Published in final edited form as:

Proteomics. 2017 January ; 17(1-2): . doi:10.1002/pmic.201600085.

Automation of PRM-dependent D3-Leu tracer enrichment in HDL to study the metabolism of apoA-I, LCAT and other apolipoproteins

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Abstract

We developed an automated quantification workflow for PRM-enabled detection of D3-Leu labeled apoA-I in HDL isolated from humans. Subjects received a bolus injection of D3-Leu and blood was drawn at seven time points over three days. HDL was isolated and separated into six size fractions for subsequent proteolysis and PRM analysis for the detection of D3-Leu signal from ~0.03 to 0.6 % enrichment. We implemented an intensity-based quantification approach that takes advantage of high resolution/accurate mass PRM scans to identify the D3-Leu 2HM3 ion from non-specific peaks. Our workflow includes five modules for extracting the targeted PRM peak intensities (XPIs): Peak centroiding, noise removal, fragment ion matching using m/z windows, nine intensity quantification options, and validation and visualization outputs. We optimized the XPI workflow using *in vitro* synthesized and clinical samples of D0/D3-Leu labeled apoA-I. Three subjects' apoA-I enrichment curves in six HDL size fractions, and LCAT, apoA-II and apoE from two size fractions were generated within a few hours. Our PRM strategy and automated quantification workflow will expedite the turnaround of HDL apoA-I metabolism data in clinical studies that aim to understand and treat the mechanisms behind dyslipidemia.

Keywords

Apolipoprotein; Endogenous labeling; Ion peak intensity; Noise removal; Targeted mass spectrometry; Neutron encoded mass signatures; Proteomics

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Conflicts of Interest
None.

INTRODUCTION

High-density lipoprotein cholesterol (HDL-C) is known as “good cholesterol” since epidemiological studies found that HDL-C is inversely associated with cardiovascular disease risk [1, 2]. However, HDL-C raising therapies have failed to show beneficial effects on coronary heart disease (CHD) prevention, [3, 4] indicating that HDL metabolism is not as well understood as previously thought [5, 6]. In order to examine more closely the kinetics of HDL in humans, we recently reported the metabolism of five HDL particle sizes, from small to large, pre- β , α 3, α 2, α 1 and α 0, using D3-Leu to endogenously label its primary protein, apolipoprotein A-I (apoA-I), and parallel reaction monitoring (PRM) as the readout [7]. This and a preceding study [7, 8] demonstrated that HDL metabolism is more complex than the size expansion model that has prevailed in the last few decades [1, 9]. These recent findings have therefore prompted us to develop further our PRM-enabled *in vivo* metabolism strategy.

D3-Leu has been frequently used to endogenously label proteins even though alternatives, such as carbon-13 and nitrogen-15, that provide better separation between the isotopologs of unlabeled and labeled peptides, are available [10]. However, isotopolog separation does not guarantee reduced interference, even in MS2 scans that contain less interference than in MS1 scans [7]. Tracer incorporation is very low in plasma proteins including the slowly turning over apoA-I (0.03 to 0.6%) [7, 11] placing the labeled peptide signal within background peaks and at the extreme limits of an instrument’s dynamic range [7]. Endogenous labeling typically use a constant tracer infusion strategy with an initial bolus, achieving maximum enrichment at the equilibrium plateau. Even with the primed constant infusion method however, tracer incorporation is very low for most plasma proteins [10, 11]. Our PRM strategy, which is built on concepts derived from data-independent acquisition (DIA) methods [12] and neutron encoded mass signatures (NeuCode) [13, 14], circumvents the technical issues above by co-isolating and fragmenting the D0- and D3-Leu peptides for PRM at the highest resolution to separate the 2HM3 ion from background ions and its M3 isotopolog [7]. This level of accuracy permits us to analyze bolus-administered D3-Leu tracer in HDL apoA-I whose peak enrichment is lower (0.3–0.6 %) than that achieved by the constant infusion method (>1 %), but is more beneficial to compartmental modeling since the decay portion of the enrichment curve provides inflection points for curve fitting. The application of PRM in this manner permits the resolution of an unprecedented level of information on HDL metabolism. The method reveals subtle changes in the descending and ascending slopes of enrichment curves, which have large metabolic implications.

