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Myocardial injury is distinguished from stable angina by a set of candidate plasma biomarkers identified using iTRAQ/MRM-based approach

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ABSTRACT

The lack of precise biomarkers that identify patients at risk for myocardial injury and stable angina delays administration of optimal therapy. Hence, the search for noninvasive biomarkers that could accurately stratify patients with impending heart attack, from patients with stable coronary artery disease (CAD) are urgently needed in the clinic. Herein, we performed comparative quantitative proteomics on whole plasma sampled from patients with stable angina (NMI), acute myocardial infarction (MI), and healthy control subjects (Ctrl). We detected a total of 371 proteins with high confidence ($\text{FDR} < 1\%$, $p < 0.05$), including 53 preliminary biomarkers that displayed ≥ 2 -fold modulated expression in patients with CAD (27 associated with atherosclerotic stable angina, 26 with myocardial injury). In the verification phase, we used label-free LC-MRM-MS-based targeted method to verify the preliminary biomarkers in pooled plasma, excluded peptides that were poorly distinguished from background, and performed further validation of the remaining candidates in 49 individual plasma samples. Using this approach, we identified a final panel of 8 novel candidate biomarkers that were significantly modulated in CAD ($p < 0.05$), including proteins associated with atherosclerotic stable angina that were implicated in endothelial dysfunction (F10 and MST1), and proteins associated with myocardial injury reportedly involved in plaque destabilization (SERPINA3, CPN2, LUM), and in tissue protection/repair mechanisms (ORM2, ACTG1, NAGLU). Taken together, our data showed that candidate biomarkers with potential diagnostic values can be successfully detected in non-depleted human plasma using an iTRAQ/MRM-based discovery-validation approach, and demonstrated the plausible clinical utility of the proposed panel in discriminating atherosclerotic stable angina from myocardial injury in the studied cohort.

KEYWORDS: Cardiovascular disease, atherosclerosis, myocardial injury, angina, plasma biomarker, iTRAQ, MRM.

INTRODUCTION

Cardiovascular disease (CVD) arising from atherosclerosis is the leading cause of death worldwide (1). While there are established methods of assessing the extent of atherosclerosis in affected patients, at least 14% of initial cardiac events occur among asymptomatic individuals lacking identified CVD risk factors (2-4). Current imaging modalities and serological indicators used in the diagnosis and monitoring of CADs are focused on the late symptomatic stages, often after irreversible myocardial injury, thus limiting treatment options (5-8). Consequently, there remains an urgent need for methods of early CAD detection and timely therapeutic interventions to prevent, delay or attenuate plaque destabilization.

Biomarker discovery is progressively moving towards the use of biomarker panels that can better predict clinical outcomes against a backdrop of extensive heterogeneity at the molecular, population, and epidemiological levels (9). Accordingly, technological advancements in mass spectrometry (MS) have led to the development of powerful new platforms for biomarker discovery studies (10-12). Studies of aberrant protein expression in diseases have been made possible by the optimization of shotgun-based quantitative proteomic methods that generate a large pool of potential biomarkers in just a single experiment (13), and have provided valuable new insights into pathophysiological events underlying CAD (14). In spite of these developments, our understanding in the triggers and mechanisms that promote plaque destabilization in CAD remains limited, hence the ability to assess patient risk of atherosclerosis-associated angina and the onset of acute clinical events remains extremely poor.

The lengthy and laborious process of verifying and validating candidate biomarkers creates a major bottleneck in the development of new diagnostic tests for use in clinical settings. While enzyme-linked immunosorbent assays (ELISAs) are often used for biomarker verification and validation, this approach can be both costly and time-consuming when needing to develop assays for multiple protein targets (15). In contrast, multiple reaction monitoring (MRM)-MS represents a rapid and cost-effective approach for measuring, verifying and validating complex panels of protein biomarkers without the limitations of antibodies quality and availability (16-20). MRM-MS is a quantitative and targeted proteomic platform that enables simultaneous monitoring of multiple peptide transitions in parallel, thereby achieving the reproducibility and level of throughput required for pre-clinical verification of large numbers of candidate biomarkers (21, 22).

In this study, we described the systematic application of isobaric tags for relative and absolute quantification (iTRAQ)-based protein expression analysis, and label-free targeted MRM-based

quantitation strategy for the discovery and validation of candidate biomarkers of CAD in plasma, sampled from patients with stable angina (NMI), acute myocardial infarction (MI), and healthy control subjects (Ctrl). Using this approach, we proposed a diagnostic panel consisting of eight novel candidate biomarkers that discriminates the multifactorial pathophysiology of atherosclerosis (F10, MST1) and myocardial injury (ORM2, SERPINA3, CPN2, LUM, ACTG1, NAGLU). Further assessment of these novel candidates in a larger patient cohort should pave the way for future clinical validation studies and potential diagnostic applications.

EXPERIMENTAL PROCEDURES

Chemicals

All water and acetonitrile (ACN) used in this experiment were of high performance liquid chromatography (HPLC) grade (Thermo Scientific, Waltham, MA). All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless stated otherwise.

Human plasma samples

Forty nine patients were recruited from 2010 to 2012 for this study. All patients were admitted to National University Heart center for investigation or interventional procedure, and consented to participate in blood collection for research. Upon admission, recruited patients underwent investigation including serial electrocardiogram echocardiogram, coronary angiogram and high sensitivity cardiac troponin I test, if appropriate. Based on clinical assessment and investigation, patients were stratified to control group (Ctrl), stable angina group (NMI), and non ST elevated myocardial infarction group (NSTEMI/MI), following AHA guidelines for diagnosis and management of coronary artery disease patients (23). We included stable angina patients (NMI, n=20) with coronary atherosclerosis confirmed by coronary angiogram (significant coronary disease with more than 60% stenosis of at least one coronary vessel), have angina symptoms but do not have accelerated symptoms or myocardial infarction within 3 months. NSTEMI/MI group (n=15) comprised patients with coronary atherosclerosis on coronary angiogram (significant coronary disease with more than 60% stenosis of at least one coronary vessel), symptoms, changes on electrocardiogram with positive high sensitivity cardiac troponin I test on serial blood sampling (minimal sampling two times with elevation of Troponin I more than 10 times) according to international guideline. Only patients with fresh NSTEMI/MI were included in this study (within 5 days from onset). Control group (n=14) were patients who presented with atypical symptoms and underwent coronary angiogram to exclude CAD, had no angina or heart failure symptoms and presented with normal electrocardiogram, and normal high sensitivity cardiac Troponin I test level (patient demographics and clinical characteristics detailed in Table 1). In this studied cohort, the cases and controls were closely matched in terms of age gender and race frequencies, to minimize the possible difference in relative risk assessment and outcomes. Plasma collected from peripheral access, were stored at -80°C until processing for proteomic analysis. Written informed consent was obtained from all study participants. The study was approved by the National Healthcare Group Domain Specific Review Board (NHG DSRB).

Protein precipitation from non-depleted plasma

In order to minimize biological variation, individual plasma samples collected from each study group were equivalently pooled to obtain a final sample volume of 200 μ L. Plasma proteins were precipitated in 80% acetone for 4h at -20°C, before pelleted by centrifugation (16,000 x g, 10min). The recovered protein pellets were quantified using the bicinchoninic acid assay according to the manufacturer's protocol. For each study group, approximately 200 μ g proteins were extracted for downstream proteomic processing.

