v]) in 30 min. The nanoHPLC column was drawn to a tip of 5 µm, which acted as the electrospray needle of the MS source. The Lumos mass spectrometer was operated in data-dependent MS/MS mode for a cycle time of 3 s, with an HCD normalized collision energy of 32 % and recording of the MS2 spectrum in the Orbitrap. In the master scan (MS1), the resolving power was 120,000, the scan range was m/z 400 to 1,500, at an automatic gain control target of 400,000 at maximum fill time of 50 ms. Dynamic exclusion was set after n=1 with exclusion duration of 10s. Charge states 1 to 5 were included. For MS2, precursors were isolated with the quadrupole with an isolation width of 1.2 Da. The MS2 scan resolution was 30,000 with an automatic gain control in standard mode at a maximum fill time of 60 ms.

LC-MS/MS data analysis

MS data were imported in XCalibur Qual Browser (version 2.2; Thermo Fisher Scientific) for manual interpretation and annotation of the spectra. For database searches, XCalibur raw files were converted into peak lists using Proteome Discoverer 2.4.0.305 (Thermo Fisher Scientific) and searched against the human proteome using the Mascot search algorithm (version 2.2.07; Matrix Science). The MS tolerance was set at 10 ppm, and the MS/MS tolerance was set at 20 milli mass units. Trypsin was selected as enzyme (allowing two missed cleavages), and carbamidomethylation was set as fixed modification on cysteine residues and oxidation (methionine) as a variable modification. A target false discovery rate of 0.01 at the peptide level (based on a target-decoy analysis) was used.

LC–MRM-MS analysis

Apolipoprotein(a) measurements were performed within the standard operating procedure for sample preparation and tryptic digestion of serum apolipoprotein panel (including apoA-I and B-100) as published previously [30]. Multiple rection monitoring (MRM) MS measurements were performed on a 1,290 ultraperformance liquid chromatography system coupled to a 6495A triple–quadrupole mass spectrometer equipped with electrospray ionization (Agilent Technologies, Santa Clara, CA) operating in positive ion mode. Peptide separations were performed on a Zorbax SB-C18 column 2.1 × 50 mm, 1.8 µm (Agilent Technologies, Santa Clara, CA) and the column oven was set at 70 °C. Mobile phase A consisted of 5 % (v/v) methanol and 0.05 % (v/v) formic acid in water and mobile phase B of 95 % (v/v) methanol and 0.05 % (v/v) formic acid in water. The LC-system was operated at constant flow of 0.3 mL/min with a non-linear gradient profile over 10 min followed by a isocratic recondition step of 1 min prior to system re-equilibration. The collision energy (CE) was optimized per transition to obtain a maximum signal intensity.

SIL-peptides, SIL-isotopologues and MRM transitions

All MRM transitions with regard to quantification of apo(a), apoB-100 and apo-AI have been reported previously [29]. Four SIL peptide isotopologues (“SIL-1”, “SIL-2”, “SIL-3” and “SIL-4”) for each different quantifier peptides (LFLEP, GISST and TPENY) were synthesized and added into nine different in-house standards L3-L11 (containing various apo(a)-concentrations with various numbers of kringle repeats as indicated in Supplemental Table 1) to a concentration of 10, 20, 40 and 100 nmol/L, respectively. In a similar manner these isotopologues were added to eight different routine serum samples that previously demonstrated >15 % differences between immunoturbidimetric assay (ITA) and MS-based apo(a) quantification. Transitions of SIL-peptides (“SIL-1”, “SIL-2”, “SIL-3” and “SIL-4”) are summarized in Supplemental Figure S1. In order to setup transitions for additional peptides, namely apo(a)-peptides that contain a missed cleavage, Expasy and Protein Prospector were used (https://web.expasy.org/peptide_mass/) (https://prospector.ucsf.edu/prospector/mshome.htm).

For verification of previously reported post-translational modifications the UniProt database was used for P08519 (https://www.uniprot.org).