In our previous study [7], the XIC-based approach was suitable for enrichment quantification, however significant time was spent validating all quantified candidate ions per fraction per subject in order to ensure that subtle changes in enrichment curve shapes were biological in origin and not due to incorrect peak assignment. One factor of incorrect 2HM3 peak assignment is the retention time shift incurred by deuterium labeling. Overall, manual validation or manual quantification of the XICs ensured that the correct peaks were chosen, minimizing the impact of quantification inaccuracy and associated variability in the data. Despite the inconvenience of manual validation, it was necessary in order to be confident of the very low enrichment data; as a consequence, potential sources of variation

in the data could be evaluated elsewhere, such as the mass spectrometric method acquisition strategy, HDL sample preparation and donor-to-donor variability. Taken together the results from two independent studies [7, 8], the variation in HDL metabolism among apolipoproteins is greater than the technical variability provided by either analytical workflow (GC/MS or PRM).

In this current study and leveraging our recently acquired knowledge of HDL apoA-I metabolism [7, 8], we have implemented a bioinformatic pipeline that aims to develop an automated, unbiased and efficient way to identify candidate peptides for PRM-assisted tracer enrichment studies. We capitalized on the availability of the multiple high resolution/accurate mass (HR/AM) scan data acquired in PRM to perform tracer enrichment quantification using a PRM peak intensity-based strategy [7]. In doing so, quantification is not affected by the retention time shift incurred by deuterium labeling as it is when using the area under the curve (AUC) of the XIC-based approach. We evaluated the candidate sources of technical error that could compromise a transition from the reliable and trusted manual quantification approach to one that is automated. Our workflow permitted us to analyze within hours the enrichment profiles of six HDL apoA-I size fractions in three subjects and the enrichment profile of the low abundant lecithin-cholesterol acyltransferase (LCAT), an enzyme whose activity plays a significant role in HDL metabolism [15–17]. XPI thus provides an efficient means for increased throughput for future *in vivo* metabolism studies that capitalize on the advent of HR/AM-PRM based technologies.

2 Materials and methods

Additional materials/methods are provided in the Supplemental Information

2.1 Software packages

The extracted PRM peak intensity (XPI) software was programmed in Python (<https://www.python.org/>) version 3.4.3. We employed Numpy (<https://www.numpy.org/>) and Scipy (<http://www.scipy.org/>) for mathematical and statistical calculations, Pymzml (<http://pymzml.github.io/>) for data extraction from mzML files and noise reduction, Pyteomics (<https://pythonhosted.org/pyteomics/>) for calculation of peptide molecular masses and Matplotlib (<http://www.matplotlib.org/>) for data visualization. ProteoWizard (<http://proteowizard.sourceforge.net/>) was used to convert RAW to mzML files. Skyline (<https://skyline.gs.washington.edu>) or Proteome Discoverer 1.4 (PD, Thermo Scientific) was used to quantify the area under the curve (AUC) of the extracted ion chromatograms (XICs) of the MS1 signals in data-dependent acquisition (DDA) experiments.

2.2 XPI software

The peptide library workflow (Fig.1A) - The initial DDA peptide library consists of peptides containing leucines within first/last five residues [7]. The library is refined by ranking the peptides based on their monoisotopic peak XIC intensities. The top-ranked peptides are then reanalyzed using PRM (R=17.5K) to classify peptides considering the quality and intensities of their leucine-containing fragment ions. The final candidate peptide library comprises the

inclusion list for PRM at the higher resolutions settings (e.g. R=140K) in order to detect D3-Leu tracer.

Detection and quantification of light and heavy target ions (Fig. 1B) - XPI is an automated pipeline for, but not limited to, PRM-dependent D3-Leu tracer enrichment in clinically derived HDL apoA-I. From the peptide library, XPI generates all the possible leucine-containing fragment ions including light (M0) and heavy (2HM3) by considering the mass shift (3.01883025 Da) induced by D3-Leu labeling. XPI extracts data from mzML files and carries out pre-processing steps including intensity centroiding, background subtraction, and noise removal using Pymzml. With the collected data, XPI finds target fragment ions by matching the scan number, the mass window ($=|\text{theoretical mass} - \text{observed mass}|$) and the RT range. In order to accommodate the RT shift incurred by the deuterium, and to identify the low abundant 2HM3 peak efficiently, XPI performs RT refinement steps by reference to M0 ion detection since the M0 peaks are readily identified. This extra refinement step excludes non-specific peaks that appear in a larger RT window, and uses the LOWESS algorithm, the local maxima and minima method, to do so. XPI provides nine methods for the quantification: the sum, max, top3, median, average, qsum, qtop3, qmax and qaverage (Fig. 1B). The sum, max, top3, median and average options calculate a representative value from the whole set of collected PRM intensities, while the others, qsum, qtop3, qmax and qaverage, calculate the intensity from the second and third quartiles. XPI finally calculates ratios between M0 and 2HM3 ions in two ways: 1) heavy/light or light/heavy, or 2) enrichment ($\text{Heavy}/(\text{Heavy}+\text{Light})$).

