TITLE

Development and Validation of Apolipoprotein A-I Associated Lipoprotein Proteome Panel for the Prediction of Cholesterol Efflux Capacity and Coronary Artery Disease

RUNNING HEAD

Proteomic Assay for Lipoprotein Function

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ABBREVIATIONS

CEC – cholesterol efflux capacity, pCE – predicted cholesterol efflux, pCAD – predicted coronary artery disease probability, CAD – coronary artery disease, AALP – apolipoprotein A-I associated lipoprotein particle, hsCRP – high sensitivity C-reactive protein, ApoA-I – Apolipoprotein A-I, ApoB – Apolipoprotein B-100

STRUCTURED ABSTRACT

Background

Cholesterol efflux capacity (CEC) is a measure of HDL function that, in cell-based studies, has demonstrated inverse association with cardiovascular disease. The cell-based measure of CEC is complex and low-throughput. We hypothesized that assessment of the lipoprotein proteome would allow for precise, high-throughput CEC prediction with clinical validity.

Methods

After isolating lipoprotein particles from serum, we used LC-MS/MS to quantify 21 lipoprotein-associated proteins. A bioinformatic pipeline was used to identify proteins with univariate correlation to cell-based CEC measurements and generate a multivariate algorithm for CEC prediction (pCE). Using logistic regression, protein coefficients in the pCE model were reweighted to yield a new algorithm predicting coronary artery disease (pCAD).

Results

Discovery using targeted LC-MS/MS analysis of 105 training and test samples yielded a pCE model comprised of 5 proteins (Spearman r=0.86). Evaluation of pCE in a case/control study of 231 healthy and coronary artery disease (CAD) specimens revealed lower pCE in cases (P=0.03). Derived within this same study, the pCAD

model significantly improved classification (P<0.0001). Following analytical validation of the multiplexed proteomic method, pCE and pCAD models were examined in a case/control study of myocardial infarction in 137 post-menopausal women (P=0.015 and P=0.001, respectively).

Conclusions

We developed, validated and tested a multiplexed proteomic assay to predict CEC and CAD. Specimens from healthy subjects demonstrated modest, but statistically significant, higher pCE values than specimens from subjects with CAD. The pCAD model improved stratification compared to pCE. Further studies to assess clinical validity of the pCE and pCAD models are warranted.

INTRODUCTION

High density lipoproteins (HDL) are macromolecular assemblies that play a key role in lipid transport but also exert effects in endothelial function, thrombosis, and inflammation. A recent focus by a number of groups on HDL function, rather than HDL cholesterol (HDL-C), revealed that efflux capacity is inversely associated with coronary artery disease (CAD)(1–3).

Traditionally cholesterol efflux is measured using a cell based assay where cultured macrophages are loaded with ^{3}H-labeled cholesterol and subsequently exposed to a cholesterol acceptor, typically LDL-depleted serum (4). Modifications to the assay have been developed which make use of fluorescent or stable isotope labeled cholesterol to simplify sample handling (5,6). Although successfully deployed for clinical studies, the assay remains complex and an easier method to enable larger studies would be welcome.

Proteomic surveys of HDL have provided a consensus view that 60-90 proteins are reliably associated with HDL and quantitative differences in protein abundance have been associated with inflammation, cardiovascular, and kidney disease (7–11). Furthermore, proteomic changes have been proposed to have significant functional consequences (12). We have previously demonstrated that rapidly enriched lipoprotein particles using ApoA-I affinity possessed physical and chemical properties similar to those of HDL (13). In this work, we explored quantitative relationships between the

ApoA-I associated lipoprotein particle (AALP) proteome and cholesterol efflux capacity (CEC) with the goal of deriving a predictor of CEC (pCE). We further explored modification of pCE to develop a model that would serve as a proteomic indicator of coronary artery disease (pCAD).

