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Protein Biomarkers of New-Onset Cardiovascular Disease: A Prospective Study from the Systems Approach to Biomarker Research in Cardiovascular Disease (SABRe CVD) Initiative

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Abstract

Objective—Incorporation of novel plasma protein biomarkers may improve current models for prediction of atherosclerotic cardiovascular disease (ASCVD) risk.

Approach and Results—We utilized discovery mass spectrometry (MS) to determine plasma concentrations of 861 proteins in 135 myocardial infarction (MI) cases and 135 matched controls. We then measured 59markers by targeted MS in 336 ASCVD case-control pairs. Associations with MI or ASCVD were tested in single marker and multimarker analyses adjusted for established ASCVD risk factors.

Twelve single markers from discovery MS were associated with MI incidence (at $p<0.01$) adjusting for clinical risk factors. Seven proteins in aggregate (cyclophilin A, CD5 antigen-like, cell surface glycoprotein MUC18, collagen-alpha 1 [XVIII] chain, salivary alpha-amylase 1, C-reactive protein, and multimerin-2) were highly associated with MI ($p<0.0001$) and significantly improved its prediction compared to a model with clinical risk factors alone (C-statistic of 0.71 vs. 0.84). Through targeted MS, twelve single proteins were predictors of ASCVD (at $p<0.05$) after adjusting for established risk factors. In multimarker analyses, four proteins in combination (alpha-1-acid glycoprotein 1, paraoxonase 1, tetranectin, and CD5 antigen-like, predicted incident ASCVD ($p<0.0001$) and moderately improved the C-statistic from the model with clinical covariates alone (C-statistic of 0.69 vs. 0.73).

Conclusions—Proteomics profiling identified single and multimarker protein panels that are associated with new onset ASCVD and may lead to a better understanding of underlying disease

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Disclosures

Drs. Muntendam, Adourian, and Juhasz contributed to this project while affiliated with BG Medicine, Inc., a biomarker discovery company.

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mechanisms. Our findings include many novel protein biomarkers that, if externally validated, may improve risk assessment for MI and ASCVD.

Keywords

Biomarker; cardiovascular disease; epidemiology; myocardial infarction; proteomics

Introduction

Atherosclerotic cardiovascular diseases (ASCVD) are a leading cause of morbidity and death globally.[1] A more accurate assessment of ASCVD risk could lead to earlier treatments to delay disease onset and prevent the occurrence of clinical events and death. Efforts to improve the detection of ASCVD include the use of risk assessment algorithms that take into consideration age, sex, and several established clinical risk factors.[2] Numerous studies have attempted to identify plasma protein biomarkers of ASCVD to improve the detection of individuals at increased risk for ASCVD.[3, 4] The extent to which plasma protein biomarkers improve the prediction of risk for ASCVD beyond established risk factors remains controversial.[5]

As part of the Systems Approach to Biomarker Research in Cardiovascular Disease (SABRe CVD) Initiative, which seeks to discover biomarkers of ASCVD and its major risk factors, we sought to identify plasma protein biomarkers of new-onset myocardial infarction (MI) and ASCVD in participants from the Framingham Heart Study. Identification of novel biomarkers that individually or in aggregate predict risk of ASCVD could provide insight into the biology of the disease and could aid in developing targeted prevention strategies during the preclinical phase of ASCVD, when intervention may be more likely to alter disease progression.

Methods

Detailed information about study design, experimental strategy, and statistical methods including single marker analyses, multiple imputation and multiple marker analyses are available in the online-only Methods.

Results

iTRAQ discovery

Baseline characteristics of 135 pairs of MI cases and controls with protein levels are summarized in Table 1. Systolic blood pressure, total cholesterol, and body mass index (BMI) were higher and HDL cholesterol was lower in cases than controls. Diabetes and hypertension treatment were more common in cases than controls. The median follow up time (from the baseline examination to event occurrence) was 2.34 years with lower and upper quartiles 1.11 years and 4.06 years. In total, 861 proteins were measured by iTRAQ.

Single marker analysis—Among the iTRAQ measured proteins, 34 had no known protein annotation (i.e. classified as unknown) and for 39 proteins multiple isoforms were identified; this left 753 unique known markers, among which results for 168 markers were