Validation and visualization (Fig. 1C) - Since XPI has nine options for quantification, XPI shows which method fits best to a given dataset by drawing box-plots based on the R^2 between the intended and observed ratio (Fig. 1C). When possible, we recommend using standard mixtures to evaluate the best quantification method. The user can choose the best quantification method by comparing the average or median R^2 of the standard data. XPI provides a standard curve plot with a regression line to check the linearity of the standard data (Fig. 1C). XPI also provides the summed M0 ion intensity versus accumulated ratio plots for filtering/excluding low and outlier enrichment PRM ions (Supplemental Information). XPI draws various plots at the protein and peptide level. Two- and three-dimensional mass profiles with a color scheme using the mass are supplied for visualization and validation of the target ion identification if needed. Since XPI uses Python scripts, it is executable regardless of the operating system as long as Python 3 and the required packages are installed. XPI can process 7 mzML files (~1.6 gigabytes) within 12 minutes with Intel Core i7 (2.6 GHz), 16Gb memory and OS X 10.11.2 environment. XPI is downloadable at <http://cics.bwh.harvard.edu/software>.

3 RESULTS

3.1 Rationale for the extracted PRM peak intensity quantification strategy

The overall aim of this study is to automate the quantification and enrichment curve output for, but not limited to, bolus-administered D3-Leu tracer enrichment in circulating apoA-I containing HDL particles, a method used to study HDL metabolism (Fig. 2A,B). The D0-Leu and D3-Leu peaks are measured as the M0 (light) and 2HM3 (heavy) PRM ions

respectively. Traditionally, light and heavy peaks are quantified using the XIC method. However, deuterium labeling causes a slight shift in the peptide RT (Fig. 2C). Automation of XIC-based quantification assumes that unlabeled and labeled ions co-elute which is typical for the majority of proteomics experiments that use carbon-13 or nitrogen-15 labeling strategies. Moreover, due to the very low signal intensity of D3-Leu in apoA-I and the RT shift, enrichment calculation is vulnerable to interference by non-specific signals. For this particular study, we turned to the intensity-based approach that quantifies the relative intensities of the 2HM3 and M0 ions directly from the PRM spectra. Our software extracts the centroided PRM peak intensities (XPIs) derived from the multiple scans across the scan cycle and performs noise-filtering steps (Fig. 2D), permitting precise quantification that is not affected by the RT shift (Fig. 2E).

3.2 Cell-free synthesized apoA-I D0-Leu/D3-Leu standard curve

We optimized the XPI workflow (Fig. 1) using an *in vitro* synthesized (FLEXIQuant) [18, 19] apoA-I standard [7] (Supporting Information Fig. 1). We prepared D0-Leu-apoA-I and D3-Leu-apoA-I protein mixtures spanning a 1:1 to 10,000:1 (D0:D3) mixing ratio (Supporting Information Fig. 1A) that overlaps with the very low D3-Leu signal observed in circulating apoA-I (0.03 to 0.6 % of the D0-Leu signal) [7, 8]. Since the scan range limit for the Q Exactive is 5,000:1 the 10,000-fold dilution was the baseline/noise control. DDA analysis of the D0-Leu-apoA-I sample resulted in fifty precursor ions accounting for 70% of the protein sequence (Supporting Information Fig. 1B). Thirty of the fifty apoA-I precursor ions contained leucines within first/last five residues. We previously demonstrated that fragment ions <600 *m/z* are ideal candidates for 2HM3 tracer enrichment since their 2HM3 ions can be readily resolved from interfering peaks when the scan resolution (*R*) is set to 140K (Q Exactive) [7]. We selected the top 18 peptides for fragment ion screening based on the AUC of monoisotopic XIC. Overall, the more intense the precursor's signal intensity, the more likely the fragment ions will produce intensities high enough to detect the 2HM3 peak in the PRM scan.