In-solution tryptic digestion, peptide labeling, and peptide fractionation

Extracted proteins were solubilized in lysis buffer (8M urea, 50mM triethylammonium bicarbonate [TEAB], pH 8.0), supplemented with protease inhibitors (1:50, v/v) and phosphatase inhibitors (1:10, v/v) (Roche Diagnostics, Mannheim, DE). For each study group, approximately 200 μ g plasma proteins were reduced with 5mM tris 2-carboxyethyl phosphine hydrochloride for 3h at 30°C, followed by alkylation with 10 mM methyl methanethiosulfonate for 1h in the dark at room temperature. The urea concentration was then diluted to less than 1 M prior to overnight digestion at 37°C with sequencing-grade modified trypsin (trypsin 1:100 protein w/w ratio; Promega, Madison, WI). The tryptic peptides were desalted using a Sep-Pak C18 cartridge (Waters, Milford, MA) and the eluted peptides were dried in a vacuum concentrator. The dried peptides were then reconstituted in 50 mM TEAB and labeled with 8-plex iTRAQ isobaric tags according to manufacturer's protocol (Applied Biosystems, Foster City, CA) respectively; 113_{Ctrl}, 114_{MI} and 115_{NMI}. The labeled plasma peptides were combined and dried using a vacuum concentrator. The dried iTRAQ-labeled peptides were reconstituted in 200 μ L mobile phase A (85% ACN, 0.1% acetic acid [HAc]) and fractionated using a PolyWAX LP anion-exchange column (4.6 $\times$ 200mm, 5 μ m, 300Å, PolyLC, Columbia, MD) on a Shimadzu Prominence UFLC system (Kyoto, JP). The UV spectra of the peptides were collected at 280 nm. Mobile phase A and Mobile phase B (30% ACN, 0.2% formic acid [FA]) were used to perform a 60 min gradient elution as follows; 0-36% B for 30min, then 36–100% B for 20 min, and finally 100% B for 10 min (flow rate 1 mL/min). 30 separate fractions were collected, vacuum dried, and reconstituted in 3% ACN, 0.1% FA for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

iTRAQ-labeled quantitative proteomics by LC-MS/MS

The dried iTRAQ-labeled peptides were dissolved in 40 μ L solvent A (2% ACN, 0.1% FA) and 1 μ L of sample per fraction were loaded into a trap column (0.5 mm x 200 μ m) at a flow rate of 3 μ L/min for 10 min, and resolved on an analytical column (15 cm x 75 μ m) with a linear gradients

of solvent B (98% ACN, 0.1% FA) from 5%-12% in 2 min; 12% to 30% in 57 min and 30-90% in 2 min at a flow rate of 300 nL/min on the Nanoflex cHiPLC system. The nanoLC column was rinsed with 90% solvent B for 7 min and equilibrating with 95% solvent A for 13 min. For information dependent acquisition (IDA) on AB SCIEX TripleTOF® 5600 system, 250-ms survey scan (TOF-MS) and 100-ms automated MS/MS product ion scan for the top-20 ions with the highest intensity was performed with a cycle time of 2.3s. The MS/MS triggering criteria for parent ions were as follows: precursor intensity (> 125 cps), 2- 5 charge states with dynamic exclusion time of 8s and collision energy (CE) set as rolling CE script based on m/z and charged state of the precursors. Triplicate LC-MS/MS runs per fractions were performed. The peak areas of the iTRAQ reporter ions reflect the relative abundance of the corresponding proteins in the samples.

iTRAQ data analysis

Protein identification and peptide quantification were performed by searching all spectra generated from the IDA acquisitions in Triple-TOF® against the UniProt database (version Aug 2011, 446597 sequences, 188463640 residues) using the Paragon™ (24) and Pro Group™ (25, 26) algorithms found in ProteinPilot™ V4.1 (AB SCIEX, Framingham, MA). User-defined parameters were configured as follows; (i) Sample Type, iTRAQ 8-plex (Peptide Labeled); (ii) Cysteine alkylation, MMTS; (iii) Digestion, Trypsin; (iv) Instrument, TripleTOF 5600; (v) Species, Human; (vi) ID Focus, Biological modifications; (vii) Search effort, Thorough; (viii) Specific Processing, Quantitate, Bias correction, Background correction; (ix) Results quality, Detected protein threshold [Unused Protscore (Conf)] >: 0.05 (10.0%). Peptides were automatically selected for quantification by the Pro Group algorithm in ProteinPilot™ software, which then calculated the reporter peak area, error factor (EF), and corresponding p-value. The resulting data were auto-normalized for bias correction and background correction to eliminate variations due to loading error or co-elution of non-target peptides using the Paragon™ (24) algorithm method within ProteinPilot™. Search results were exported into Microsoft Excel for further comparison of replicate runs. A 2-fold change cut-off was set such that up-regulated proteins were identified by expression ratios ≥ 2 and down-regulated proteins by ratios ≤ 0.5 . The false discovery rate (FDR) for each search was generated by ProteinPilot™ and the numbers of proteins reported in this study were based on global protein FDR < 1%. Differentially expressed proteins were extracted for further analyses. The iTRAQ LC-MS/MS data and the ProteinPilot generated group file are deposited in the Proteome Xchange Consortium (27) via the PRIDE partner repository with the dataset identifier PXD006333.

Assay development for MRM

For MRM, 3-5 unambiguous tryptic peptides and 3-5 most intense fragment ions (transitions) for each candidate protein were selected. Selection of peptides and transitions for the candidate proteins were derived from the discovery MS/MS data collected on AB SCIEX TripleTOF® 5600 system. For candidate proteins that were identified with less than 3 peptides in the discovery phase, proteotypic peptides and the best MRM transitions were generated by in silico digestion using Thermo Scientific™ Pinpoint™ V1.3 software (Thermo Fisher Scientific, Cambridge, MA), and by SRM Atlas (<http://www.srmatlas.org/>), a publicly accessible database of SRM/MRM transitions acquired in previous targeted proteomics studies (28). The MRM instrument acquisition parameters predicted by Pinpoint™ V1.3 were then imported directly into the instrument method setup program. For each study group (MI, NMI or Ctrl), preliminary screening was performed via unscheduled MRM analysis of 53 preliminary candidate biomarkers in unfractionated pooled plasma digests. Positive peptide identification was based on the detection of at least three co-eluting transitions in each of the preliminary LC-MRM-MS runs (visualized in Pinpoint™ V1.3). Only peptides with consistent transition profiles were selected for the scheduled LC-MRM-MS analysis of individual plasma samples.

LC-MRM-MS

The same set of individual patient plasma used in iTRAQ experiment was individually digested, desalted and vacuum dried as described earlier, without peptide labelling and fractionation. The targeted peptides were assayed in triplicate in a TSQ Vantage triple quadrupole mass spectrometer coupled to an EASY-nLC™ 1000 nanoflow UHPLC system (Thermo Scientific Inc., Bremen, Germany). The retention time (RT) for each peptide was determined by full acquisition (unscheduled) MS/MS analysis. The predicted RT (± 5 min error) for each transition was then used to determine a 10min isolation window for dynamic exclusion (scheduled) analyses. For each run, a total of 1 μ g tryptic peptides was loaded onto an Acclaim® PepMap100 trap column (75 μ m x 2cm; nanoViper C18, 3 μ m, 100Å) and resolved on an Acclaim® PepMap RSLC C18 column (75 μ m x 15cm; nanoViper C18, 2 μ m, 100Å) (Thermo Scientific, USA), at a flow rate of 300nL/min. Mobile phase A (0.1% FA in HPLC water), and mobile phase B (0.1% FA in ACN) were used to establish a 60min gradient as follows; 3-30% B for 45min, 30-50% B for 9min, 50-60% B for 1min, 60% B for 2min, and finally re-equilibration at 3% B for 3min. The TSQ Vantage was set to perform data acquisition in positive ion mode. An electrospray potential of 1.5kV and capillary temperature of 250°C were used for ionization. The selectivity for both Q1 and Q3 were set to 0.7 Da (full-

width at half-maximum). A collision gas pressure of 1mTorr of argon was used for Q2. Transition scan times were 10ms for full MRM and 50ms for the isolation window MRM.

MRM data analysis

All MRM raw data files generated by the TSQ Vantage were processed using Pinpoint™ V1.3, which automatically detected the integrated peak areas of all targeted transitions and aligned these for data visualization, as well as performing relative quantification at the levels of transition, peptide and protein. Global normalization of the peak intensities from the triplicate measurements were also computed by the software. The extracted ion chromatogram of all transitions for each targeted peptide were examined manually to confirm accurate peak integration. The integrated peak areas of all considered transitions were summed to generate mean peptide areas, across the triplicate LC-MRM-MS runs. Consequently, the respective mean peptide areas were summed to determine the relative abundance of corresponding candidate protein.

The final list of 49 proteotypic peptide sequences selected for MRM validation were submitted to NCBI BLASTP (29, 30) for searching using default settings against non-redundant protein sequences (nr), to confirm uniqueness (detailed information available in supplemental information). The MRM transitions used to validate the 8 candidate biomarkers are listed in Table 4 (details available in supplemental data S3; worksheet MRMStat). The LC-MRM-MS raw data files of the 8 candidate biomarkers are also deposited in the PRIDE data repository database with the dataset identifier PXD006333.

Bioinformatic analyses and data annotation

The open access tool FunRich V2.1.2 (31) was used for gene ontology (GO) annotation and functional enrichment and interaction network analyses. Scatter dot plots were generated and statistically analyzed using GraphPad Prism V6.0 (Graphpad Software, San Diego, CA). Parametric analyses and unpaired student's *t*-test were used to evaluate differences between numerical variables and $p < 0.05$ was considered statistically significant. Data are presented as mean $\pm$ standard deviation (SD) of triplicate measurements.