Results

General MS-based strategies to verify equimolar release of quantifier peptides

Detailed knowledge on the digestion characteristics of a protein into proteotypic peptides is crucial when aiming for

accurate protein quantifications. Comprehensive databases such as NextProt and ProteinAtlas provide a valuable source of previously observed digestion products of a specific protein, nevertheless due to lab-to-lab variations CLSI-C64 recommends "experimental verification" on site [8]. A workflow to address digestion outcome in an exploratory *survey* manner is proposed in Figure 1.

In a *survey* all proteolytic peptides are identified in two types of protein digests, namely one obtained using non-optimal conditions (for example short digestion times) and one from optimal conditions as defined in the RMP-conditions [30]. The first sample contains partially digested proteins and is suitable for detailed mapping of areas in the protein backbone that are not fully cleaved or unexpectedly proteolyzed. To this end, an untargeted data-dependent or data-independent MS-analysis of the complex digest is performed on a high resolution MS system to identify ragged and miscleaved peptides. In the same *survey* analysis regular (expected) proteotypic peptides are identified and compared to previously reported peptides in single/multiple reaction monitoring (SRM/MRM) databases (*in silico*) [32–34]. Next, peptide candidates are selected for further evaluation of their performance using a *targeted* liquid chromatography (LC) MRM-MS set-up [9]. From the combined results from databases and *survey* analysis a set of candidate peptides is selected and monitored in digestion time courses to differentiate between slow and quickly forming peptides and determine peptide stability after release from the corresponding protein backbone [8, 9, 18, 35]. Furthermore the digestion efficiency and reproducibility are used to select the most optimal surrogate peptide(s). Although thus obtained results are highly informative, it is noted that a digestion plateau does not warrant *full conversion* of the protein into peptides. Commonly, interpeptide disagreement points towards issues on digestion efficiency provided that two or more quantifier peptides from one protein are available, but excellent agreement does not necessarily imply equimolar conversion. The absence of ragged and miscleaved peptides suggests equimolar release of corresponding target peptides, provided it is demonstrated that such species potentially can be detected in a specific MRM set-up. To this end specific miscleaved peptides are synthesized and used to develop and optimize MRM transitions. For specific purposes these transitions can be included in a targeted strategy to detect potential protein digestion anomalies. In addition, stable isotope-labeled (SIL) peptides (with heavy R or K incorporated) are used for normalization purposes [36, 37].

Survey evaluation of apo(a) proteolysis

The commercially available immunoassay-based Lp(a) tests exhibit large between-test result variations, which impedes meta-analysis of epidemiological research and pharmaceutical trials and stresses the importance of standardization with a new RMP [27]. It is postulated that one Lp(a) particle (present in the circulation) contains one apo(a) and one apoB100 molecule [38, 39]. Apo(a) contains various N-and O-glycosylation sites and is furthermore characterized by a size-polymorphism due to inheritance of different apo(a) alleles with varying numbers of K-IV$_2$ (kringle) repeats (Figure 2 exemplifies six repeats) [22, 40, 41]. The total number of N-glycosylation sites partly depends on kringle repeats (grey arrows in Figure 2), resulting in an overall molecular weight of apo(a) ranging from 250 to 800 kDa. Early studies of apo(a) glycopeptides have revealed that O-glycosylation is clustered in the kringle K-IV$_2$ linker domains [40, 42]. At that time it was already noted that "the factors influencing apo(a) proteolysis are uncertain" and that O-glycosylation could play a role in restricting