The use of mass spectrometry for protein analysis in the clinical laboratory has gained traction as demonstrated with assays for thyroglobulin, apolipoproteins, lipoprotein associated phospholipase A2, insulin and its C-peptide (14–17)However, multiplex proteomic panels are in their infancy, have been less extensively reported, and can present unique challenges in analytical validation. To facilitate studies aimed at investigating clinical validity of pCE/pCAD we followed Institute of Medicine recommendations regarding discovery and analytical validation by establishing a "bright line" in which the method and its associated algorithms are locked before analytical validation, with any modifications warranted by the results of subsequent clinical studies requiring a return to the discovery/development phase and use of specimen sets naïve to previous analysis (18). Here we describe the discovery and development of the pCE and pCAD models and the validation of the underlying analytical method. Subsequently, the performance of the locked and validated pCE and pCAD models were evaluated in a case/control study in a population of post-menopausal women.

MATERIALS AND METHODS

All study methods were approved by local Institutional Review Boards, as appropriate. His$_6$-tagged and ^{15}N-labeled ApoA-I was purchased from Genscript. Ni-NTA immobilized metal affinity micro columns were purchased from Phynexus. Stable isotope labeled peptides were ordered from New England Peptides. Charcoal stripped serum was obtained from Golden West Biologicals. Other reagents were purchased at highest available quality from Sigma-Aldrich.

Study Specimens

Discovery and development experiments were performed specimens selected from the coronary artery disease arm of the Fairbanks Institute for Healthy Communities biobank. Clinical specimens for post-bright line evaluation of clinical validity were obtained from the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKTOCS) (19–21). Specimens for evaluation of longitudinal stability were obtained from BioServe Biotechnologies.

Cell-Based Assessment of Cholesterol Efflux Capacity

Human serum samples were LDL-depleted and cell based assays to measure the efflux of ^{3}H-labeled cholesterol from J774 macrophages was performed by Vascular Strategies, Inc. using the method described by de la Llera-Moya(22). All measurements were reported as normalized cholesterol efflux capacity values (% efflux/4hr).

Affinity Enrichment and LC-MRM Analysis of ApoA-I Associated Lipoproteins

Utilizing a semi-automated workflow implemented on a FreedomEvo automated liquid handler (Tecan Group, Ltd.), metal chelate affinity chromatography was used to isolate AALP from human serum. AALP eluent was heat treated for denaturation, followed by LysC digestion and internal standard addition. Manual intervention was only required to transfer plates to a centrifuge or incubator during the process. Samples were transferred to a multiplexed LC-MS/MS system (StreamSelect, Agilent Technologies), where 3 individual LC-systems comprising a quaternary and binary pump were coupled to a CTC-PAL autosampler and a single Agilent 6495 mass spectrometer. Quantitative analysis was performed using MassHunter (Agilent). Extensive experimental details including all LC-MS/MS parameters are provided in the **Supplemental Data File (Part I)**.

Informatics Workflow for pCE and pCAD Model Development

Prior to computational analysis, the calibrated response of each targeted peptide was normalized to the calibrated response of ^{15}N-His$_6$ApoA-I to account for variations in recovery during the enrichment process. For proteins with two peptides measured by LC-MS/MS, the relative amount of protein was established using the peptide with higher intensity. An analytical pipeline encompassing a series of sequential steps for feature selection was applied to normalized data to discover proteins associated with CEC [23]. For univariate analysis, robust linear regression was applied to each protein to predict CEC on 70 training samples and proteins with p-value < 0.1 were selected. Multivariate selection of proteins was performed using elastic net, and a model was built on the selected proteins using a partial least-squares regression. To assess model

performance, Spearman correlation and median absolute difference (cost) between pCE and measured CEC were calculated. The final analytical method utilizes a well-defined mixture of stable isotope-labelled peptides as a single point calibrator to ensure stability of the computational algorithm in the presence of any interferences or other matrix effect. Finally, the panel was tested with 35 serum specimens. To assess pCE performance in differentiating subjects with and without CAD, we tested the model on 74 healthy controls and 157 CAD specimens.