RESULTS AND DISCUSSION

Workflow for iTRAQ-based quantitation of the plasma proteome

Comparative quantitative proteomics analyses of plasma are useful for biomarker discovery. In this study, we employed an iTRAQ labeling approach to conduct multiplex quantitative analyses using a single reagent (32). In order to probe for specific proteins associated with atherosclerotic stable angina and/or myocardial injury in human plasma, the study subjects were carefully grouped into defined cohorts as follows; patients with myocardial infarction (MI; n=15), patients with stable angina (NMI; n=20), and control subjects without angina (Ctrl; n=14). As outlined in Figure 1, for each study group an equal concentration of proteins (200 µg) were isolated from pooled plasma, proteolytically digested with trypsin, labeled with distinct isobaric tags, and mixed prior to fractionation in the first dimension. The fractionated labeled peptides were subsequently analyzed in triplicate using LC-MS/MS, and the relative abundance of specific peptides were determined based on the intensities of the iTRAQ reporter fragment ions (detected in the 113-115 m/z region of the peptide product ion spectra). Open-source public tools were used to conduct differential quantitative proteomic analyses and select proteins-of-interest for subsequent verification and validation by LC-MRM-MS.

Quality assessment of the quantitative MS data

The run-to-run technical variation was determined in terms of percentage coefficient variation (%CV), the number of protein, peptide and spectral identified (FDR < 1%) in replicate 01 (R01), replicate 02 (R02) and replicate 03 (R03) were compared as summarized in Table 2. Detailed search and quantitation information are provided in supplemental data S1 (worksheets PPRepPro and PPRepPep). The overall %CV achieved across the triplicate measurements were 8.36% for identified proteins, 2.00% for peptides, and 1.43% for spectra, thus confirming minimal variation and good system reproducibility. In addition, ~77% (356) of total proteins identified (FDR < 1%) were quantified in at least two of the triplicate analyses, and ~62% (286) were detected in all three LC-MS/MS runs (Supplemental Figure S1), suggesting good overall protein complementation between triplicates. Next, we assessed the reliability of iTRAQ quantitation for each protein (FDR < 1%) by comparing the tag ratios of 114_{MI}:113_{Ctrl}, 115_{NMI}:113_{Ctrl} and 114_{MI}:115_{NMI} (Figure 2). When analyzed on scatter log₂ plots, these pairwise comparisons confirmed that the iTRAQ ratios generated were highly consistent between triplicates (Figure 2A-C; G-I) and analytical runs (Figure 2D-F), confirming the reliability and confidence of our iTRAQ quantitative dataset. The detailed statistical calculations are provided in supplemental data S1 (worksheet PPRepStat).

Identification and quantification of differentially expressed plasma proteins

The combined triplicate LC-MS/MS database search returned with a core set of 371 quantified proteins (FDR < 1%, unused protein score > 2 correspond to 99% confidence). The detailed search and quantitation information are provided in supplemental data S2 (worksheets PP2DPro and PP2DPep). Of the total 371 proteins detected, ~92% were identified from ≥ 2 constituent peptides, 71% from ≥ 5 peptides, and just 8% from single peptide (95% peptide confidence level throughout), indicating robust identification of the quantified proteins. In order to identify potential biomarkers of specific CAD phenotypes, atherosclerosis-specific markers were determined using the iTRAQ ratios 114_{MI}:113_{Ctrl} and 115_{NMI}:113_{Ctrl}, while specific markers of myocardial injury were flagged using the iTRAQ ratio 114_{MI}:115_{NMI}.

Differentially expressed proteins were selected using the following criteria; (a) protein ratio fold-change ≥ 2.0 (up-regulation) or ≤ 0.5 (down-regulation) and $p < 0.05$; (b) parallel protein regulation trend in combined triplicate search results and in average of individual replicate search results; (c) protein identified based on ≥ 2 peptides with 95% confidence and present in ≥ 2 replicates. Based on these criteria, the initial list of 371 proteins included 53 potential biomarkers that exhibited significant differential expression between study groups (Table 3). Detailed quantitation information are provided in supplemental data S2 (worksheet PP2DSL). Proteins that did not meet these stringent criteria were excluded from subsequent analyses. Of the 53 candidates shortlisted in the discovery phase, 27 proteins displayed comparable modulation in both the MI and NMI study groups relative to controls (26 being up-regulated and 1 down-regulated in disease), and were therefore considered as potential biomarkers of atherosclerosis. Conversely, proteins that were differentially modulated in MI and NMI (26 proteins exhibiting ≥ 2 fold-change in the MI group only) were selected as putative indicators of myocardial injury.

Functional analyses of the differential plasma proteome

Differentially expressed proteins were functionally compared between study groups and classified using FunRich V2.1.2 to (31) identify key biological processes, and pathways that might discriminate atherosclerosis from myocardial injury. The complete functional analyses are provided in supplemental data S2 (worksheet PP2DEnrich). Functional enrichment analyses were ranked using the Benjamini–Hochberg method, and categories with a corrected p-value < 0.05 were identified as key functional categories. The biological process and pathway distribution of the proteins identified as being modulated in atherosclerosis, and/or myocardial injury are shown in Figure 3. In atherosclerosis, the differentially expressed proteins were enriched in processes

and pathways known to be associated with CAD, including the host immune response (37%), protein metabolism (33.3%), and coagulation (3.7%) (Figure 3A), as well as the clotting cascade (52.9%), common pathway of coagulation cascade (35.3%), gamma-carboxylation of protein precursors (17.6%), and cell surface interactions with the vascular wall (17.6%) (Figure 3B).

In myocardial injury, the differentially expressed proteins were associated with processes and pathways including cell growth and/or maintenance (37%), host immune responses (19.2%), and lipid transport (3.78%) (Figure 3C), as well as platelet activation (30.8%), integrin function in angiogenesis (23.1%), cell-extracellular matrix interactions (15.4%) and smooth muscle contraction (15.4%) (Figure 3D). Collectively, these comparative functional enrichment data predicted some subsets of biological processes and pathways that were distinctive to proteins associated with atherosclerosis or myocardial injury, suggesting that these proteins may be able to differentiate clinically important sub-groups of CAD. These findings also served to confirm that our patient groups and controls were appropriately classified for use in this biomarker discovery study.

Selection of peptides for MRM verification

Moving forward from the discovery phase to the verification phase, label-free targeted multiplex MRM-MS approach was used to confirm the disease-specific modulation of 27 candidate biomarkers of atherosclerosis, and 26 putative biomarkers of myocardial injury (Table 3). MRM-MS assays can be coupled with label-free or stable isotope dilution (SID) to achieve relative or absolute protein quantification, respectively. While SID method is more common, several studies have now demonstrated that label-free, MRM-based quantitation represents a highly efficient approach as first-level biomarker verification, by facilitating the low-cost screening of large numbers of individual candidates (33-35).

In the preliminary LC-MRM-MS experiment, to verify 53 candidate biomarkers, unlabeled protein digests of pooled plasma from each study group (MI, NMI and Ctrl) plasma were assayed in duplicates to evaluate the large number of targeted transitions (255 unmodified peptides, 999 transitions) derived from Pinpoint™ V1.3 software and SRM Atlas (28). A detailed description of the initial MRM-method is provided in supplemental data S3 (worksheet MRMPrelim). The optimal representative peptides that have the strongest signal and reproducibility in MRM runs in verification phase and their transitions were carefully assembled, and the poorly performing proteins and peptides flagged by Pinpoint™ were excluded from further analyses. Positive

confirmation and consistent detection were manually checked by comparing peptide relative abundance, elution time, and number of co-eluting transition peaks (min. 3) between duplicate LC-MRM-MS analyses of the pooled plasma protein digests from each study group. Poor quality peptides with low intensity, inconsistent transitions and high background interferences, likely constrained by sample complexity, which limits detection of lower abundance analyte, were eliminated by Pinpoint™. Based on reported experience in the field, it is expected that only a small fraction of the potential biomarkers identified by proteomics will become clinically useful (33). Thus to further refine the list of potential biomarkers, proteins represented by fewer than two positive unambiguous peptides in the initial MRM assay were excluded, to increase overall confidence in accurate identification and quantification.