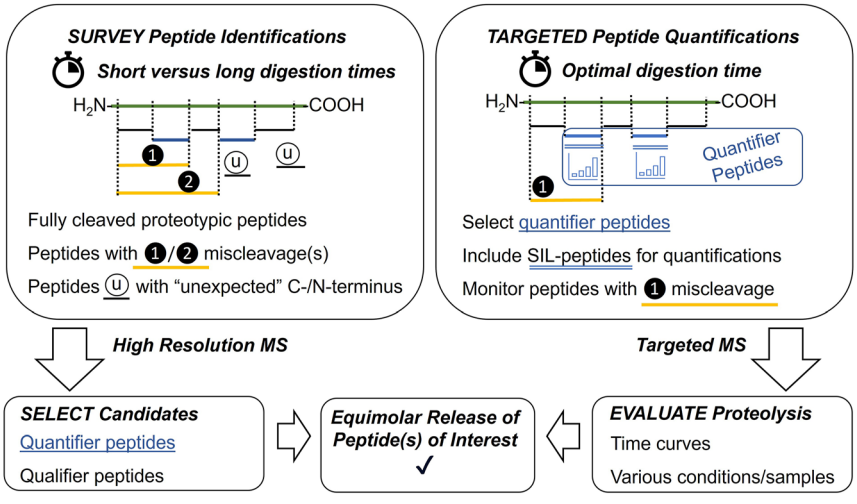


Figure 1: MS-based strategies to collect evidence for equimolar proteolysis. *Survey* peptide identifications include mapping fully cleaved peptides as well as miscleaved peptides in samples that are subjected to either short or optimal digestion times. In addition, the occurrence of ragged peptides with unexpected termini is verified. Targeted peptide quantifications includes the use of SIL-peptides for the quantifier and qualifier peptides and monitor specific miscleaved peptides.

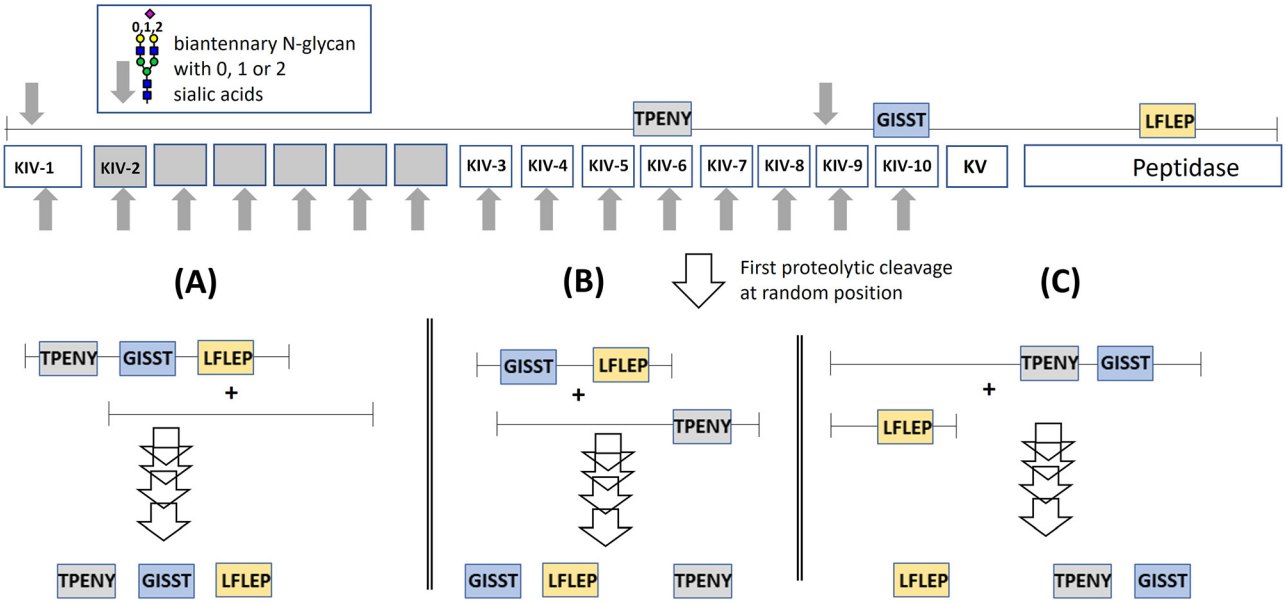


Figure 2: Schematic of apo(a) with varying numbers of K-IV_2 repeats and inherent size-polymorphisms. The multiple N-glycosylation sites are indicated with grey arrows, of which the site-occupancy and structure analysis have been overviewed [41]. The three quantifier peptides used for LC-MRM-MS are depicted with their abbreviated sequences. Three different scenarios are illustrated that start with a first cleavage at three different positions in the intact protein. In an optimal situation all scenarios result in the same end-point with release of three target peptides in an equimolar manner. In practice the scenarios may result in a non-equimolar outcome due to loss of one of the partially digested protein (chemical or physical instability) on the way to the end-points.

proteolytic cleavages of the interkringle linkers, stressing the importance of a careful assessment of digestion efficiency [40].