To explore the potential for improving classification performance, we refined the coefficients for the proteins in the pCE model using logistic regression based on clinical status (pCAD, **Table 1**). Cross-validation was used to generate biomarker scores that reflect CAD probability for the CAD and control subjects, by reweighting protein coefficients using a subset of subjects and applying this to the left-out subjects. The pCAD biomarker score can be simply converted to a probability score between 0 and 1 by means of the following equation:

$$CAD\ probability = \frac{1}{1 + e^{-pCAD}}$$

All statistical analysis of the data was performed using R version 3.2.3 (24)

Method Validation

The LC-MS/MS assay which provides quantitative values for 5 apolipoproteins used in the two biomarker panels (pCE and pCAD) was validated. To achieve high-throughput quantitative analysis, a multiplex HPLC system was employed in which three independent HPLC systems were coupled to the mass analyzer. During validation, each HPLC was

evaluated independently. Test materials included three QC pools with high, medium, low

pCAD values, and individual specimens as needed. Validation figures-of-merit represent

total imprecision and bias which consisting of inter- and intraday as well as inter-HPLC

measurements. The following parameters were validated: assay precision and accuracy,

sample stability at selected temperatures, high/low mixing to demonstrate measurement

linearity, limit of analytical sensitivity, matrix effect, impact of interferences, and in-process

stability. Additionally, a short-term longitudinal study was undertaken for 29 individuals

sampled weekly over 8 weeks to assess intra-individual variability. Detailed validation

experimental protocols and results are presented in the Supplemental Data file (part II).

RESULTS

The overall strategy for discovery, optimization, analytical validation, and evaluation of a

multiplexed proteomic assay to predict cholesterol efflux capacity and coronary artery

disease is depicted in **Figure 1**. First, serum samples were used to obtain both cell-

based CEC measurements and quantitative analysis of 21 AALP associated proteins

using LC-MS/MS. Using a bioinformatic pipeline, an algorithm consisting of a weighted,

linear combination of 5 apolipoproteins was derived and validated for prediction of CEC

(pCE). The 5 apolipoprotein coefficients were reweighted using logistic regression

against CAD and healthy control specimens to yield a predictor of CAD (pCAD). The

LC-MS/MS analytical method enabling both pCE and pCAD models was then

extensively refined to optimize for the 5 proteins included in each model. The standard

operating procedure was locked, and method was then validated establishing the bright

line for examining clinical validity in a population naïve to assay discovery and validation.

Targeted Quantitation by Multiple-Reaction Monitoring Mass Spectrometry

We used a quantitative LC/MS/MS method to explore the relationship between the AALP proteome and CEC. Based on a literature search and results from previous data-dependent proteomics experiments, 21 proteins with known associations with lipid transport, reverse cholesterol transport and/or cardiovascular disease were selected (lipid metabolism (Apolipoproteins A-I, A-II, A-IV, C-I, C-II, C-III, C-IV, D, E, F, J, L-I, M), enzymes (Phospholipid Transfer Protein – PLTP, Cholesteryl ester transfer protein - CETP, lecithin cholesterol acyl transferase – LCAT, paraoxonase 1 – PON1), and acute phase response proteins (Complement C3, Haptoglobin, Serum Amyloid A 1 and 2 - SAA1/2, and SAA4)). Two optimal peptides from each protein (where possible) were identified and the entire workflow was optimized. Digest conditions were obtained which yielded stable peptide abundances for all proteins within 4 hours.

Development and Validation of a Multivariate Algorithm for CEC Prediction

A set of 70 training and 35 independent test samples (**Supplemental Table 3**) were randomly selected without respect to any disease diagnosis but were carefully matched on lipoprotein measurements. Normalized CEC and mass spectrometry data for the compliment of 21 proteins were determined for each specimen. After univariate analysis, 9 proteins with p-value < 0.1 (Apolipoproteins A-I (p=3.07E-5), A-II (p=5.4E-3), C-I (p=1.08E-3), C-II (p=4.7E-10), C-III (p=2.13E-11), C-IV (p=1.41E-4), D (p=7.71E-2),

CETP (p=3.47E-2), and SAA (9.84E-2))were identified by robust linear regression. Subsequent elastic net regression selected 5 proteins (ApoA-I, ApoC-I, ApoC-II, ApoC-III, ApoC-IV) on which partial linear regression was applied to establish the final predictive CEC (pCE) model (**Table 1**). A typical chromatogram for target peptide quantifier ions is shown in **Figure 2A**. Peptides used in the pCE model are denoted by an asterisk and span approximately 3 orders of magnitude of dynamic range in measured abundance (and calibrated response). The pCE model performed well (training set spearman r=0.67, p<0.001 (**Figure 2B**); validation set Spearman r=0.86, P<0.001 (**Figure 2C**)).