The refined list of MRM targets included 49 peptides and 168 MRM transitions, representing 8 potential candidate biomarkers of atherosclerosis and 15 candidate markers of myocardial injury (details available in supplemental data S3; worksheet MRMFfinal). Based on our iTRAQ dataset (supplemental data S2; worksheet PP2DPro), we selected a subset of housekeeping proteins that exhibited stable expression across the MI, NMI and Ctrl groups; serum albumin (ALB), serotransferrin (TF) and alpha-2-macroglobulin (A2M). These house-keeping proteins (10 peptides, 37 transitions) were then included in the MRM-assay and quantified alongside with the 23 candidate biomarker targets in unfractionated digests of individual plasma samples. Details of the house-keeping transition list are provided in supplemental data S3 (worksheet MRMHseKp).

MRM validation of candidate biomarkers

Simultaneous targeted label-free quantification of 23 candidate biomarkers, were performed on the same set of 49 individual plasma samples (MI, n=15; NMI, n=20; Ctrl, n=14) that were pooled for discovery-phase iTRAQ experiment. The RT and normalized peak areas computed by Pinpoint V1.3 were used to assess reproducibility (%CV) for each of the 59 peptides assayed here (147 separate LC-MRM injections). We observed that 56 of 59 peptides exhibited CV values < 5%, and the remaining 3 peptides displayed CVs < 10%, demonstrating excellent within-run instrument reproducibility and HPLC system stability. Details of these assessments are provided in Supplemental data S3 (worksheet MRMPinPt) and the peptide CV calculations are displayed in Table 4. Next, we probed for potential inconsistencies in the peptide and protein relative abundance (peak area) results generated by each of the triplicate LC-MRM-MS runs performed in each study group. Again, we obtained low %CV values for both the overall dataset and individual peptide peak areas; Ctrl < 16%, MI < 15%, and NMI < 6% (Table 4). Accordingly, we

also observed low median %CV values for the 26 protein peak areas; Ctrl=4.51%, MI=4.71%, and NMI=1.52%. These data indicated that even in complex digests of individual human plasma samples from highly heterogeneous donors, our relative protein quantification data were highly consistent and reliable.

In order to render the quantitative data more comparable between study populations, each of the individual protein peak areas was normalized to the arithmetic mean of three separate house-keeping proteins (Supplemental data S3; worksheet MRMNorm). In Figure 4 A-C, the log₂ box-and-whiskers plots showed the relative abundance of the house-keeping proteins ALB, A2M, and TF across individual plasma samples in each study group, and demonstrated that the median expression levels were comparable between Ctrl, MI and NMI samples (consistent with our earlier iTRAQ results). The detailed statistical results are provided in supplemental data S3 (worksheet MRMStat). Next, we assessed whether expression levels of the eight candidate markers of atherosclerosis were modulated in the MI and NMI groups relative to controls. Unlike the iTRAQ results, the MRM analysis indicated that expression levels of ATRN, TTR, AMBP and LPA were not significantly different in either the MI or NMI patient groups when compared with controls, hence these proteins were excluded. In contrast, we detected that both MI and NMI plasma samples contained significantly increased levels of F10 ($p < 0.0001$), VTN ($p < 0.0001$), and MST1 ($p < 0.0001$) relative to that of control samples (Figures 4D-F), and were considered as potential biomarkers of atherosclerosis. While MI plasma was also found to be significantly enriched in AFM when compared with controls ($p = 0.0121$), we were unable to detect AFM enrichment in NMI patients ($p > 0.05$), hence this marker was excluded from further consideration.

Finally, the relative expression levels of 15 candidate proteins of myocardial injury were examined in individual plasma from both MI and NMI patients. Among these candidates were 6 proteins that displayed upregulation in MI relative to NMI; SERPINA3 ($p < 0.0001$), CRP ($p = 0.00691$), SAA1 ($p = 0.00116$), ORM2 ($p < 0.0001$), CPN2 ($p < 0.0001$), and ACTG1 ($p < 0.0001$) (Figure 4G-L). We also observed significantly reduced expression of 4 proteins in MI compared with NMI; APOA4 ($p < 0.0001$), LUM ($p < 0.0001$), NAGLU ($p < 0.0001$) and TLN1 ($p = 0.000769$) (Figure 4M-P). Despite being highlighted as potential biomarkers in the earlier discovery phase experiment, a subset of the proteins assessed by MRM were found to exhibit comparable expression levels in both MI and NMI plasma samples (APOE, LRG1, FLNA, SAA4 and ITIH3). The detailed statistical results are provided in supplemental data S3 (worksheet MRMStat). Table 5 shows a comparison of the discovery-phase iTRAQ results with the proteins identified by MRM as being differentially

expressed in MI and/or NMI patients. The final ratios of protein fold-change between patient and control populations were then determined by calculating the average normalized peak area for the remaining candidate biomarkers in each separate study group (Supplemental data S3; worksheet MRMStat).

We observed that 12 proteins exhibited similar patterns of differential expression in both the iTRAQ and MRM datasets (excluding TLN which had earlier been rejected as a potential biomarker of myocardial injury). The discrepancies in relative protein quantitation between the iTRAQ and MRM measurements could perhaps be attributed to the pooling strategy used in the discovery phase, which averages protein abundance across samples prior to quantitation. Consequently, when individual plasma samples were analyzed by MRM, the true biological variation in protein abundance would become apparent and conflict with the iTRAQ results. The differences in the complexity of fractionated and unfractionated plasma analyzed for iTRAQ and MRM respectively may have influenced the outcome of the quantitation. Furthermore, the inclusion of all contributing peptides, including splicing isoforms and post-translation modified peptides for quantitation in iTRAQ, versus the use of targeted unmodified peptides for MRM quantitation may also have contributed to these variations. Nonetheless, our MRM verification analyses confirmed that 12 of 23 protein candidates exhibited significant differential expression patterns ($p < 0.05$, > 2 -fold change) that were in good agreement with the discovery proteomics data. These observations confirmed that multiplex MRM analyses can be used to efficiently and reproducibly verify numerous disease biomarker candidates in crude human plasma.

Candidate biomarkers displaying significant differential expression

Label-free MRM verification of the discovery-phase findings across 49 independent plasma samples identified 12 candidate biomarkers as priority targets for further clinical evaluation in larger patient cohorts. We showed that the relative expression of VTN, F10 and MST1 did not differ between CAD phenotypes but was significantly up-regulated in both MI and NMI patients compared with controls, suggesting that these proteins could represent potential biomarkers of coronary atherosclerosis but are unable to distinguish recent MI.

Increased plasma levels of VTN have already been reported to correlate with severity of CAD (36), as such it will not be elaborated. F10, a mediator of the coagulation cascade, plays an important role in endothelial integrity maintenance, and in atherosclerosis progression (37). Hence, increased levels of F10 (~4-fold) in CAD likely reflects an induction of thrombin formation

at sites endothelial inflammation and/or injury, thereby promoting atherosclerosis. It has been shown that MST1 exert inhibition of inducible nitric oxide synthase (iNOS) in macrophages (38, 39), typically induced by inflammatory mediators as part of host immune defense (40). It is therefore reasonable to hypothesize that the observed up-regulation of MST1 in atherosclerosis (~23-fold in MI, ~13-fold in NMI), may indicate host suppression of iNOS expression in attempt to limit further endothelial damage. F10 and MST1 are novel candidate biomarkers of atherosclerosis, given their known influences on endothelial dysfunction, it is possible that dual up-regulation of these proteins in plasma could be used to identify early atherosclerosis in patients.

In addition, we have identified nine proteins that displayed differential modulation in patients with MI compared with stable angina; SAA1 (~86-fold change), CRP (~15-fold), ORM2 (~2.1-fold), SERPINA3 (~2.7-fold), CPN2 (~2.2-fold), ACTG1 (~4.4-fold), APOA4 (~0.2-fold), LUM (~0.2-fold) and NAGLU (~0.26-fold). Previous reports have already documented the potential clinical utility of the association between MI and serum levels of CRP (41-43) and SAA1 (44-47). Recent studies on ORM2 have revealed roles in protection against ischemia-reperfusion (IR) injury, anti-inflammatory (48, 49) and immunomodulatory functions (50). Our data suggest that ORM2 up-regulation in MI may serve to restrain the host inflammatory response and limit tissue damage arising from IR injury. SERPINA3 inhibits extracellular (ECM) degrading neutrophil cathepsin G and mast cell chymase (51). Herein, elevated SERPINA3 in plasma of MI patients could be associated with remodeling and/or destabilization of atherosclerotic plaques, corresponding patient risk of heart attack.