In Figure 2 three apo(a) quantifier peptides "TPENYPNAGLTR", "GISSTTVTGR" and "LFLEPTQADIALLK" are further abbreviated as TPENY, GISST and LFLEP [29]. The selection of these peptides for quantification purposes is based on previous studies in our Leiden Apolipoprotein Reference Laboratory that included evaluation of the digestion time courses [9, 30]. Alternatively, apolipoproteins can be measured by MS in their intact form (top-down approach), but the quantitative performance of this approach is still in its infancy [43]. Aiming to collect additional evidence on the completeness of apo(a) digestion three different scenarios are plotted with digestion trajectories that all lead to these three quantifiers (i.e. theoretical end-products of the digestion). It is hypothesized that these scenarios may exhibit different digestion efficiencies due to formation of various large "intermediate polypeptides" that can complex with other intermediate structures (proteins) that are present in a complex serum digest. On top of this, apo(a) digestion scenarios *inherently* differ due to the earlier explained size-polymorphisms. The outcomes were further studied by performing a *survey* analysis on eight different serum digests that were obtained after Lp(a)-enrichment using two different anti-Lp(a) antibodies of two different serum samples ("L8" and "L3") to yield samples L8M, L8A, L3M and L3A (Supplemental Table 1, Lp(a)-enrichment was necessary for in-depth identification of all apo(a) peptides). These samples were subjected to short or optimal digestion conditions. In order to study potential steric hindrance from the presence of multiple N-glycans a third set of samples was analyzed after N-deglycosylation. The *survey* analysis was used to focus on three quantifier peptides TPENY, GISST and LFLEP. In a previous study the digestion kinetics of these proteotypic peptides of apo(a) were reported and it was found that all three peptides readily reach a plateau [30]. Nevertheless, the appearance of a plateau does not necessarily mean that all protein is converted into peptide. Therefore, all apo(a)-peptides were evaluated in samples that were subjected to a short digestion, including those that contained a miscleavage. More than 95 % of peptide intensities (derived from peptide spectral matches) corresponded to "regular" apo(a) proteotypic peptides, and only two miscleaved peptides were found in proximity of the quantifier peptides (Supplemental Figure S2). MS/MS-data unequivocally identified these miscleaved peptides in the sequences of quantifier peptides LFLEP or GISST (Figure 3). Although the fully cleaved quantifying peptides indeed are the dominant species this also demonstrates that digestion at this part of the protein is not complete after 15 min.