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available in fewer than 40 pairs of individuals. We used markers with data available on at least 40 case-control pairs because for covariate adjusted analysis we required that at least 5 to 10 events per variable in a model were present.[6] Of 587 unique markers tested for association with MI, none had a p-value that attained overall significance after Bonferroni correction (0.05/587; p<8.5E-05). The top ten iTRAQ proteins associated with MI (Table 2) were glycoprotein 5 (OR per 1 SD = 0.44, 95%CI [0.27, 0.71]), CD5 antigen-like (0.55 [0.38, 0.79]), myoglobin (0.55 [0.37, 0.84]), inhibitor protein 1 (0.55 [0.36, 0.84]), C-reactive protein (1.75 [1.18, 2.59]), cyclophilin A (0.56 [0.37, 0.85]), contactin-1 (0.62 [0.45, 0.88]), albumin (0.43 [0.36, 0.85]), neural cell adhesion molecule 1 (0.61 [0.41,0.89]), and selenium-binding protein 1 (0.63 [0.42, 0.95]). Glycoprotein 5 was not associated with MI in the paired t-test (p=0.13) due to confounding by diabetes. Among diabetic subjects, the mean ± standard deviation (SD) of glycoprotein 5 for cases and controls were −0.30±0.89 and 0.04±1.02; among subjects without diabetes, the mean ± standard deviation (SD) of glycoprotein 5 for cases and controls were 0.46±1.00 and 0.71±0.54, respectively. Protein markers with p-value between 0.01 and 0.05 in either paired t-test or conditional logistic regression are available in online Supplementary Table I.

Most, if not all of the unidentified proteins are believed to be antibody fragments generated from the sample preparation workflow and not related to endogenous biomarkers. Different isoforms could be from post translational modification or a cleaved or truncated version of the same marker. In statistical analysis we prioritized isoforms with complete data if possible. However due to funding and laboratory restrictions and limited non-renewable plasma samples, we did not attempt to further identify the exact nature of these unidentified products or isoforms.

Multiple marker analysis—To identify a multimarker protein panel from iTRAQ that was significantly associated with MI, we conducted analyses for 544 proteins (measured in 60% or more of samples) using stepwise selection within bins as detailed in the Methods section. The number of times that a marker was retained in the final model varied from 1 to 259 (out of 260 runs).

Using multiple imputation-stepwise selection based on 26 protein markers that were selected more than 50% of time, we identified a multimarker panel of 7 proteins associated with MI status. The multimarker panel included cyclophilin A, CD5 antigen-like, cell surface glycoprotein MUC18, collagen alpha-1 (XVIII), salivary alpha-amylase 1, C-reactive protein, and multimerin-2 (Table 3). The C-statistic from the model with clinical covariates only was 0.71. The median C-statistic for the multiple marker model (based on each of 20 imputed datasets) was 0.84 (limits across imputations, 0.81 to 0.87). The combination of these 7 protein biomarkers was associated with MI (likelihood ratio test p<0.0001). Among cases, the gain in predicted probability of being a case from the clinical model to multiple marker model had a mean of 0.16 (standard error 0.02). The two markers with largest effect on MI case status were cyclophilin A (OR per 1 SD = 0.34, 95% CI [0.18, 0.63], p=0.0008) and CD5 antigen-like (0.48 [0.29, 0.79], p=0.0040). In single marker analysis (Table 2), glycoprotein 5 was the strongest predictor of MI. This maker was among the top 26 markers that were selected over 50% of the time, but it narrowly missed stepwise selection (p=0.056) adjusting for variables chosen in the final model.

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MRM and Depletion MRM

Single marker analysis—The top proteins in single- or multiple-marker analysis from iTRAQ were assayed on 336 pairs of ASCVD cases and controls. MRM or depletion MRM was not attempted on any markers identified from iTRAQ discovery if their plasma concentrations were below the detection limits of MRM technology (see Supplementary Methods section). A total of 32 protein markers were measured by MRM and 27 by depletion MRM. In individual marker analyses, the top MRM markers associated with ASCVD were alpha-1-acid glycoprotein 1, C-reactive protein, ceruloplasmin, serum amyloid A-1 protein, gelsolin, tetranectin, hemopexin, paraoxonase 1, protein Z-dependent protease inhibitor, and leucine-rich alpha-2-glycoprotein ($p<0.05$ in the risk factor adjusted conditional logistic regression model; Table 4). The top proteins associated with ASCVD among those measured by depletion MRM included protein Z-dependent protease inhibitor and neural cell adhesion molecule 1 (Table 4). The remaining markers we measured using MRM and depletion MRM are available in online Supplementary Table II.

Multiple marker analysis—In risk factor adjusted multiple biomarker analysis from MRM and depletion MRM, alpha-1-acid glycoprotein 1 (OR per 1 SD = 1.45, 95% CI [1.17,1.80], paraoxonase 1 (0.75 [0.60,0.94]), tetranectin (0.76 [0.60,0.95]), and CD5 antigen- like (0.81 [0.67,0.98]) jointly predicted ASCVD risk (Table 5). The C-statistic of the model with clinical covariates only was 0.69; with the addition of the panel of four protein biomarkers, the C-statistic rose to 0.74. The likelihood ratio test showed that these proteins were associated with ASCVD ($p<0.0001$). Among cases, the gain in predicted probability of being a case from the clinical model to multiple marker model had a mean of 0.032 (standard error 0.007).