CPN2, the subunit of carboxypeptidase N enzyme that inactivates bradykinin and complement anaphylatoxin (52-54) It has been suggested that CPN inhibits of fibrinolysis (55), and may be up-regulated in MI during clot formation over a ruptured plaque. The observed elevated expression of CPN2 in MI could possibly be an attempt to limit complement activation and restrict inflammation. ACTG1 maintains cytoskeletal integrity (56), contributes to the regulation of epithelial gap junctions (57), and plays key roles in both endothelial cell motility and neo-vessel angiogenesis (58). Though no concrete association with MI has been reported, the elevation of ACTG1 observed post-infarction may indicate its involvement in myocardial wound healing and tissue repair.

Of the three candidate biomarkers that were down-regulated with myocardial injury, the major HDL component APOA4 has already been documented for its inverse associations with risk of

CADs (59-61). The collagen-associated ECM protein LUM is involved in collagen fibril assembly and tissue repair (62, 63). It seems likely that LUM effects on collagen assembly could influence risk of plaque rupture, hence decreased levels of LUM may represent a clinically useful indicator of plaque destabilization and relative risk of MI. NAGLU is an acid hydrolase that facilitates the degradation of heparan sulfate (HS) glycosaminoglycans within lysosomes (64). It has been reported that the HS content of the blood vessel walls is decreased in atherosclerosis (65-67), leading to increased retention of lipoprotein in the subendothelial matrix (65). We would therefore speculate that decreased NAGLU expression could represent a physiological response to HS degradation in the atherosclerotic vessel walls.

Our functional linkage analyses indicated that the novel putative biomarkers of myocardial injury are broadly associated with two major processes; either plaque destabilization (SERPINA3, CPN2, LUM), or mechanisms of protection against further damage (ORM2, ACTG1, NAGLU), although their precise roles will require further study. We postulate that decreased expression of LUM and elevated levels of F10, SERPINA3, and CPN2 are associated with the pathogenesis of atherosclerosis and/or myocardial injury, whereas reduced NAGLU expression and up-regulation of MST1, ORM2, and ACTG1 are instead associated with mechanisms of atheroprotection and/or tissue healing. In addition to confirming the disease association of previously identified biomarkers of CAD (VTN, CRP, SAA1 and APOA4), we now describe potentially new diagnostic panel consisting of eight candidate biomarkers that can differentiate between the complex multifactorial pathophysiologies of atherosclerosis (F10, MST1) and myocardial injury (ORM2, SERPINA3, CPN2, LUM, ACTG1, NAGLU).

Although our preliminary findings clearly suggest that all eight protein candidates are potential diagnostic biomarkers for CAD and MI, we would like to underline some clinical limitations. The relatively small sample size and single cohort used for this study, may have weakened the diagnostic association of the proposed candidate marker with CAD, as we do not know the effect of CAD risk factors and medication on protein levels in plasma. Nevertheless, with relevant experience and stable platforms, reliable statistical difference of numerous potentially useful plasma biomarkers of CAD was achieved. In addition, there are no plasma-based screening assays that effectively discriminate underlying atherosclerotic conditions from impending myocardial injury at present. It is hoped that further assessment of these putative biomarkers in large-scale clinical validation studies will reveal high predictive values with genuine clinical utility for stratifying patient risk of infarction and accurate diagnostic testing.

CONCLUSION

The identification of sensitive and specific biomarkers that effectively stratify patients at risk for CAD remains as one of the greatest challenges in modern medicine. Here, we identified a putative panel of eight novel plasma proteins that can reliably distinguish myocardial injury from underlying atherosclerosis in human patients. The usage of MRM-MS-based targeted approach for pre-clinical biomarker verification and validation, have facilitated the screening of all 53 discovery candidates, and the validation of 23 verified candidates on 49 individual patients. This cost-effective approach possesses the capacity and efficiency, to screen and prioritize initial long candidate list in a single workflow at low cost and high throughput. Functional pathological linkage of the eight prioritized biomarkers to CAD, have suggested their involvement in endothelial dysfunction (F10, MST1), plaque destabilization (SERPINA3, CPN2, LUM) and protective mechanisms against further damage (ORM2, ACTG1, NAGLU).

Considering the small sample size used in this study, these eight candidates would warrant further statistical assessment in larger scale clinical validation on orthogonal patient cohort, that possess adequate power to correct for potential confounders (e.g. medication). Depending on the availability of resources, immunoassay-based or targeted absolute quantitation of protein (AQUA) MRM-MS-based approach can be utilized in clinical validation studies, to achieve sensitive and accurate quantitative measurements for potential clinical utility. Though with limitations, we showed that the combination of iTRAQ and MRM proteomics are efficient, robust and convenient platforms, that fulfils the requirement for the high confident identification (low FDR), verification and validation of potentially useful plasma biomarkers on MI and NMI CAD patient cohorts. This systematic and high throughput strategy presented here is well orchestrated for unbiased multiplexed quantitative discovery, the prioritization of candidates with the use of well-characterized technologies, will facilitate the development of clinically relevant plasma protein biomarker panel for CAD.

AVAILABILITY OF DATA AND MATERIAL

The iTRAQ LC-MS/MS and LC-MRM-MS proteomics raw data files along with the ProteinPilot generated group files have been deposited to the Proteome Xchange Consortium (<http://proteomecentral.proteomexchange.org>) (33) via the PRIDE partner repository with the dataset identifier PXD006333.

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SUPPORTING INFORMATION

The following files are available free of charge at ACS website <http://pubs.acs.org>:

Supplemental information. NCBI BLASTP results of all 49 peptide sequences used for MRM validation in individual patient plasma. The top 20 hits sequence identity when examined among species were 100%. The peptides used for MRM validation assay are proteotypic.

Supplemental Figure S1. Venn diagram showing the overlap in proteins identified (FDR <1%) across the three iTRAQ technical replicates (R01, replicate 1; R02, replicate 2; R03, replicate 3).

Supplemental data S1, Worksheet PPRepPro lists all non-redundant iTRAQ-quantified plasma proteins (FDR < 1%) obtained from individual triplicates LC-MS/MS dataset. The respective iTRAQ ratios of $114_{\text{MI}}:113_{\text{Ctrl}}$, $115_{\text{NMI}}:113_{\text{Ctrl}}$ and $114_{\text{MI}}:115_{\text{NMI}}$ for each protein (FDR < 1%) were exported from ProteinPilot™ V4.1 software. Unused ProtScore > 1.5 correspond to FDR < 1% in R01, Unused ProtScore >2.01 correspond to FDR < 1% in R02 and Unused ProtScore > 2.05 correspond to FDR < 1% in R03. MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control; Replicate 01; R02, Replicate 02; R03, Replicate 03. **Worksheet PPRepPep** lists all non-redundant iTRAQ-quantified plasma peptides (FDR < 1%) obtained from individual triplicates LC-MS/MS dataset. The respective iTRAQ ratios of $114_{\text{MI}}:113_{\text{Ctrl}}$, $115_{\text{NMI}}:113_{\text{Ctrl}}$ and $114_{\text{MI}}:115_{\text{NMI}}$ for each protein (FDR < 1%) were exported from ProteinPilot™ V4.1 software. Unused ProtScore > 1.5 correspond to FDR < 1% in R01, Unused ProtScore > 2.01 correspond to FDR < 1% in R02 and Unused ProtScore > 2.05 correspond to FDR < 1% in R03. MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control; Replicate 01; R02, Replicate 02; R03, Replicate 03. **Worksheet PPRepStat** contains the tabular results of statistical analyses between individual triplicates LC-MS/MS dataset. The respective iTRAQ ratios of $114_{\text{MI}}:113_{\text{Ctrl}}$, $115_{\text{NMI}}:113_{\text{Ctrl}}$ and $114_{\text{MI}}:115_{\text{NMI}}$ for each protein (FDR < 1%) were exported from ProteinPilot™ V4.1 software. All statistical results were generated using GraphPad Prism V 6.0. Measured iTRAQ ratios of all matched proteins were used in Pearson's correlation analyses. MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control; Replicate 01; R02, Replicate 02; R03, Replicate 03.