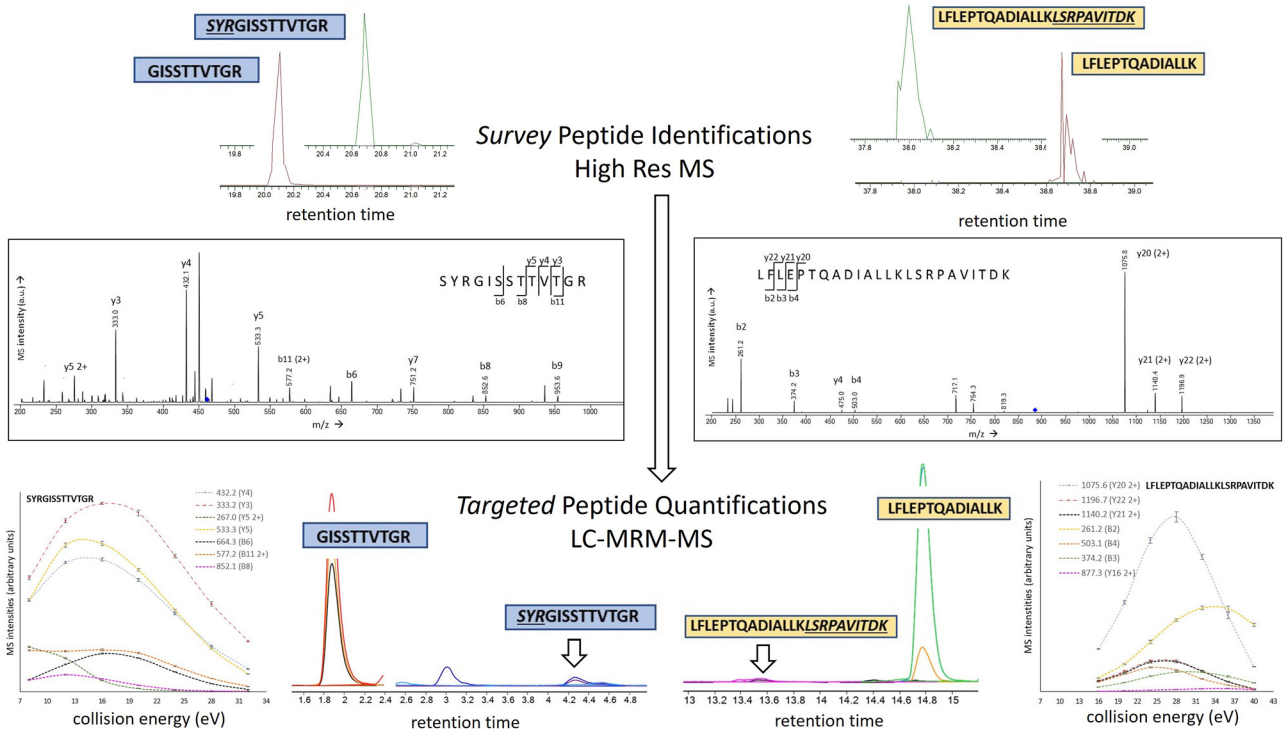


Figure 3: Identification of two apo(a) quantifier peptides that contain a miscleavage followed by targeted peptide quantification of synthetically prepared surrogate peptides. In a *Survey* two apo(a) peptides were detected when applying a short proteolysis (15 min), namely "LFLEP" (in yellow), and "GISST" (in blue), as well as two miscleaved peptides, namely SYRGISSTTVTGR and LFLEPTQADIALLKLSRPAVITDK. Quantifier peptide TPENY was identified solely as end-product upon both short and optimal digestion (not shown). The miscleaved surrogate peptides were used for optimization of transition collision energies (left and right corner) in LC-MRM-MS. Thus developed transitions can be included in a targeted peptide quantification workflow to identify proteolysis anomalies.

The miscleaved peptides SYRGISSTTVTGR and LFLEPTQADIALLKLSRPAVITDK were not observed in any of the samples that were digested according to the candidate RMP digestion protocol. Their absence suggests that digestion had been "completed" at that position in the protein. From this we conclude that, although the digestion of the measurand apo(a) may not be complete for the full part of the protein sequence, the conversion into the quantifying peptides is equimolar. The sequence coverage results from the *survey* analysis of apo(a) are summarized in Supplemental Figure S2. Finally, the presence of non-tryptic peptides (both on N- and C-termini) in the *survey* data was evaluated. Such peptides would interfere with accurate quantification of the corresponding protein in case their sequence would overlap with quantifying peptides. As an example all non-tryptic peptides (some with multiple peptide spectrum matches (PSMs)) that were detected in sample L8A are summarized in Supplemental Table 2. Importantly, just one "odd peptide" from apo(a) was detected, however this peptide "E_{20}QSHVVQDCYHGDGQSYR_{37}" is explained by expected protein processing (AA 1–19 is the signal peptide). This demonstrates that "odd peptides" could be detected and identified in the database search. The data from the other apolipoproteins also demonstrate that if odd cleavages would occur these indeed are detected.