We then evaluated the candidacy of leucine-containing fragment ions for enrichment analysis by performing PRM on the 18 precursors. The precursors were divided into two injection-sets so that their RT overlaps could be minimized (Supporting Information Fig. 1D,E), dedicating scan time (*R*=17.5K) on a single target at a time when possible [20]. We ranked the precursors based on their summed M0 fragment ion intensities using XPI. The precursors contained between two to five detectable candidate fragment ions. Only three precursors did not yield lower mass fragment ions with discernible signal (Supporting Information Fig. 1D,E). The final PRM library for apoA-I comprised 15 precursor ions with 52 leucine-containing fragment ions (Supporting Information Figs. 1G).

In order to quantify the D3-Leu-to-D0-Leu ratio we reanalyzed the 15 precursors using PRM (*R*=140K) however, the isolation window was centered on the average *m/z* of M0 and M3 peaks (or M6 for 2 leucines etc.), $\pm 5m/z$ (Fig. 1A). Fig. 3A displays the three-dimensional plots of the extracted PRM peak intensities for seven M0 and 2HM3 ion pairs of the peptide, [THLAPYSDELR]³⁺, over its RT. The color scale indicates mass. The plots are scaled to the M0 peak intensities therefore the 2HM3 peaks are only discernible primarily at the 0.2

and 1 mixing ratios. However, upon closer inspection, the peak apices can be seen at the 0.002, 0.01 and 0.02 mixing ratios. These plots highlight the extreme differences in relative intensities between the M0 and 2HM3 peaks. On the other hand, the two-dimensional plots in Fig. 3B are scaled to each M0 and 2HM3 ion's maximum extracted PRM intensity (y_5). In this view, it can be seen that the 2HM3 ion is reliably identified across all mixing ratios.

With the collected 52 fragment ions, we compared nine quantification methods (Materials and methods 2.2) by computing the R^2 between the intended (mixing) and observed (2HM3/M0) ratios and chose the 'sum' method because it showed the highest mean R^2 (Materials and methods 2.2; Supporting Information Table 1). The $\log_{10}$ 2HM3 and M0 ion intensities are positively correlated and there is a relatively higher variation at low 2HM3 ion intensity (Supporting Information Fig. 2A). The standard data's R^2 is 0.95, showing high accuracy of combining PRM with the XPI workflow (Supporting Information Fig. 2B). Supporting Information Fig. 2C shows the standard curve ribbon plots for precursors, [ATEHLSTLSEK] 3^+ , [THLAPYSDELRL] 3^+ , [WQEEMELYR] 2^+ and [AKPALEDLR] 2^+ that were all quantified at a high R^2 (0.98–0.99). For example, the y_5 ion of the [THLAPYSDELRL] 3^+ peptide (Fig. 3 A and B) has a 0.96 R^2 (Supporting Information Table 1).

3.3 Validation using XIC-quantified HDL apoA-I enrichment clinical data

The ultimate application of our intensity-based quantification method is to automate apoA-I D3-Leu tracer enrichment calculations in HDL metabolism clinical studies (Fig. 2A, B). To ascertain whether we could quantify HDL apoA-I enrichment *in vivo*, we re-analyzed our recently published XIC-based quantified and manually inspected apoA-I data using the XPI method (Supporting Information Fig. 3). In order to identify and extract the intensities of the fragment ions' M0 and 2HM3 peaks, we used a 0.005 Da mass window and the 'sum' quantification method. A comparison between automated quantification using XPI and manually controlled analysis demonstrates that XPI is comparable with manual inspection of the data. (Supporting Information Fig. 3).

3.4 Evaluating sources of technical noise in PRM intensity based quantification data

A major challenge for the automation of D3-Leu tracer detection and quantification *in vivo* is the confidence that subtle variations in enrichment curves are due to biological and not technical variations. For instance, we previously demonstrated that HR/AM MS1 based enrichment analysis (increased interference) resulted in enrichment curve compression [7]. In addition, manual analysis of the enrichment data enabled us to rule out PRM peak detection and quantification as a source of technical variability, as well as ascertain that increased interference, as provided by the MS1 experiment, was directly responsible for curve compression, and not quantification error. Before using XPI to automate apoA-I enrichment analysis from three new HDL apoA-I subjects, we re-analyzed apoA-I enrichment curves from the HDL alpha3 size fraction of one subject described in our previous study (Supporting Information Fig. 4). We introduced technical variability to the sample by re-collecting the enrichment curve data at varying PRM resolution settings, 140K, 70K and 35K. Decreasing the resolution increases interference by background ions. Not surprisingly, decreasing the PRM scan resolution compressed the enrichment curves. Taking