Supplemental data S2, Worksheet PP2DPro lists all non-redundant iTRAQ-quantified plasma proteins (FDR < 1%, obtained from combined triplicate LC-MS/MS dataset. The respective iTRAQ

reporter ions of $114_{\text{MI}}:113_{\text{Ctrl}}$, $115_{\text{NMI}}:113_{\text{Ctrl}}$ and $114_{\text{MI}}:115_{\text{NMI}}$ ratios for each protein were calculated and exported from ProteinPilot™ V4.1 software. Unused ProtScore > 2.02 correspond to FDR < 1% in combined searched dataset. MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control. **Worksheet PP2DPep** lists all non-redundant iTRAQ-quantified plasma peptides (FDR < 1%) obtained from combined triplicate LC-MS/MS dataset. The respective iTRAQ reporter ions $114_{\text{MI}}:113_{\text{Ctrl}}$, $115_{\text{NMI}}:113_{\text{Ctrl}}$ and $114_{\text{MI}}:115_{\text{NMI}}$ ratios for each protein were calculated and exported from ProteinPilot™ V4.1 software. Unused ProtScore >2.02 correspond to FDR < 1% in combined searched dataset. MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control. **Worksheet PP2DSL** lists the shortlisted iTRAQ-quantified plasma proteins (FDR < 1%) obtained from 2D combined and 1D individual triplicate LC-MS/MS dataset. The respective iTRAQ reporter ions $114_{\text{MI}}:113_{\text{Ctrl}}$, $115_{\text{NMI}}:113_{\text{Ctrl}}$ and $114_{\text{MI}}:115_{\text{NMI}}$ ratios for each protein were calculated and exported from ProteinPilot™ V4.1 software. Unused ProtScore > 2.02 correspond to FDR < 1% in 2D combined searched dataset. MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control. **Worksheet PP2DErich** contains the complete analyses of GO Gene ontology (GO)-based biological process and biological pathway enrichment using statically significant deregulated atherosclerotic-specific proteins and myocardial injury-specific proteins. The significance of the enriched categories was ranked by the Benjamini–Hochberg (BH) adjusted p-value, $p < 0.05$ indicates high enrichment. Enrichment and statistical data were generated by FunRich V2.1.2.

Supplemental data S3, worksheet MRMPrelim contains the initial MRM-method information of 255 peptides and 999 transitions representing 53 candidate protein biomarkers for multiple reaction monitoring (MRM)-assay in pooled plasma samples. All MRM transitions were derived from SRM Atlas and Pinpoint V1.3 software. **Worksheet MRMFinal** contains the refined MRM-method information of 174 peptides and transitions representing 23 candidate protein biomarkers for scheduled multiple reaction monitoring (MRM)-assay in individual plasma samples. **Worksheet MRMHseKpMRM** contains the method information of three house-keeping proteins, including serum albumin (ALB), serotransferrin (TF) and alpha-2-macroglobulin (A2M). All MRM transitions were derived from SRM Atlas and Pinpoint V1.3 software. **Worksheet MRMPinPt** contains PinPoint V1.3 generated peak area intensity, signal to noise ratio and file retention time of each targeted transition in individual Ctrl (Control, n=14), MI (myocardial infarction, n=15) and NMI (stable angina, n=20) patient plasma samples. **Worksheet MRMNorm** contains the original total peak area intensity and normalized total peak area intensity in individual Ctrl (Control, n=14), MI (myocardial infarction, n=15) and NMI (stable angina, n=20) patient plasma samples. Data

were normalized to the mean of the three housekeeping proteins (A2M, ALB, TF). **Worksheet MRMStat** contains the tabular results of statistical analyses on targeted plasma proteins generated by GraphPad Prism V 6.0. Statistical results were tabulated based on individual patient peak areas computed by Pinpoint V1.3 software, normalized to the mean to three housekeeping proteins.

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TABLES AND FIGURES

Table 1. Patient demographics and clinical characteristics

Variables	Categories	MI (n=15)	Non-MI (n=20)	Control (n=14)	p-value
Mean Age $\pm$ SD		63.6 $\pm$ 11.81	62.0 $\pm$ 7.88	53.5 $\pm$ 8.62	0.012 [^]
Gender	Male, n (%)	10 (66.67)	15 (75)	11 (78.57)	0.753*
	Female, n (%)	5 (33.33)	5 (25)	3 (21.43)	
Race	Chinese, n (%)	8 (53.33)	10 (50)	10 (71.43)	0.355*
	Malay, n (%)	4 (26.67)	5 (25)	4 (28.57)	
	Indian, n (%)	2 (13.33)	5 (25)	0 (0)	
	Others, n (%)	1 (6.67)	0 (0)	0 (0)	
Diabetes Mellitus	No, n (%)	6 (40)	6 (30)	10 (71.43)	0.052*
	Yes, n (%)	9 (60)	14 (70)	4 (28.57)	
Hypertension	No, n (%)	1 (6.67)	1 (5)	6 (42.86)	0.006*
	Yes, n (%)	14 (93.33)	19 (95)	8 (57.14)	
Hyperlipidaemia	No, n (%)	0 (0)	0 (0)	8 (57.14)	0.000*
	Yes, n (%)	15 (100)	20 (100)	6 (42.86)	
Ejection Fraction	1-good (>45%)	8 (53.33)	13 (65)	10 (71.43)	0.569*
	2-fair (30-45%)	6 (40)	7 (35)	4 (28.57)	
	3-poor (<30%)	1 (6.67)	0 (0)	0 (0)	
Smoking	No, n (%)	10 (66.67)	10 (50)	10 (71.43)	0.394*
	Yes, n (%)	5 (33.33)	10 (50)	4 (28.57)	
Renal Impairment	No, n (%)	15 (100)	20 (100)	14 (100)	NA
	Yes, n (%)	0 (0)	0 (0)	0 (0)	
Peripheral vascular disease	No, n (%)	15 (100)	18 (90)	14 (100)	0.221*
	Yes, n (%)	0 (0)	2 (10)	0 (0)	
Previous Stroke	No, n (%)	15 (100)	20 (100)	14 (100)	NA
	Yes, n (%)	0 (0)	0 (0)	0 (0)	
Mean HbA1c Level $\pm$ SD		7.25 $\pm$ 1.87 (n=13)	6.93 $\pm$ 1.25 (n=19)	6.12 $\pm$ 1.12 (n=13)	0.020 [^]
Mean Creatine Kinase (U/L) $\pm$ SD		3280.42 $\pm$ 5591.37 (n=12)	102.67 $\pm$ 74.44 (n=6)	161.25 $\pm$ 170.80 (n=4)	0.249 [^]
Mean CKMB (μ g/L) $\pm$ SD		82.26 $\pm$ 74.80 (n=12)	3.73 $\pm$ 6.70 (n=6)	1.85 $\pm$ 1.03 (n=4)	0.015 [^]
Mean Troponin I (μ g/L) $\pm$ SD		19.86 $\pm$ 19.12 (n=13)	0.75 $\pm$ 1.81 (n=6)	0.02 $\pm$ 0.02 (n=4)	0.019 [^]
Aspirin	No, n (%)	5 (33.33)	5 (25)	13 (92.86)	0.000*
	Yes, n (%)	10 (66.67)	15 (75)	1 (7.14)	
Clopidogrel	No, n (%)	12 (80)	14 (70)	14 (100)	0.083*
	Yes, n (%)	3 (20)	6 (30)	0 (0)	
Beta Blockers	No, n (%)	5 (33.33)	6 (30)	11 (78.57)	0.011*
	Yes, n (%)	10 (66.67)	14 (70)	3 (21.43)	
ACE Inhibitors	No, n (%)	9 (60)	11 (55)	11 (78.57)	0.356*
	Yes, n (%)	6 (40)	9 (45)	3 (21.43)	
Ca Antagonists	No, n (%)	12 (80)	16 (80)	13 (92.86)	0.546*
	Yes, n (%)	3 (20)	4 (20)	1 (7.14)	
Angiotensin Receptor Blockers	No, n (%)	15 (100)	19 (95)	13 (92.86)	0.602*
	Yes, n (%)	0 (0)	1 (5)	1 (7.14)	
Statins	No, n (%)	3 (20)	0 (0)	12 (85.71)	0.000*
	Yes, n (%)	12 (80)	20 (100)	2 (14.29)	

n, number of individuals; SD, standard deviation; *, p-value is calculated based on Chi-Square test for the categorical variables; [^], p-value is calculated based on ANOVA for the continuous variables.

Table 2. Experimental variation of protein, peptide and spectral identification.

Identification n	iTRAQ_R0 1	iTRAQ_R0 2	iTRAQ_R0 3	SD	%CV	Mean
Protein	464	450	395	36.47	8.36	436
Peptide	14848	15352	15394	303.84	2.00	15198
Spectral	159793	155425	156673	2249.86	1.43	157297

R01, Replicate 01; R02, Replicate 02; R03, Replicate 03; SD, Standard deviation; %CV, Percentage coefficient variation

Table 3. List of proteins exhibiting significantly modulated expression in disease.