Targeted evaluation of apo(a) proteolysis

In quantitative protein MS workflows a SIL-peptide is added for each quantifier (and qualifier) peptide to correct for ionization differences and ion suppression [8, 12]. The use of SIL peptides enables a careful evaluation of various sample preparation conditions and the "behavior" of different samples (such as lipemic vs. regular serum), as well as the acquisition of a precise digestion time course. Furthermore, for the example of apo(a), two miscleaved peptides that were found in the *survey* analysis were synthesized that contain quantifier peptide sequences with one elongation, namely SYRGISSTTVTGR and LFLEPTQADIALLKLSRPAVITDK. These peptides were used to develop and optimize MRM-transitions that were used to verify the presence of miscleaved quantifier peptides. It is noted that a SIL-peptide does not correct for sample-to-sample variations with regard

to protein extraction or digestion efficiency. Intuitively, the optimal internal standard (IS) would be a properly folded (equivalent) full-length isotope-labeled protein of interest that would exhibit the same biological interactions as the native protein in matrix. Such an IS could control for all potential sources of error in the assay, especially in the case where proteins are first enriched from the matrix before quantification by MS. Unfortunately, such standards are often not available and as an alternative winged or concatenated peptides have been proposed, albeit that are not identical to the protein of interest [44]. In the current study we have evaluated the application of multiple SIL-peptides, namely isotopologues with various molecular masses, to gain a qualitative insight into proteolysis with regard to linearity and potential concentration biases [36, 37]. Here it is emphasized that these isotopologues are only used for a sub-set of samples and that their application does not replace the traditional external calibration curves. Four SIL peptide isotopologues ("SIL-1", "SIL-2", "SIL-3" and "SIL-4") of three different quantifier peptides (LFLEP, GISST and TPENY) were added into nine different in-house standards L3-L11 (containing various apo(a)-concentrations with various numbers of kringle repeats as indicated in Supplemental Table 1) to a concentration of 10, 20, 40 and 100 nmol/L, respectively. Regression analysis (R^2-values >0.99) demonstrated that MRM-derived peak areas were linear at the applied concentration range, indicating that the isotopologues are perfectly suited for normalization of signal intensities (Supplemental Figure S3). Within one sample the three slopes of the three quantifier peptides differed from each other, explained from the fact that all peptides exhibit different MS response factors. When comparing samples L3-L11 it was observed that the slopes of a certain quantifier were sample-specific and it was hypothesized that the addition of SIL isotopologues into other samples could be used to pinpoint specific aberrances. To further test this hypothesis we spiked the four isotopologues of three different apo(a)-quantifier peptides into serum samples that previously demonstrated >15 % differences between immunoturbidometric assay (ITA) and MS-based apo(a) quantification. It is noted that serum Lp(a) levels are routinely quantified using commercially available immunoassays such as the ITA and we have compared Lp(a)-ITA and MS-based apo(a) quantifications in hundreds of serum samples and reported that the majority of clinical specimens were in a good agreement, whereas for some individual specimens marked discordances (>15 %) were observed [27]. Interestingly, excellent linearities of the different isotopologues were observed in the specimens that differed in Lp(a)-ITA and MS-based apo(a) concentrations (Figure 4). It is clear that the slopes differed from sample-to sample as was observed for samples L3-L11, but so far no specific correlations are seen with regard to ITA-lower or ITA-higher apo(a) concentrations when comparing to MS.

Discussion

The results that are obtained from quantitative protein MS applications should be traceable to higher order reference measurement systems, such as described in ISO 17511:2020. Currently an IFCC working group on MS-based quantification of apolipoproteins (WG APO-MS) collaborates on the calibration of various serum apolipoproteins [26, 30, 45, 46]. Here we have presented strategies to address the challenge of verifying equimolar protein-to-peptide conversion and