all PRM ion data of two peptides that were used for manual quantification (Supporting Information Fig. 3), apoA-I peak enrichment decreased from about 0.25 % (R=140K) to < 0.1 % (R=35K) (Supporting Information Fig. 4A). We then applied the PRM ion filtering strategy (Supplemental Information) and the resulting apoA-I enrichment curves were more similar to each other across the three resolution settings; peak enrichment was closer to 0.3 % (Supporting Information Fig. 4A). As the resolution decreased, more fragment ions were filtered out (Supporting Information Fig. 4B and 4C). However, compensating for the lower number of fragment ions at 35K was the increase in XPIs across the RT, due to the increase in scan speed at the lower resolution. Although 70K and 140K resolution data resulted in a similar number of fragment ions (134 and 138 ions, respectively), and the 140K data contained fewer XPIs (2092 in 140K vs. 2906 in 70K), there was less variability in the 140K data in terms of the coefficient of variation (0.81 in 70K vs. 0.75 in 140K) (Supporting Information Fig. 4B). These data demonstrate the ability for our PRM-XPI strategy to evaluate sources of technical noise for reliable automated quantification of very low tracer enrichment.

3.5 ApoA-I enrichment across six HDL size fractions in new subjects

For three new subjects, XPI quantified the enrichment curves for the five HDL size fractions, alpha0, alpha1, alpha2, alpha3, and prebeta, plus an additional size fraction that we have annotated as <prebeta since its size is smaller than that of prebeta (Supplemental Information). Two PRM acquisition sets, each separated by six months to evaluate variability between acquisition sets, were acquired and analyzed using XPI. The first set targeted apoA-I alone, the second targeted apoA-I plus lecithin-cholesterol acyltransferase (LCAT), apoA-II and apoE (more below). For apoA-I, the enrichment data from both acquisition sets were combined to plot the summed M0 ion intensity versus accumulated enrichment (Fig. 4A, B), and the log10 (M0 ion intensity) versus log10 (2HM3 ion intensity) before and after filtering of unreliable apoA-I enrichment data (Fig. 4C, D). The acquisition sets were comparable in enrichment results as indicated by the correlation coefficient, R, that ranged between 0.44 to 0.84 across the HDL size fractions (Fig. 4E). As a measure of confidence that these correlations are significant, we performed a random simulation of the data using the Monte Carlo method and calculated the empirical P-value (eP) [21](Fig. 4E, Supplemental Information). The simulation demonstrated that all of the correlations are significant (eP < 0.05) except one, <prebeta fraction of subject 3 (eP=0.0919), which means negligible inter-day variation in almost all observations (Fig. 4E).

For any given HDL size, 19 to 28 of the 58 PRM ion measurements survived the ion filtering step (Fig. 4D). ApoA-I intensity itself varies across the HDL sizes and in all the subjects. When compared to alpha1, alpha2 and alpha3, the alpha0, prebeta and <prebeta fractions are one to two orders less abundant in absolute signal (Fig. 4F). As a consequence, the latter sizes tended to retain fewer ions after filtering - between 19 to 25 versus 23 to 28 ions for alpha1, alpha2 and alpha3 (Fig. 2D). Overall, the [THLAPYSDELRL]³⁺ peptide remained in all fractions for all three subjects, followed by [ATEHLSTLSEK]³⁺ and [THLAPYSDELRLRQR]³⁺ in most fractions/subjects.

Using XPI, we calculated the median enrichment of each peptide at each time point, and then the mean value of the peptide medians (Fig. 5A). We then determined the time of peak enrichment and calculated the slopes of the ascending, descending and convergent lines for each of the six HDL size fractions. We found that the time of peak enrichment for <prebeta is markedly delayed, between 6 and 22 hours, when compared to the larger fractions whose peak enrichments range between 2 and 6 hours (Fig. 5B). We also observed a decrease in ascending and descending slope from the alpha to prebeta and <prebeta sizes (Fig. 5B). Furthermore, the converging slope of prebeta and <prebeta tends to be similar to, or slightly higher, than the alpha category (Fig. 5B). Overall, the slope and peak enrichment data of prebeta are consistent with our previous findings that the majority of prebeta appears in circulation later than the larger alpha fractions [7, 8]. Interestingly, the marked delay in time of peak enrichment and low ascending slope of <prebeta compared to the larger size fractions, indicate that this newly reported size fraction appears the latest in circulation.