Accession ^{a,b}	Protein Description ^{a,b}	Peptides (95%) ^a	MI(114):Ctrl (113)			NMI(115):Ctrl (113)			MI (114):NMI(115)			
			2D ratio ^a	1D mean ratio ^b	P value ^a	2D ratio ^a	1D mean ratio ^b	P value ^a	2D ratio ^a	1D mean ratio ^b	P value ^a	
<i>Shortlisted biomarkers of atherosclerosis</i>												
41.91	P02760	Protein AMBP	57	18.20	7.83	0.010	14.86	7.79	0.008	1.43	1.11	0.109
3.31	P59666	Neutrophil defensin 3	2	14.59	6.98	0.040	12.36	6.85	0.012	1.13	1.02	0.925
12.49	P20851	C4b-binding protein beta chain	10	10.57	12.78	0.047	10.47	14.01	0.037	1.14	0.97	0.677
					<							
33.65	P08519	Apolipoprotein(a)	58	10.00	8.01	0.0001	4.13	4.59	0.002	2.68	1.97	0.000
18.55	P00742	Coagulation factor X	14	9.38	8.72	0.020	9.73	9.70	0.012	0.98	0.91	0.489
7.28	P02776	Platelet factor 4	7	6.43	7.00	0.004	6.25	7.98	0.010	1.03	0.89	0.102
43.05	P02749	Beta-2-glycoprotein 1	69	6.43	6.67	0.000	4.53	5.26	0.002	1.46	1.36	0.025
41.22	P04004	Vitronectin	71	5.92	4.29	0.018	3.50	3.07	0.009	1.82	1.41	0.064
53.78	P00736	Complement C1r subcomponent	50	5.81	4.99	0.004	3.56	3.82	0.007	1.69	1.37	< 0.0001
18.81	P07357	Complement component C8 alpha chain	19	4.66	4.46	0.006	2.49	3.55	0.002	1.89	1.23	0.010
48.40	P43652	Afamin	47	4.53	2.81	0.004	7.45	5.08	0.001	0.55	0.50	< 0.0001
8.86	P17936	Insulin-like growth factor-binding protein 3	7	4.53	2.65	0.009	5.70	3.42	0.017	0.84	0.82	0.009
17.88	P03951	Coagulation factor XI	17	4.45	3.55	0.020	3.84	3.16	0.009	1.11	1.06	0.453
					<							
56.31	P10643	Complement component C7	67	4.41	3.25	0.0001	4.13	3.37	0.004	1.07	0.95	0.462
38.29	O75882	Attractin	29	4.02	3.11	0.020	3.80	3.56	0.020	1.00	0.82	0.017
11.08	P27918	Properdin	7	3.87	3.68	0.004	4.17	4.30	0.000	0.92	0.85	0.214
36.15	P05160	Coagulation factor XIII B chain	22	3.87	4.23	0.005	3.63	3.86	< 0.0001	1.11	1.06	0.625
38.56	P07225	Vitamin K-dependent protein S	38	3.84	3.47	0.000	2.99	3.01	0.002	1.29	1.17	0.391
24.93	P05156	Complement factor I	23	3.80	4.05	0.002	3.05	3.74	0.004	1.29	1.12	0.058
66.13	P03952	Plasma kallikrein	56	3.66	2.74	0.001	4.53	3.80	0.001	0.79	0.69	0.000
78.44	P00734	Prothrombin	137	3.60	4.00	0.006	2.42	3.55	0.000	1.60	1.14	0.086
20.31	Q14520	Hyaluronan-binding protein 2	22	3.16	7.62	0.019	2.54	6.56	0.003	1.24	1.16	0.390
47.18	P00748	Coagulation factor XII	43	3.05	2.79	0.017	4.66	4.62	< 0.0001	0.63	0.57	0.018
13.77	Q961Y4	Carboxypeptidase B2	15	2.83	2.61	0.002	2.31	2.53	0.010	1.22	1.04	0.634
18.12	P26927	Hepatocyte growth factor-like protein	13	2.81	4.21	0.004	2.63	4.13	0.000	1.12	1.07	0.731
9.08	Q9UGM5	Fetuin-B	11	2.09	2.74	0.002	2.27	3.38	0.019	0.95	0.86	0.444
					<							
68.16	P02766	Transthyretin	105	0.10	0.12	0.0001	0.15	0.22	< 0.0001	0.54	0.43	0.000
<i>Shortlisted biomarkers of myocardial injury</i>												
11.58	P02735	Serum amyloid A protein	10	22.91	18.26	0.003	0.05	0.13	< 0.0001	59.16	43.96	0.001
4.62	P02741	C-reactive protein	3	10.86	6.29	0.027	0.39	0.46	0.028	21.88	11.97	< 0.0001
53.50	P01011	Alpha-1-antichymotrypsin	80	3.63	3.19	0.001	0.46	0.58	0.000	7.45	5.17	0.001
25.01	P02750	Leucine-rich alpha-2-glycoprotein	32	2.91	3.23	0.006	0.46	0.62	0.011	5.75	4.86	0.001
11.89	P12814	Alpha-actinin-1	7	0.79	0.66	0.009	0.17	0.23	0.000	5.01	3.48	0.085
					<							
37.07	P63261	Actin, cytoplasmic 2	53	0.55	0.56	0.0001	0.12	0.17	< 0.0001	4.92	3.52	0.006
25.52	P18206	Vinculin	21	0.67	0.51	0.000	0.14	0.15	< 0.0001	4.88	4.00	0.008
					<							
7.75	P35542	Serum amyloid A-4 protein	23	1.39	1.43	0.0001	0.29	0.45	0.004	4.61	3.35	0.043
33.31	P02748	Complement component C9	35	3.98	4.10	0.004	0.90	1.06	0.768	4.57	3.99	0.015
38.20	P22792	Carboxypeptidase N subunit 2	37	1.63	1.41	0.001	0.39	0.54	0.015	3.91	2.85	0.024
3.44	P78417	Glutathione S-transferase omega-1	2	1.85	1.38	0.198	0.50	0.60	0.010	3.70	2.33	0.023
13.70	P19652	Alpha-1-acid glycoprotein 2	43	1.47	1.18	0.007	0.39	0.59	0.002	3.66	1.97	0.001
31.49	P02649	Apolipoprotein E	44	1.47	1.28	0.036	0.42	0.52	0.001	3.31	2.36	0.017

2D, iTRAQ ratio of each protein derived from the combined results of triplicate LC-MS/MS runs; 1D, Mean iTRAQ ratio of each protein calculated from the individual LC-MS/MS results; Cov, Coverage; Pval, P value; EF, Error factor; CI, Confidence interval; R01, Replicate 01; R02, Replicate 02; R03, Replicate 03; MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control; 113, 114 and 115, 8-plex isobaric tags.

Table 3. List of proteins exhibiting significantly modulated expression in disease.(Continued)

Unused ^a	Accession ^{a,b}	Protein Description ^{a,b}	Peptides (95%) ^a	MI(114):Ctrl (113)			NMI(115):Ctrl (113)			MI (114):NMI(115)		
				2D ratio ^a	1D mean ratio ^b	P value ^a	2D ratio ^a	1D mean ratio ^b	P value ^a	2D ratio ^a	1D mean ratio ^b	P value ^a
3.83	P07195	L-lactate dehydrogenase B chain	5	1.56	1.21	0.187	0.45	0.48	0.002	3.31	2.52	0.011
54.35	P21333	Filamin-A	36	0.88	0.63	0.001	0.29	0.25	< 0.0001	3.25	2.74	0.000
41.24	Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	42	4.02	3.68	< 0.0001	1.27	1.73	0.023	3.19	2.24	0.007
17.19	P18428	Lipopolysaccharide-binding protein	17	3.16	2.56	0.002	1.16	1.03	0.720	2.81	2.40	0.001
68.17	Q9Y490	Talin-1	66	0.60	0.61	0.003	0.22	0.27	< 0.0001	2.81	2.29	0.015
10.43	P07737	Profilin-1	9	0.67	0.65	0.000	0.27	0.30	0.000	2.47	2.29	0.013
5.77	P54802	Alpha-N-acetylglucosaminidase	3	1.22	1.26	0.659	3.05	4.34	0.192	0.40	0.37	0.014
30.80	P29622	Kallistatin	27	0.49	0.46	0.000	1.24	1.29	0.099	0.38	0.34	< 0.0001
19.18	P02753	Retinol-binding protein 4	34	0.32	0.30	< 0.0001	0.81	0.87	0.103	0.37	0.33	0.001
31.67	Q96PD5	N-acetylmuramoyl-L-alanine amidase	40	0.97	1.11	0.560	2.99	3.14	0.005	0.29	0.33	< 0.0001
19.39	P51884	Lumican	15	0.20	0.21	0.000	0.64	0.76	0.009	0.28	0.26	0.000
68.37	P06396	Gelsolin	73	0.15	0.18	< 0.0001	1.10	1.27	0.155	0.13	0.14	< 0.0001
60.12	P06727	Apolipoprotein A-IV	84	0.15	0.22	< 0.0001	1.60	1.81	0.024	0.10	0.14	< 0.0001

2D, iTRAQ ratio of each protein derived from the combined results of triplicate LC-MS/MS runs; 1D, Mean iTRAQ ratio of each protein calculated from the individual LC-MS/MS results; Cov, Coverage; Pval, P value; EF, Error factor; CI, Confidence interval; R01, Replicate 01; R02, Replicate 02; R03, Replicate 03; MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control; 113, 114 and 115, 8-plex isobaric tags.