The associations between CEC determined by cell-based assay and proteomic estimation and other clinical measurements (Total Cholesterol, HDL-c, LDL-c, non-HDL-c, Triglycerides, ApoA-I, ApoB, and hsCRP) were examined. The most significant associations were with ApoA-I (Pearson r=0.57, **Supplemental Figure 1A**) and HDL-c (Pearson r=0.45, **Supplemental Figure 1B**), in agreement with previous reports (2,25). We also observed a significant negative correlation between CEC and hsCRP (Pearson r=-0.23, **Supplemental Figure 1C**), consistent with previous observations that CEC is negatively impacted in inflammation response (12).

Testing and Refinement of pCE Algorithm for CAD Diagnosis

Assuming predicted CEC should have similar inverse association with cardiovascular disease as reported for cell-based CEC measurements, we tested the pCE model on 157 CAD specimens and 74 age and sex-matched apparently healthy controls (**Supplemental Table 4**) from the Fairbanks Institute biobank. We found lower median predicted CEC for patients with CAD (case, 9.91 % efflux/4hr; control, 10.2 % efflux/4 hr;

p=0.03) (**Figure 3A**). Using predicted CEC as a classifier of case vs. control, the AUC of the ROC curve was 0.62 (p=0.02) (**Figure 3D**). We also performed a comparison of the predicted CEC and cell-based CEC measurements in a small subset of Fairbanks specimens. Cell based CEC measurements were obtained after 15 highest and lowest predicted CEC values. No significant difference was evident when comparing samples across predicted and cell-based measurements (p=0.39 for high pCE-specimens, p=0.08 for low-pCE specimens) (**Supplemental Figure 2**).

The pCAD results revealed a significant difference in the median probability scores between the CAD and control cohorts (p<0.0001) (**Figure 3B**) yielding an AUC of 0.73 (**Figure 3D**). Using Youden's index as the numeric threshold, the pCAD model demonstrated sensitivity and specificity of 71% and 76%, respectively. A second, and separate, sampling of age and sex-matched CAD (n=92) and Control samples (n=92) from the Fairbanks biobank, not used for pCAD algorithm derivation, was conducted several weeks later, demonstrating similar performance (p<0.0001, AUC=0.71)(**Figure 3C**).

Analytical Validation Summary

In the design of this assay, some traditional metrics like spike recovery cannot be assessed. For the purposes of this manuscript, the most critical figures of merit are presented here but additional data regarding key validation metrics (linearity, stability, interferences, etc.) are found in the Supplemental Data File (Part II)

To estimate assay imprecision, test materials were analyzed in four replicates each over 15 days, across three HPLC systems coupled to the same mass spectrometer. Estimates

of total imprecision for the assay were determined at the peptide level. The coefficients of variation (CV) for ApoA-I, ApoC-I, ApoC-II, and ApoC-III ranged from 5 - 8% for three pooled and two individual specimens (**Table 2 and Supplement Table 5**). The CV of the lowest abundance protein, ApoC-IV, ranged from 7.7 to 14%, below the established acceptance criteria of 20%.

In the absence of a reference method or material with a known value, bias is evaluated as the difference from initially assigned results. Prior to start of validation, test materials were extensively characterized, and values were assigned for each of the 5 proteins. As shown in **Supplement Table 6**, no protein other than ApoC-IV in the contrived high QC pool demonstrated a bias > ±20%. Patient sample 2, having the same assigned ApoC-IV levels, demonstrated bias within the acceptance criteria. The modest performance of ApoC-IV is likely because it is the least abundant protein in the assay, nearly 4 orders of magnitude lower in abundance than ApoA-I.