3.6 LCAT metabolism in alpha2 and alpha3 HDL size fractions

In addition, we investigated the enrichment profile for LCAT, the enzyme that esterifies free cholesterol for subsequent internalization into the HDL particle core [17]. LCAT metabolism has not been previously characterized. LCAT is primarily associated with alpha3 HDL (~70 % of total signal) with the remaining mostly residing on alpha2 HDL [7]. For comparison, we also investigated the enrichment profiles of apoA-II and apoE, two abundant HDL apolipoproteins whose metabolism on HDL have been shown to be distinct from one another [8]. For apoA-II, apoE and LCAT, two candidate peptides (Fig. 5C) were analyzed; however, enrichment was only reliably detected for one LCAT peptide, SSGLVSNAPGVQIR. The second peptide, even after replicate PRM acquisitions (below), did not pass the ion filtering step.

Our initial analysis, which included apoA-I, apoA-II, apoE and LCAT target peptides in a 30-minute gradient, revealed less than 1% peak enrichment for LCAT and apoA-II, and up to 4% for apoE. The low apoA-II and the relatively high apoE enrichment is consistent with our previous findings [7]. Interestingly, the LCAT MS1 signal is approximately 50- to 100-fold less than that of apoA-II and apoA-I, respectively, and 10-fold less than that of APOE (Fig. 5D); yet LCAT enrichment is in the same range as that of apoA-I and apoA-II, in contrast to apoE whose enrichment is 10-fold greater. Considering the combined low absolute and low enrichment signal for LCAT, we reanalyzed the alpha2 and alpha3 sizes, selecting for LCAT (and apoE as a control) in order to get replicate measurements. To gain even more enrichment data points, we extended the XPI analysis to the *a4* and *a5* ions whose signal intensities surpass those of their *b*-ion counterparts (Fig. 5E). By combining the enrichment data collected from multiple replicates, including those examining intra-day variability (Supporting Information Fig. 5), we confirmed the peak enrichment for LCAT to be 0.4% to 0.6 % for alpha3 and 0.1% to 0.4 % for alpha2, between 6 to 22 hours across the subjects and size fractions (Fig. 5F). LCAT's enrichment profile is more similar to apoA-I and apoA-II when compared to that of apoE whose peak enrichment is reached by two hours, followed by a steep descent to nearly baseline by 12 hours.

4 DISCUSSION

Clinical studies aimed to understand, manage or treat dyslipidemia have sometimes included endogenous labeling with a tracer isotope in subjects to monitor the metabolism of target apolipoproteins [8, 22, 23]. Until work conducted in our laboratory, these studies have primarily depended on MRM or GC/MS as the readout for the very low tracer incorporation into apolipoproteins, which, due to the low-resolution measurements of these approaches, has limited the scope of these *in vivo* metabolism studies. PRM is a powerful alternative to MRM since it not only places tracer detection in the MS2 where there is less interference than in MS1 (as does MRM), but also provides HR/AM measurements that differentiate the 2HM3 ion from interference, for several fragments at once. This increase in specificity permitted us to split total HDL apoA-I signal into six HDL size populations without compromising tracer detection. The ability for the Q Exactive to reliably detect tracer less than one percent is very useful, especially since a PRM scan contains several candidate ions for quantification. However, not all fragment ions are equally suitable for tracer enrichment, thus we established an automated and systematic method to measure, evaluate and choose PRM ions for enrichment studies. Our data analysis throughput was facilitated by our automated extracted PRM peak intensity (XPI) workflow, which allowed a relatively fast turnaround (minutes) for the enrichment calculation and evaluation, compared to the time required to do the same analysis using the XIC method (hours). Moreover, the XPI quantification strategy is not directly sensitive to the shift in RT incurred by deuterium labeling as is the XIC method, other than ensuring that the 2HM3 search space is confined to the RT established by the M0 peaks (Fig. 3).

We evaluated the candidacy of the PRM ions based on a comparison between the accumulated enrichments and the M0 intensities of all PRM ion pairs. In this analysis we observed that there is a peptide enrichment bias that is conserved between PRM acquisition replicates and across all HDL samples, irrespective of the subject. Interestingly, although the PRM M0 ion intensities vary across the fractions, their relative abundances and enrichment properties are conserved (Fig 4A). Future studies that will collect more PRM data may help verify the source of this bias.