We repeated multiple marker analysis on the 135 MI case-control pairs included in the MRM and depletion MRM analysis. Protein Z-dependent protease inhibitor (OR per 1 SD = 1.7, 95% CI [1.16, 2.50]), C-reactive protein (1.51 [1.03, 2.23]), and CD5 antigen-like (0.7 [0.50, 0.98]) in combination predicted the new onset of MI. The addition of these three markers to the clinical risk factors increased the C-statistic from 0.72 to 0.76. The likelihood ratio test showed these protein biomarkers to be jointly associated with ASCVD ($p<0.0001$). Among cases the mean gain in predicted probability of being a case from the clinical model to multiple marker model was 0.068 (standard error 0.015). We acknowledge that some markers contribute to only either MI or only ASCVD, and some markers contribute to both outcomes in multiple marker analysis but with different effect sizes. This could be due to the following reasons. One is that the proteins might play different roles in MI than in the other ASCVD diseases (e.g. acute thrombotic events vs. chronic atherosclerotic disease). The second reason is that the two analyses were based in part on different samples. The analysis for MI was based on 135 pairs of MI cases and controls; ASCVD analysis was based on 336 pairs of ASCVD cases and controls (including the MI pairs). The third reason is that the set of candidate markers is not identical for different outcomes. In joint modeling, the effect size and direction of a specific marker is affected by other markers in the same model.

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Discussion

Through discovery and targeted proteomic studies, we identified single protein biomarkers that were associated with risk of MI or ASCVD. We also identified panels of proteins that in aggregate improved MI and ASCVD risk prediction above and beyond established risk factors. We included up to 587 protein biomarkers assayed by iTRAQ discovery mass spectrometry of MI cases and controls. After adjusting for established risk factors, the top iTRAQ derived protein biomarkers of MI in single marker analyses were glycoprotein 5, CD5 antigen-like, myoglobin, protein kinase C inhibitor protein 1, C-reactive protein, cyclophillin A, contactin-1, and albumin (p=0.007). Cyclophilin A, CD5 antigen-like, cell surface glycoprotein MUC18, collagen alpha-1(XVIII) chain, salivary alpha-amylase 1, C-reactive protein, and multimerin-2 emerged as a multimarker protein panel for MI (p<0.0001). This panel of seven proteins improved MI risk prediction compared with clinical risk factors only, with a model C-statistic of 0.84 (versus 0.71 for the clinical risk factor only model, p<0.0001). Further studies are warranted to determine if the multimarkers we identified can be replicated, and if so, whether adding protein panels to models based on conventional risk factors demonstrates clinical utility for the multimarker panel.

We then targeted 32 and 27 proteins for measurement by MRM and depletion MRM mass spectrometry, respectively. Alpha-1-acid glycoprotein, C-reactive protein, ceruloplasmin, serum amyloid A-1 protein, gelsolin, tetranectin, hemopexin, paraoxonase 1, protein Z-dependent protease inhibitor, and leucine-rich-alpha-2-glycoprotein were the top MRM markers of ASCVD in single marker analyses (at p<0.05). The top proteins associated with ASCVD among those measured by depletion MRM were protein Z- dependent protease inhibitor and neural cell adhesion molecule 1 (at p<0.05). In multiple marker analysis of the combined MRM platforms, alpha-1-acid glycoprotein, paraoxonase 1, tetranectin, and CD5 antigen-like in combination predicted incident ASCVD (p<0.0001) and increased the C-statistic from the model with clinical covariates (0.69 to 0.73, p<0.0001). The panel of three MRM markers that predicted MI (which included CD5 antigen-like, C-reactive protein, and protein Z- dependent protease inhibitor) increase the C-statistic from 0.72 to 0.76 (p<0.0001).

Our proteomic work identified several novel biomarkers of MI and ASCVD; nine of our markers were significant in a prior proteomic study of cardiovascular disease related outcomes. [7] Prentice et al, used proteomic screening of plasma from cases and controls in the Women's Health Initiative in an effort to identify proteomic biomarkers of coronary heart disease (CHD) and stroke. The main difference between their proteomics study and ours is that they pooled plasma samples from cases (and controls), precluding individual participant level analyses or complete adjustment for multiple ASCVD risk factors. They identified 37 proteins that were nominally associated with CHD and 47 proteins associated with stroke. [7] In their study, CD5 antigen-like, cyclophilin A, monocyte differentiation antigen CD14, multimerin-2, sulfhydryl oxidase 1, extracellular superoxide dismutase [Cu-Zn], apolipoprotein A-II, granulin, and insulin-like growth factor-binding protein 5 all had nominal p-values <0.05.