Studies of calibrator performance demonstrated that linear dynamic ranges spanned about two orders of magnitude for all five proteins (Supplemental Data File, Part II). Limits of quantification were evaluated by dilution of calibrator peptides in a digest matrix (**Supplemental Data File, Part II**, **Supplemental Figure 3**) in which lower limits of quantification were established upon visual inspection of departure of calibrator from linearity for each peptide. A five-point mixing experiment demonstrated that the response of pCAD and pCE values were linear as function of dilution for three high/low pCAD pairs (**Supplemental Figure 4**). The mean post-IMAC enrichement recovery of the spiked capture reagent, ^{15}N-His$_6$ApoA-I, from matrix was 78 ± 7% (SD) relative to direct digestion of the same amount of ^{15}N-His$_6$ApoA-I in PBS buffer. Recovery of endogenous ApoA-I

was found to be highly correlated with automated immunoturbidimetric results (**Supplemental Figure 5**).

Best practice for protein LC-MS/MS assays is to assess the agreement between two or more peptides from each protein (when possible) and evaluating adherence to fragment ion intensity ratios established from characterization of synthetic peptides. For each protein with two peptides, stable linear relationships were observed across all experimental measurements. Using 233 random serum samples, representative data for linear correlation between the two ApoA-I peptides revealed a slope of 0.88 with r^2=0.94. Data for peptides from ^{15}N-ApoA-I, ApoC-I, ApoC-II can be found in the supplemental section (**Supplemental Figure 6 and 7**). Likewise, fragment ion ratios for all measured peptides were well below the established acceptance criteria of ±30%.

Matrix effect studies demonstrated that no substantial ion suppression or enhancement were observed (**Supplemental Figure 8**). Unconjugated bilirubin and hemoglobin at 5 mg/dL and 200 mg/dL, respectively do not affect peptide quantification. However, unacceptable interference by intralip at ≥150 mg/dL was observed (**Supplemental Figure 9)**.

Longitudinal Evaluation of Biological Variability

We investigated the biological variation of the pCAD and pCE measurement in specimens collected once per week from 29 subjects over an eight-week period. Clinical measurements (HDL-c, LDL-c, triglycerides) were evaluated to assess quality of the specimens. One subject was rejected due to abnormally high triglycerides (an identified interference) for all collected specimens (>900 mg/dL). Four other individual specimens

were rejected due to greater variation (> 2 times SD) in two or more of three measurements (HDL-c, LDL-c, triglycerides) from the mean of 8 observations. The pCE and pCAD scores were then determined for the remaining 216 specimens from 28 subjects (**Supplement Figure 12**). For pCE, biological variation overs eight-weeks was observed to be 7.1%. Evaluation of intra-individual variability of pCE/pCAD in a modest longitudinal study gave positive indication that the lipoprotein proteome and therefore pCE/pCAD for an individual is generally stable over eight weeks. In such situations where the biological variability is low and analytical precision of the test method is high, measurements are more likely to reveal clinically relevant changes.

pCE and pCAD Model Evaluation in UKCTOCS Cohort

After analytical method validation, the performance of the pCE and pCAD models was assessed in United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) biobank, a population distinct from development and Fairbanks cohorts (detailed in Supplemental Data File). The cohort was post-menopausal women diagnosed with myocardial infarction (MI) 1 to 2 years after sample collection as an indicator of CAD (n=69) and healthy controls matched on age, time to sample processing, blood pressure, and BMI. The pCE (**Figure 4A**) model yielded significantly lower predicted CEC values among cases relative to controls (median pCE=10.66 vs 11.15, p=0.01). Likewise, pCAD (**Figure 4B**) values for the cases were significantly elevated relative to the controls (median predicted probability of CAD=0.51 vs 0.37, p=0.001).

DISCUSSION

The interest in cholesterol efflux capacity as a cardiovascular biomarker was stimulated by a series of studies that demonstrated an inverse relationship between serum cholesterol efflux capacity and cardiovascular risk. A substantial challenge in realizing the benefit of this measurement lies in the technical challenges of running a cell-based assay. We proposed that a proteomic approach to estimating serum CEC might provide an avenue to enable large scale studies with an assay that is more amenable to clinical use. The pCE model performance in both the Fairbanks (**Figure 3A**) and UKCTOCS (**Figure 4A**) cohorts replicates previously observed trends suggesting that pCE can recapitulate biological function that has been explored using an orthogonal method. Despite the differing statistical significance in this study, both pCE and pCAD may present advantages depending on the context of future studies, as pCE is directly correlative to a biological function, and pCAD was developed as a predictor of disease state.