We analyzed the apoA-I enrichment across six HDL size fractions in three subjects (Fig. 5), including a novel and smallest size fraction-containing apoA-I signal that we refer to as, <prebeta. The enrichment curves have a steady decrement in slope from alpha0 to the <prebeta class (Fig. 5A), large to small, consistent with our previous findings that demonstrated that prebeta appears later than and originates predominantly from alpha3 HDL [7, 8]. In addition, we found that the enrichment curve for <prebeta was distinct from the prebeta fraction itself. Enrichment curves from two of three subjects exhibited a large delay in peak enrichment (12 and 18 hours) and a slower ascending slope (Fig. 5A). This suggests that <prebeta is appearing in circulation even later than prebeta, perhaps due to generation from alpha3 HDL [8], or from another larger HDL size fraction. Further investigation is necessary to characterize the metabolism of this unique HDL size fraction. The observation that the prebeta and <prebeta HDL apoA-I fractions appear the latest in circulation further do, however, support our recent findings that challenge the canonical HDL metabolism model that posits prebeta is released by the liver first, and converted to alpha3, then alpha2,

and eventually alpha1 [24]. The canonical model would therefore predict that the prebeta ascending slope would be greatest of the size fractions, followed by alpha3, alpha2, etc., which is opposite to what we observe.

LCAT is a hallmark HDL protein whose cholesterol esterification activity inspired the reverse cholesterol transport theory [16, 17], which in turn led to the HDL size expansion model [25]. HDL size expansion is thought to occur with the LCAT-dependent formation of cholesterol esters followed by their internalization into the particle core. LCAT resides primarily in the alpha3 size and to a lesser extent, in alpha2 [7], we therefore monitored its D3-Leu enrichment in these two size fractions. Since this is the first time LCAT metabolism has been reported and since we were limited to a single peptide for its enrichment analysis, we extended enrichment quantification to include the *a4* and *a5* ions, in addition to their *b*-ion counterparts, in order to increase the number of measurements. The ability to adjust the data analysis strategy without re-acquiring the data underscores the advantage of the full scan in PRM. Our XPI output revealed that LCAT turnover is slow, comparable to that of apoA-I and apoA-II when considering the contrasting rapid metabolism of apoE. The significance of the slow turnover of LCAT remains to be determined.

In closing, we have presented an automated data analysis and output workflow that capitalizes on PRM technology to measure D3-Leu-dependent *in vivo* metabolism studies. Although D3-Leu has traditionally presented technical challenges for stable isotope-enabled relative quantification studies, such as the light and heavy isotope overlap, and the RT shift in the XIC, our workflow takes advantage of the merits of co-isolation used in DIA and NeuCode [12–14], and HR/AM for 2HM3 detection (NeuCode) to make D3-Leu the more tractable tracer for endogenous labeling. Moreover, our XPI approach provides us with a more rapid turnaround for data analysis when compared to the XIC approach, which will facilitate future clinical studies aimed to increase the throughput and productivity of HDL apolipoprotein metabolism research. Finally, our workflow can be applied to any type of *in vivo* or *in vitro* labeling study that uses human or animal samples, and to any study that uses PRM for quantitative proteomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

This study was supported by research grants from Kowa Company, Ltd. (to M.A.) and the National Institutes of Health (R01HL107550 to M.A.; UL1 RR 025758-01; and R01HL095964 to F.M.S.).

Abbreviations

AUC	area under the curve
DIA	Data-independent acquisition
HDL-C	high-density lipoprotein cholesterol

HR/AM	high resolution/accurate mass
LDL	low-density lipoprotein
RT	retention time
XIC	extracted ion chromatogram
XPI	extracted PRM peak intensity

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SIGNIFICANCE OF STUDY

ApoA-I is closely related to the concentration of HDL (HDL apoA-I) in clinical studies on dyslipidemia. Subjects participate in metabolism studies using endogenous labeling of apoA-I by, most commonly, D3-Leu. The ability to monitor the properties of HDL metabolism in conjunction with a candidate HDL raising therapy increases the likelihood of understanding the mechanism(s) through which the therapy acts. However, D3-Leu enrichment in apoA-I is very low, less than 1 %. In this study, we combine our recently developed HR/AM-PRM-based 2HM3 detection strategy with an automated enrichment quantification informatics workflow to expedite HDL apoA-I metabolism data analysis. The workflow is sensitive enough to automate quantification of D3-Leu enrichment in HDL apoA-I signal that is split into six size fractions, and to quantify low enrichment in LCAT, a key enzyme for HDL metabolism. Using LCAT, we also demonstrated that not only *b*- and *y*-ions, but also *a*-ions can be used for enrichment if they are available. Our automated method is spectra intensity-based and circumvents technical challenges associated with using the XIC/AUC-based quantification of deuterium labels. Our method generates apolipoprotein enrichment curve data within minutes, permitting clinicians with a means to increase throughput and scope of *in vivo* HDL metabolism studies.