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Our MI prediction model included novel proteins for which associations with ASCVD have not previously been reported at the population level. Such markers include: collagen alpha-1(XVIII) chain, cyclophilin A, CD5 antigen-like, and salivary alpha-amylase 1. Cyclophilin A (coded for by the gene *PPIA*) was protective in our study (OR per 1 SD = 0.34, 95% CI [0.18, 0.63], p=0.0008 in multiple maker analysis). It is a ubiquitous, intracellular protein that plays a role in protein folding and trafficking. It is secreted by cells in response to inflammatory stimuli, especially oxidative stress. [8] Nigro et al. found that atherosclerosis was greater in *APOE* knockout mice vs. double knockouts for *APOE* and *PPIA*.[9] Human studies have shown a positive association between cylclophilin A and atherosclerosis[10] and congestive heart failure.[11] In a cross-sectional study, [12] cyclophilin A levels were associated with coronary atherosclerosis. To our knowledge, there has been no prior prospective study of the association of circulating cyclophilin A with the incidence of MI.

CD5 antigen-like (CD5L) is a cell-surface ligand on activated lymphocytes,[13] and its function in atherogenesis largely remains unknown.[14] Collagen alpha-1 (XVIII) chain (*COL18A1*) is a basement protein and its C-terminus encodes for endostatin, a 20kDa proteolytic fragment that inhibits angiogenesis and atherosclerosis.[15] When atherosclerosis prone *APOE* knockout mice were bred with *COL18A1* knockout mice, there was extensive intimal neovascularization in the double knockouts. [16] This may suggest a mechanistic relation between collagen alpha-1 (XVIII) and atherosclerosis, because plaque neovascularization is thought to promote atherosclerosis. Salivary alpha-amylase 1 cleaves starch glycosidic linkages to produce smaller saccharides; alpha-amylase 1 is higher in populations that have evolved under high-starch diets and may modulate glycemic response after glucose intake.[17]

Among the proteins identified by MRM as being associated with ASCVD, alpha-1 acid glycoprotein is an acute phase protein that is secreted by the liver and measurable in plasma. It is an abundant plasma protein that increases in response to infection, inflammation, tissue injury, or cancer.[18, 19] Its biological function remains unknown. Paraoxonase is a component of HDL cholesterol and protects LDL from oxidative modification, and thus delays the progression of atherosclerosis. Lower circulating levels of paraoxonase 1 have been reported to be associated with risk of MI,[20–24] and in Framingham, we detected an association with ASCVD with similar directionality (OR per SD= 0.79, 95% CI [0.64, 0.98], p=0.031) and an association with MI (cases' level lower than controls 0.24±0.09 SD, p=0.015). Tetranectin is an adhesion molecule found on endothelial cells and platelets.[25] Tetranectin is released by platelets and binds to the plasminogen kringle 4 domain; it enhances plasminogen activation and inhibits the proliferation of endothelial cells.[26] Population studies have shown that decreased plasma tetranectin levels are associated with coronary artery disease.[27] Our study shows similar results, albeit using a prospective study design; higher tetranectin levels were inversely associated with risk of ASCVD (OR 0.76 [0.61, 0.95], p=0.017).

Our study has several limitations. Despite the overlap of several of our ASCVD biomarkers with those reported in the literature (e.g., C-reactive protein, alpha-1-acid glycoprotein 1, paraoxonase 1), many of our protein biomarkers are novel and our results require independent validation. Additionally, there is bias towards more abundant proteins via the

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proteomics approach, and a trade-off exists between how many proteins can be identified and how accurately they can be quantified. Random factors play a role in our multiple marker analysis of iTRAQ data. Despite these limitations, our study has several strengths. This is one of few proteomic studies of incident MI in an observational study setting. Our proteomics platforms enabled us to individually compare plasma samples of cases and controls and conduct multimarker analyses. Additionally, we discovered single protein biomarkers and protein multimarkers and evaluated their performance in prediction of MI and ASCVD.

Methods

Study sample

Framingham Heart Study (FHS) offspring cohort participants (n=5124) have undergone periodic clinic examinations approximately every four years since their enrollment in 1971.[1] Onsite clinic examinations included medical history, questionnaires focused on CVD symptoms and risk factors, medication use, and lifestyle factors.[2] During each clinic visit, a 12-lead electrocardiogram was obtained as well as measurements of blood pressure, height and weight, and collection of fasting blood specimens for glucose and lipoprotein measurements.[3] FHS offspring participants who attended examination 5 (1991–1995), 6 (1995–1998), 7 (1998–2001), or 8 (2005–2008) and were free of ASCVD at that examination, were eligible for this study. Participants at these examinations who developed a qualifying ASCVD event (defined below) during follow up were selected as cases. Individuals who attended the same examination but remained free of ASCVD during the same follow-up period were eligible as controls. Among 3799 FHS offspring cohort participants who attended examination cycle 5, 3639 were free of prevalent ASCVD, and 338 developed incident ASCVD (cases) during follow up through December 31, 2008. ASCVD events included myocardial infarction (MI, n=136), death due to coronary heart disease (n=28), atherothrombotic brain infarction (n=70), coronary artery bypass grafting (n=57), and percutaneous transluminal coronary angioplasty (n=47). Clinical data and biological specimens for cases were selected from the FHS clinic examination visit immediately preceding ASCVD event occurrence (examination cycle 5 [n=85], 6 [n=66], 7 [n=150] or 8 [n=37]). For each case, one control was selected based on the following features: a) attended the same baseline examination cycle as the case; b) matched for age (±5 years), sex, smoking status, and statin use; c) free of ASCVD on the date of the event for the case. Clinical data and biological specimens for each control were selected from the same examination cycle as its matched case.