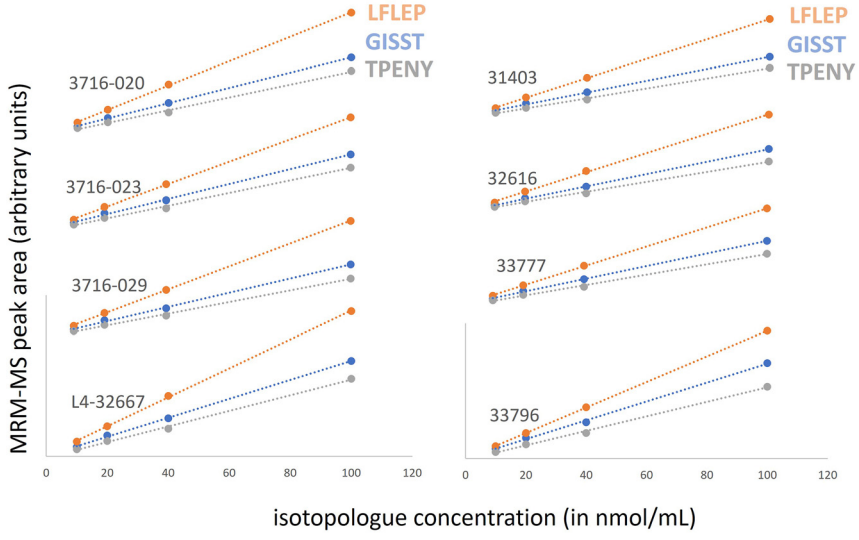


Figure 4: Application of four isotopologues of three different apo(a)-quantifier peptides that were spiked into serum samples that previously demonstrated >15 % differences between the ITA- and MS-based apo(a) quantification (see Supplemental Table 1). For clarity reasons the MRM-MS peak areas that are used as an intensity measure are not depicted on the y-axis. On the left-hand side the ITA-quantity was higher than MS, on the right-hand side the MS-quantity was higher than ITA.

exemplified these for quantification of apo(a). So far, all studies that have been performed at the Leiden Apolipoprotein Reference Laboratory with targeted LC-MRM-MS point toward an equimolar release of apo(a) quantifier peptides from the protein backbone. This findings corroborate with the previously determined digestion curves, where it was observed that quantifying peptides LFLEP and GISST were quickly "released" from the protein backbone. It was also found that in all samples peptide TPENY was solely detected as an end-product, independent of digestion times. Potential steric hindrance from the presence of multiple N-glycans was assessed through N-deglycosylation prior to optimal digestion. No differences in apo(a) sequence coverage nor identity of miscleaved peptides was observed between samples that were N-deglycosylated prior to digestion and samples that did not go through this PNGase-F step (Supplemental Figure 2). Furthermore, it was found that apo(a) is exclusively cleaved in the "expected" manner by trypsin, implying that neither *in vivo* nor *in vitro* non-tryptic cleavages had occurred. Finally, the use of various SIL peptide isotopologues could not explain for specific deviations in ITA-vs. MS-based quantification, not for ITA-lower nor for ITA-higher apo(a) concentrations.

In the MS-based proteomics community it is often assumed that the conversion of a protein into surrogate peptides is either equimolar (full conversion) or the digestion efficiency of specific protein is identical in all samples of interest. This assumption is sufficient for biomarker discovery studies, but for the implementation of a reference measurement procedure (RMP) at the Leiden Apolipoprotein Reference Laboratory the quality bar is raised to the level that equimolar formation of peptide measurands from the protein of interest is *required*. Incomplete proteolysis can be inferred from protein digestion time courses and the presence of miscleaved peptides. In this paper we have reported a combination of MS-based strategies to evaluate completeness of the conversion of a protein into quantifier peptides. These strategies were exemplified for quantitative measurement of apo(a) to provide complimentary evidence for our candidate RMP. Multiple miscleaved peptides were detected when applying short digestion time on apo(a), including at regions that contain the quantifier sequence. When applying the candidate RMP digestion protocol most of these miscleaved peptides had disappeared, implying a more complete (although not full) proteolysis of the protein. Importantly, no miscleaved peptides were observed that originated from regions close to the quantifier peptides GISST and LFLEP. Although four different isotopologues of each apo(a)-quantifier peptide demonstrated excellent linearity of internal standards in multiple serum samples with varying apo(a) concentrations, their application could not explain for observed differences between ITA- and MS-quantification. From the current results it is concluded that apo(a)-quantifier peptides are solely detected as end-products upon using the candidate RMP digestion protocol. These data further corroborate the validity of the IFCC-endorsed RMP as a robust method for apo(a) quantification and standardized readout for Lp(a) particles.

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Data availability: The raw data can be obtained on request from the corresponding author.

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