Figure 1. Workflow for D3-Leu peptide library and automated extracted PRM peak intensity (XPI) quantification steps

A, Flowchart for Leucine-containing peptide selection and PRM enabled 2HM3 enrichment detection. B, Outline of the file types and software used for PRM peak detection and noise/outlier removal. C, Quantification, validation and visualization modules used for XPI.

Figure 2. Outline of the D3-Leu bolus-dependent endogenous labeling of HDL apoA-I strategy

A, Three subjects were administered D3-tracer and their blood was collected over three days. B, Workflow for HDL isolation and peptide sample preparation for PRM analysis. C, The XICs of an example apoA-I peptide's M0 and 2HM3 fragment ions. Standard AUC calculations assume that unlabeled and labeled ions co-elute, however deuterium labeling causes a shift in RT resulting in missing peak areas when a narrow AUC window is used to increase specificity. D, Before and after ion filtering using the XPI-based quantification – the M0 and 2HM3 peak intensities in each PRM scan are extracted and plotted. [blue line: peak smoothing line, red line: acquired peak, red circle: local maximum and yellow circle: local minimum]. The 2HM3 ion search space is limited to the scan numbers in which the M0 is identified. E, Superimposition plot of the XPIs versus RT.

Figure 3. Extracted PRM peak intensity plots from the apoA-I standard curve

A, Three-dimensional plots of the extracted peak intensities (“XPIs”) of THLAPYSDELRL fragment ions, *b*₃, *b*₄, *b*₅, *y*₃, *y*₄, and *y*₅. Heavy, 2HM3 ion and light, M0 ion. X-, Y- and Z-axes correspond to RT, ion series and ion intensity respectively. B, Two-dimensional mass profile of the *y*₅ ion, showing the non-overlapping properties of the M0 and 2HM3 peaks.

Figure 4. Ion filtering of APOA1's extracted PRM peak enrichments

A, PRM ion legend for panels B-D. Since two PRM technical replicates are merged in panels B-D, the replicate number is indicated in the fragment ion (e.g. ATEHLSTLSEK+3-b5_1, is derived from replicate 1). B, Scatter plot of PRM M0 ion intensity sum versus the enrichment sum that is used to evaluate enrichment data consistency versus PRM ion type. Data points to the left of the blue and below the red lines were excluded from quantification. A third filter for excessive enrichment outliers (yellow line) is available but no ions were filtered using this criterion. C and D, Logged PRM M0 versus 2HM3 intensity, before (C) and after PRM ion filtering (D). E, Scatter plots with a regression line comparing the

averaged peptide enrichments at each time point between acquisition 1 and 2 for PRM ion filtered data. Pearson's r (R) and empirical p-value (eP) and are calculated to show similarity and significance, respectively. F, The measured quantity (fmol on column) of apoA-I in the six HDL fractions isolated from three subjects.

Figure 5. XPI-determined D3-Leu tracer enrichment curves for apoA-I, apoA-II, apoE and LCAT in HDL

A, ApoA-I enrichment curves with the combined data from two replicates of PRM acquisition. Open circle, median enrichment per peptide; red circle, the mean of open circles at each time point. The ascending, descending and convergent slopes of the fitted blue curve are indicated. B, Violin plots comparing the peak enrichment times and slopes calculated in (A), across the size fractions. C, Peptides and fragment ions analyzed for LCAT, apoA-II and apoE. D, The extracted ion chromatograms for the indicated apolipoprotein precursor peptides from Subject 1, alpha 3 size fraction. E, Three-dimensional plots of the XPIs for the indicated LCAT peptide, Subject 1. The PRM ions used for LCAT were, *b4*, *b5*, *a4* and *a5*. F,

Enrichment curves for LCAT, apoA-II and apoE in the alpha2 and alpha3 HDL size fractions.

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