The proteomics pipeline

The experimental strategy was a discovery-validation pipeline[4] as illustrated in Supplementary Figure 1. In the discovery phase, we utilized iTRAQ® technology (Applied Biosystems, Foster City, CA) with multidimensional LC-MS/MS to analyze 136 MI case-control pairs. Complete data collections was available for 135 case-control pairs. In the validation phase, the most promising protein biomarkers from the iTRAQ discovery phase and from literature review were chosen for multiple reaction monitoring (MRM) targeted measurements (32 by MRM and 27 by depletion MRM) based on a stepwise selection

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model. This targeted analysis was conducted on 338 qualifying ASCVD case-control pairs (676 samples total); proteomic data on 336 pairs was successfully completed. A detailed description of the iTRAQ and MRM platforms is provided in the Supplementary Materials.

Statistical analyses

Single marker analysis—All statistical analyses were performed using SAS software version 9.2 (Copyright, SAS Institute Inc., Cary, NC, USA.). We quantile-normalized values for each biomarker by Blom's method. [5] We then performed single marker and multiple marker analyses for each analytic platform (iTRAQ and MRM). We performed two analyses for single markers: 1) paired t-test to compare means between cases and controls, and 2) conditional logistic regression (CLR) models with case status as the outcome, matched pairs as strata, normalized biomarker score as the main predictor, and adjusted for clinical covariates (systolic blood pressure, hypertension treatment status, total cholesterol, HDL cholesterol, diabetes status and BMI).

Multiple marker analysis—We performed multiple marker analysis using stepwise selection in conditional logistic regression models to identify a panel of markers (i.e. a multimarker) that was associated with case status. We used a threshold level of p= 0.05 to enter and remain in stepwise selection. Because we did not have complete data for many markers from the iTRAQ platform (600 out of 861 protein markers had at least one missing value), we used multiple imputation[6] to avoid sample size reduction due to missing values. We set a threshold of 60% completeness as an eligibility criterion for joint analysis. This resulted in 544 eligible markers (283 with partially missing values and 261 completely observed).

For the 544 eligible markers, we repeatedly and randomly shuffled markers into 20 bins to generate imputed datasets, followed by stepwise selection within bins using Chen's method. [7] From each shuffle, we obtained a list of important markers (yielding $p<0.05$ in bin-specific final models). The frequency of selection across all rounds of randomizations was used as an importance scale, with markers selected more frequently deemed more important. In total, 260 shuffles were performed. Proteins chosen in at least 50% of stepwise selection models were considered key biomarkers. The list of key markers was short enough to enter into one imputation and stepwise selection model, allowing for selection of a final multimarker model. The detailed methods used for imputation is provided in the Supplementary Methods.

For MRM and depletion MRM measurements, we performed stepwise selection in conditional logistic regression from all markers for association with MI (n=135 pairs) and with CVD (n=336 pairs).

Evaluation of multimarker panels—We evaluated the performance of the final multiple marker models using C-statistics. With paired data, a C-statistic is defined as the proportion of pairs in which the case has a higher predicted probability of being a "case" than its matched control. Under the null hypothesis that the predictor is not associated with case status, the C-statistic is 0.50. We calculated C-statistic for the model with clinical covariates only and the model with multiple protein biomarkers. We used the likelihood ratio test to

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evaluate the significance of joint effect comparing the conditional logistic regression models with and without the panel of markers. In addition, among cases we calculated the gain in predicted probability of being a "case" from the clinical covariates model to the clinical covariates plus multiple protein biomarkers (i.e. multimarker) model.

Supplementary Methods

Discovery Workflow

The proteomics workflow has been described previously.[8] Briefly, a dual-stage protein depletion strategy was implemented to accommodate the quantitative analysis of the plasma proteome at a depth spanning nearly seven orders of circulating concentration (60 mg/mL - 10 ng/mL).