The trajectory for development of a new biomarker is arduous, requiring success in discovery, reduction of the process to a laboratory workflow, analytical validation and finally sufficient studies to demonstrate clinical validity and utility(18,26). Here we have described discovery and refinement of a method for the estimation of cholesterol efflux and CAD risk to a validation bright line where subsequent studies can formally evaluate clinical validity.

We have shown that a multiplexed proteomic assay with satisfactory figures-of-merit for analytical performance can be achieved. This is an incremental step toward demonstrating the broader feasibility of high-throughput multiprotein analysis in the

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clinical laboratory and presents the opportunity to rigorously explore clinical validity and ultimately utility of the pCE and pCAD biomarkers.

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Table 1: pCE and pCAD model coefficients.

Variable	pCE	pCAD
Intercept	7.57	5.81
ApoA-I	-1.74	-12.48
ApoC-I	-9.76	-112.05
ApoC-II	27.36	238.66
ApoC-III	146.76	24.11
ApoC-IV	-377.07	927.51

To calculate pCE or pCAD, each protein concentration (nM) is normalized to ^{15}N-His$_6$ApoA-I response (nM) prior to multiplication by its coefficient

Table 2. LC-MS/MS method validation performance summary.

Specimen Type	Human serum
Lower Limits of Quantitation	^{15}N-ApoA-I: 15 nM ApoA-I: 15 nM ApoC-I: 10 nM ApoC-II: 5 nM ApoC-III: 5 nM ApoC-IV: 0.2 nM
Calibrator performance (Linear Dynamic Range)	^{15}N-ApoA-I: 15 - 935 nM ApoA-I: 15 - 935 nM ApoC-I: 10 - 325 nM ApoC-II: 5 – 85 nM ApoC-III: 5 – 160 nM ApoC-IV: 0.2 – 11 nM
^{15}N-ApoA-I recovery	Mean recovery of 78% of added ^{15}N-His$_6$-ApoA-I
Precision (Lab CV)	^{15}N-ApoA-I: 5.2 - 7.0% ApoA-I: 5.2 - 7.3% ApoC-I: 6.5 - 7.0% ApoC-II: 5.3 - 7.4% ApoC-III: 5.5 - 8.1% ApoC-IV: 7.7 - 14.0%
Specificity (Interferences)	Specimens with medium to heavy lipemia (corresponding to >150 mg/dL intralipid) are rejected
Freeze/thaw stability	Up to three freeze/thaw cycles are acceptable
Sample stability	Stable for 21 days at < -60 °C, -18 to -25 °C, or 2 - 8 °C; Stable for 24 hours at 20 - 26 °C
In-process stability	Stable in sealed microtiter plates for up to 3 days when stored at 2 - 8 °C

FIGURE CAPTIONS

Figure 1. Schematic overview of the discovery of a cholesterol efflux prediction model, it's refinement and the development of a proteomic predictor of CAD, analytical validation to the bright line at which point assay parameters are locked, and finally post-validation evaluation of the assay in a case control study to examine prognostic capability.

Figure 2. (A) A representative chromatogram of peptide quantifier peaks for the five proteins that feed the predictive cholesterol efflux algorithm (*) in addition to secondary peptides that serve as assay quality control indicators. (B) Correlation of cell-based CEC measurement and LC-MS/MS CEC prediction in 70 training samples and (C) 35 test samples.

Figure 3. Box and whisker plots demonstrating performance of (A) predicted cholesterol efflux and (B) prediction of CAD models in stratification of control and CAD cohorts from the Fairbanks Institute bio-bank. (C) A repeat comparison of age/sex-matched CAD and control patients from the Fairbanks Biobank. (D) Receiver operating characteristic curves comparing diagnostic performance of pCE and pCAD algorithms in the Fairbanks bio-bank.

Figure 4. Box and whisker plots demonstrating post-validation performance of (A) pCE and (B) pCAD models in control and case cohorts from the UKCTOCS bio-bank.