Abundant protein depletion was implemented in two stages: an initial depletion of 14 selected abundant plasma proteins (5-mL, IgY14 column) followed by "Supermix" depletion (1-mL column, both from Sigma-Aldrich, St. Louis, MO).[8] 100 μL of plasma aliquots were depleted in daily batches of eight samples. Proteins in the depletion flow-through were recovered and subsequently de-salted on a reversed-phase column. The protein samples were then reduced with tris(2-carboxyethyl)phosphine (TCEP) and alkylated with iodoacetamide. Following digestion with trypsin, each sample was labeled with a unique iTRAQ tag. Peptide pools labeled with eight different iTRAQ tags were then combined into an 8-plex experiment, referred to as an iTRAQ mix. Two of the eight channels were reserved for reference samples that were created by pooling the primary samples. The remaining six channels represented primary samples. ITRAQ tags producing the *m/z* 113 and *m/z* 117 reporter ions were assigned to the reference samples. The remaining tags (*m/z* 114, 115, 116, 118, 119, and 121 reporter ions) were randomized throughout all the iTRAQ mixes to eliminate age, gender, and exam bias while ensuring that case-control pairs were assayed in the same iTRAQ mix to maximize the precision of pair-wise comparisons. The peptide pool consisting of the entire ITRAQ mix was then fractionated by strong cation exchange chromatography into nine fractions. Each fraction was further fractionated into 304 fractions by reversed-phase HPLC and directly spotted onto MALDI plates for MS/MS analysis using an AB/SCIEX 4800 TOF/TOF mass spectrometer (MDS SCIEX, Concord, ON,). Acquisition of LC-MS/MS data was optimized by in-house developed algorithms to select and measure consistent sets of peptides from experiment to experiment.[8]

Relative quantification of peptides was carried out by determining relative intensities of reporter ions between the sample and (average of) reference channels.

Identification of peptides from the MS/MS spectra was achieved using the Mascot database searching tool (MatrixScience Ltd., London, UK) and BG-Medicine -based validation protocol to distinguish true and false positive peptide matches. This procedure provides false positive identification rates well below 1% if applied to sufficient number of experiments.[8] Relative quantification of proteins was achieved by assigning the median ratio from peptides mapped to the given protein. Normalization of protein expression data was carried out using a procedure described by Vandersompele et al.[9]

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Target Analysis – Multiple-Reaction Monitoring (MRM)

Qualification of marker candidates was performed through two passes of MRM analyses of 338 ASCVD case-control pairs. In the first pass plasma samples were processed without abundant protein depletion to measure the more abundant plasma proteins. The second pass targeted proteins at lower circulating levels through the MRM analysis of plasma samples following depletion of the 14 most abundant proteins. Designation of protein targets to the first or second pass MRM analysis was made based on abundance estimates from a collection of historical in-house plasma proteomics measurements.

Proteins from 10 μL neat or 30 μL depleted plasma aliquots were reduced and alkylated with tris(2-carboxyethyl)phosphine (TCEP) and iodoacetamide. Trypsin digestion was completed overnight at 1:10 enzyme:substrate ratio. Digestion was terminated by acidifying the digests with formic acid (to pH 2.5). Approximately 4 μg of peptide material (in 0.4 mg/mL solution) was analyzed by MRM (LC-MS/MS).

LC-MS/MS analyses were performed on a 4000QTrap and 5500QTrap (for Depletion MRM) linear ion trap instrument (AB/SCIEX, Concord, ON) interfaced with a U3000 HPLC system (Dionex, Sunnyvale, CA). The systems were plumbed to facilitate an in-line desalting step on a Poros R2 column with reverse flow. After desalting peptides were separated on a Targa C18 or Reprosil (for Depletion MRM) 150×1.0 mm column (Higgins Analytical and Dr. Maisch GmbH, respectively) utilizing a 200 μL/min flow rate. Peptide elution was carried out over a 21-min gradient from 2% Buffer B to 32% B (Buffer A: 5% acetonitrile, 0.1% formic acid, Buffer B: 95% acetonitrile, 0.1% formic acid). Following elution the HPLC columns were extensively washed with 95% B. The HPLC column compartment was kept at 50°C during analysis.

For each target protein, two peptides were selected with two transitions (fragments) per peptide. Selection of these fragments was preceded by screening larger numbers of peptides and transitions (typically five peptides and five fragments for each fragment). Correct identity of peptides was confirmed by observing their correlation over a large number of individual samples.

Data collection was organized to acquisition batches sized to a 48-hr sample processing window. Randomization of acquisition order ensured the absence of age, gender, and exam number bias making sure the case-control pairs were analyzed in the same acquisition batch.

This experimental design normalizes peptide measurements to their mean measurement levels in the QC replicates. In this way, no isotope-labeled peptide standards were needed ensuring optimum multiplexing capacity of the LC-MS/MS runs at the expense of slightly increased measurement variability.[10] Before normalization, trend corrections were carried out if significant trends were detected in the series of QC samples. Normalization was performed by dividing the peak areas of individual transitions by the median of the same transition in the QC samples. In this manner, peptide quantities were reported as ratios, facilitating the conversion of peptide measurements into protein abundance measurements through simple averaging.

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Multiple Imputation

Through simulation we studied an approach for identifying important predictors in multivariable logistic models when missing values exist in hundreds of candidate variables. We started with subset of complete data and randomly masked values for some markers. We then shuffled markers into bins randomly, and performed multiple imputation immediately followed by stepwise selection. We strictly implemented Rubin's rule in stepwise selection process. Markers frequently chosen were deemed important. We examined choice of bin size, number of random shuffles of markers to bins, using prior information in MCMC imputation, and different importance thresholds for selecting important predictors. Our conclusion is that 26 is the best bin size among the values we evaluated; using prior information in imputation not only improves convergence but also improves imputation quality; 200 shuffles is enough for a stable panel. Based on decisions from the masked-data explorations, we applied the approach to real data with 544 biomarkers on 135 myocardial infarction case-control pairs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ASCVD	Atherosclerotic cardiovascular disease
CLR	Conditional logistic regression
FHS	Framingham Heart Study
iTRAQ®	Isobaric tag for relative and absolute quantification
MI	Myocardial infarction
MRM	Mass spectrometric multiple reaction monitoring
MS	Mass spectrometry

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Significance

We identified multiple protein biomarkers of MI or ASCVD; some have previously been reported to be associated with ASCVD, others are novel. Combinations of protein biomarkers from multimarker analyses might aid in improving ASCVD risk prediction algorithms. While independent replication studies are needed to confirm our single biomarkers and refine our multimarker panels and risk prediction algorithms, we view our results as promising. Such knowledge could be clinically useful for predicting ASCVD risk and guiding earlier intervention in patients to delay or prevent the onset of clinical disease.

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Table 1

Baseline characteristics of myocardial infarction cases and controls

Baseline characteristics	MI Study (ITRAQ)		ASCVD Study (MRM and Depletion MRM)	
	Controls (n=135)	Cases (n=135)	Controls (n=336)	Cases (n=336)
Age, years *	65±9	65±9	65±9	65±9
Female (%) *	34	34	30	30
Smoking, current (%) *	24	24	17	17
Statin use (%)*	19	19	22	22
Hypertension treatment (%)	37	47	35	49
Diabetes, prevalent (%)	7	28	9	27
Lipid lowering therapy (%)	20	21	24	27
Systolic blood pressure, mm Hg	131±18	137±18	131±19	137±20
Total cholesterol, mg/Dl	198±34	205±44	197±33	203±44
HDL cholesterol, mg/Dl	47±15	45±12	49±15	45±14
BMI, kg/m^2	27.8±4.4	28.8±4.8	27.6±4.4	29.0±4.9

Presented are mean±SD for continuous traits, or % for dichotomous traits

*Matching factors

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Table 2

Top protein biomarkers of myocardial infarction: results of single marker analyses from iTRAQ mass spectrometry

Gene Symbol	Protein Name	# pairs	Paired t Test		Conditional Logistic Regression	
			Mean difference (case-control) ± s.e.	P value	Odds Ratio (95% CI)	P value
GP5	Glycoprotein 5	120	−0.16±0.11	0.13	0.44(0.27,0.71)	0.0010
CD5L	CD5 antigen-like	135	−0.18±0.11	0.10	0.55(0.38,0.79)	0.0012
MB	Myoglobin	123	−0.28±0.10	0.0075	0.55(0.37,0.84)	0.0053
YWHAZ	Protein kinase C inhibitor protein 1	124	−0.2±0.10	0.057	0.55(0.36,0.84)	0.0056
CRP	C-reactive protein	129	0.35±0.11	0.0016	1.75(1.18,2.59)	0.0058
PPIA	Cyclophilin A	135	−0.26±0.10	0.012	0.56(0.37,0.85)	0.0061
CNTN1	Contactin-1	135	−0.20±0.11	0.078	0.62(0.45,0.88)	0.0063
ALB	Albumin	135	−0.22±0.09	0.012	0.55(0.36,0.85)	0.0066
NCAM1	Neural cell adhesion molecule 1	135	−0.31±0.10	0.0020	0.61(0.41,0.89)	0.010
SELENBP1	Selenium-binding protein 1	106	−0.32±0.11	0.0067	0.63(0.42,0.95)	0.028
GSN	Gelsolin	135	−0.30±0.10	0.0032	0.75(0.53,1.05)	0.090
MRC2	Macrophage mannose receptor 2	109	0.26±0.09	0.0068	1.51(0.93,2.47)	0.098
CLEC3B	Tetranectin	135	−0.26±0.11	0.0046	0.77 (0.56,1.05)	0.10
SLC3A2	4F2 cell-surface antigen heavy chain	135	−0.23±0.11	0.0054	0.79 (0.56,1.11)	0.18

This table shows markers with p-value < 0.01 in either conditional logistic regression or paired t-test. For each biomarker, data were rank normalized and have mean 0, SD 1. Differences, standard errors and odds ratios are in unit of one standard deviation. The conditional logistic regression model was adjusted for age, sex, current smoking status, statin use, systolic blood pressure, hypertension treatment status, total cholesterol, HDL cholesterol, diabetes status and BMI. Results are sorted by CLR values. All analyses are based on available data.

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Table 3

Protein biomarkers of myocardial infarction: results of multimarker analyses from iTRAQ mass spectrometry

Gene Symbol	Protein Name	Frequency selected*	Final model		
			Odds Ratio	95% CI	P value
PPIA	Cyclophilin A	94%	0.34	(0.18, 0.63)	0.0008
CD5L	CD5 antigen-like	99%	0.48	(0.29, 0.78)	0.0040
MCAM	Cell surface glycoprotein MUC18	51%	0.51	(0.30, 0.86)	0.013
COL18A1	Collagen alpha-1(XVIII) chain	90%	1.78	(1.09, 2.89)	0.021
AMY1A	Alpha-amylase 1 (salivary)	98%	0.54	(0.32, 0.91)	0.022
CRP	C-reactive protein	96%	1.87	(1.09, 3.19)	0.023
MMRN2	Multimerin-2	79%	1.66	(1.00, 2.75)	0.049

These results were obtained through the multiple imputation procedure for missing values followed by stepwise selection in conditional logistic regression model, adjusting for age, sex, current smoking status, statin use, systolic blood pressure, hypertension treatment status, total cholesterol, HDL cholesterol, diabetes status and BMI. Results are sorted by final model p-value.

*Frequency selected in stage 1, across 260 shuffles.

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Table 4

Top protein biomarkers of atherosclerotic cardiovascular disease: results of single marker analyses from MRM and depletion MRM mass spectrometry

Gene Symbol	Protein Name	Paired t test		Conditional Logistic Regression	
		Mean difference (case-control) ± s.e.	P value	Odds Ratio (95% CI)	P value
MRM (n=336 pairs)					
ORM1	Alpha-1-acid glycoprotein 1	0.29±0.07	<0.0001	1.43(1.16,1.76)	0.0007
CRP	C-reactive protein	0.35±0.07	<0.0001	1.38(1.13,1.69)	0.0016
CP	Ceruloplasmin	0.15±0.06	0.023	1.37(1.11,1.70)	0.0037
SAA1	Serum amyloid A-1 protein	0.27±0.07	0.0003	1.30(1.08,1.57)	0.0065
GSN	Gelsolin	−0.30±0.07	<0.0001	0.77(0.62,0.95)	0.013
CLEC3B	Tetranectin	−0.30±0.07	<0.0001	0.76(0.62,0.95)	0.015
HPX	Hemopexin	0.23±0.07	0.0007	1.26(1.03,1.55)	0.024
PON1	Paraoxonase 1	−0.16±0.07	0.017	0.79(0.64,0.98)	0.031
SERPINA10	Protein Z-dependent protease inhibitor	0.20±0.07	0.0031	1.25(1.01,1.53)	0.037
LRG1	Leucine-rich alpha-2-glycoprotein	0.09±0.07	0.17	1.24(1.01,1.52)	0.039
Depletion MRM (n=336 pairs)					
SERPINA10	Protein Z-dependent protease inhibitor	0.20±0.08	0.0070	1.21(1.01,1.46)	0.036
NCAM1	Neural cell adhesion molecule 1	−0.19±0.07	0.0067	0.82(0.68,1.00)	0.048

This table shows markers with p-value <0.05 in conditional logistic regression (n=336 pairs). For each biomarker, data were rank normalized and have mean 0, SD 1. Differences, standard errors and odds ratios are in unit of one standard deviation.

Conditional logistic regression results are adjusted for age, sex, current smoking status, statin use, systolic blood pressure, hypertension treatment status, total cholesterol, HDL cholesterol, diabetes status and BMI. Results are sorted by CLR values.

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Table 5

Protein biomarkers of atherosclerotic cardiovascular disease and myocardial infarction: results of multimarker analyses from MRM and depletion MRM mass spectrometry

Gene Symbol	Protein Name	Odds Ratio (95% CI)	P value
ASCVD (n=336 pairs)			
ORM1	Alpha-1-acid glycoprotein 1	1.45 (1.17, 1.80)	0.0007
PON1	Paraoxonase 1	0.75 (0.60, 0.94)	0.014
CLEC3B	Tetranectin	0.76 (0.61, 0.95)	0.017
CD5L	CD5 antigen-like	0.81 (0.67, 0.98)	0.031
MI (n=135 pairs)			
SERPINA10	Protein Z-dependent protease inhibitor	1.70 (1.16, 2.50)	0.0070
CRP	C-reactive protein	1.51 (1.03, 2.23)	0.037
CD5L	CD5 antigen-like	0.70 (0.50, 0.98)	0.039

Results are all from stepwise selection in conditional logistic regression models, adjusting for age, sex, current smoking status, statin use, systolic blood pressure, hypertension treatment status, total cholesterol, HDL cholesterol, diabetes status and BMI. Candidate markers include all 59 proteins measured by MRM and depletion MRM. P value of 0.05 was used as both enter and stay criteria. For each biomarker, data were rank normalized and have mean 0, SD 1. Odds ratios are in unit of one standard deviation.