Table 4. Reproducibility of retention time and integrated peak area of targeted peptides across different samples in multiple LC-MRM-MS runs.

Protein Accession	Gene Symbol	Sequence	Parent (Q1) m/z	Mean RT ^a	%CV ^a	Ctrl_Mean Peak Area ^b	Ctrl_%CV ^b	MI_Mean Peak Area ^b	MI_%CV ^b	NMI_Mean Peak Area ^b	NMI_%CV ^b
<i>Shortlisted biomarkers of atherosclerosis</i>											
P43652	AFM	DADPDTFFAK	563.76	26.00	1.97	5.43E+05	0.83	5.75E+05	4.20	8.73E+05	2.72
		FTFEYSR	475.22	22.06	2.51	2.44E+05	8.25	2.80E+05	5.58	4.37E+05	1.94
P02760	AMBP	AFIQLWAFDAVK	704.89	43.52	1.67	3.63E+05	2.56	2.94E+05	3.30	4.36E+05	4.38
		ETLLQDFR	511.27	27.00	2.26	1.32E+06	2.62	1.47E+06	7.00	2.11E+06	1.92
O75882	ATRN	CINQSICEK	704.89	43.52	1.67	5.02E+04	1.22	2.43E+04	14.51	3.01E+04	1.07
		WSVLPRDLHHDVNR	511.27	27.00	2.26	1.28E+06	2.71	1.44E+06	7.21	2.07E+06	1.69
P00742	F10	CINQSICEK	519.24	30.80	3.33	4.85E+05	1.19	4.17E+05	2.28	4.47E+05	1.57
		WSVLPRDLHHDVNR	519.24	30.80	3.33	1.42E+05	6.14	4.18E+04	2.90	1.04E+05	1.47
P08519	LPA	MLEVPYVDR	614.32	15.96	4.63	4.14E+05	1.09	3.83E+05	2.57	3.44E+05	2.33
		SHAPEVITSSPLK	561.29	26.39	2.70	1.06E+06	5.18	1.39E+06	1.90	1.64E+06	0.61
		SHAPEVITSSPLK	683.37	37.02	1.37	1.94E+05	2.06	1.97E+05	1.76	2.51E+05	1.28
P26927	MST1	GTDSQGDSDGGLVCFEK	900.38	18.43	2.70	6.61E+04	2.12	3.46E+04	4.10	8.61E+04	3.43
		NPDAVAAPYCYTR	720.83	16.23	7.79	1.19E+06	1.46	2.29E+06	5.18	1.85E+06	2.25
P02766	TTR	CEIAGWGTEK	547.25	20.80	3.45	4.80E+05	2.69	5.37E+05	3.90	6.07E+05	2.23
		MVCGPSGSQLVLLK	716.39	34.38	1.21	7.09E+05	0.86	1.75E+06	6.05	1.24E+06	3.52
P04004	VTN	GSPAINVAVHVR	683.88	24.24	3.46	8.88E+05	5.75	7.41E+05	6.02	7.59E+05	1.37
		TSESGELHGLTTEEFVEGIYK	819.06	35.09	1.81	3.54E+05	3.77	1.73E+05	6.44	1.83E+05	1.75
		DVWGIEGPIDAAFR	819.06	35.09	1.81	6.48E+05	3.87	5.68E+05	5.90	5.97E+05	1.17
		FEDGVLDPDYPR	823.91	44.73	1.63	2.39E+06	4.37	4.36E+06	0.61	3.56E+06	1.29
		FEDGVLDPDYPR	711.83	27.62	1.82	1.45E+06	6.99	2.78E+06	0.21	2.12E+06	0.82
		FEDGVLDPDYPR	711.83	27.62	1.82	9.44E+05	0.55	1.58E+06	1.67	1.44E+06	2.41
<i>Shortlisted biomarkers of myocardial injury</i>											
P63261	ACTG1	KDLYANTVLSGGTTMYPGIADR	1172.09	33.57	1.71	8.43E+05	5.11	1.09E+06	5.62	3.14E+05	1.92
		SYELPDGQVITIGNER	895.95	38.27	1.49	4.52E+05	3.57	6.19E+05	8.00	1.12E+05	3.60
P06727	APOA4	ISASAEELR	895.95	38.27	1.49	4.32E+05	2.76	4.81E+05	4.47	2.02E+05	0.99
		SELTQQLNALFQDK	488.26	12.76	9.73	1.01E+06	2.53	6.73E+05	4.80	2.03E+06	1.48
		SLAELGGHLDQQVEEFR	817.92	39.90	1.31	1.49E+06	8.78	5.51E+05	6.21	1.67E+06	1.80
P02649	APOE	SELEEQLTPVAEETR	643.32	31.02	2.23	2.73E+05	2.02	1.23E+05	5.28	1.90E+05	1.99
		WELALGR	643.32	31.02	2.23	1.95E+05	1.07	1.82E+05	2.59	2.51E+05	0.69
P22792	CPN2	SELEEQLTPVAEETR	865.93	24.95	1.81	2.30E+06	5.85	3.57E+06	8.43	1.80E+06	5.09
		WELALGR	422.74	26.73	2.39	9.38E+05	5.13	1.40E+06	5.37	5.79E+05	4.64
P02741	CRP	LELLSLSK	422.74	26.73	2.39	1.50E+06	3.16	2.18E+06	12.49	1.22E+06	5.37
		LTVSIEAR	451.78	27.17	2.61	3.69E+06	3.81	5.55E+06	5.72	2.67E+06	2.20
P21333	FLNA	AFVFPK	444.76	20.35	2.80	2.84E+05	4.08	1.04E+05	3.65	1.55E+05	1.97
		ESDTSYVSLK	354.71	19.67	3.84	3.49E+06	4.01	5.45E+06	5.78	2.51E+06	2.56
		CSGPGLER	564.77	15.05	8.55	2.01E+05	0.50	3.25E+06	11.87	1.82E+05	3.67
		ENGYYLIDVK	564.77	15.05	8.55	2.25E+05	7.15	2.41E+06	12.36	9.30E+04	0.90
		ENGYYLIDVK	409.70	15.93	3.25	1.08E+05	15.05	1.22E+06	8.79	1.34E+05	3.02
		ENGYYLIDVK	575.31	21.74	2.42	1.85E+06	4.61	1.71E+06	5.26	1.93E+06	1.30
		ENGYYLIDVK	575.31	21.74	2.42	7.50E+05	4.21	4.84E+05	3.10	7.57E+05	1.15
		ENGYYLIDVK	575.31	21.74	2.42	1.13E+06	3.54	1.24E+06	4.87	1.18E+06	1.69

m/z, mass-to-charge ratio; RT, retention time; ^a, values derived from individual 147 LC-MRM runs; ^b, mean total peak intensity derived from triplicate LC-MRM-MS runs.

Table 4. Reproducibility of retention time and integrated peak area of targeted peptides across different samples in multiple LC-MRM-MS runs. (Continued)

Protein Accession	Gene Symbol	Sequence	Parent (Q1) m/z	Mean RT ^a	%CV ^a	Ctrl_Mean Peak Area ^b	Ctrl_%CV ^b	MI_Mean Peak Area ^b	MI_%CV ^b	NMI_Mean Peak Area ^b	NMI_%CV ^b
Q06033	ITIH3	DYIFGN YIER	645.31	34.32	1.15	4.13E+05	5.77	7.14E+05	1.26	4.97E+05	1.05
		EVSFDVELPK	581.80	30.21	1.87	1.92E+05	5.48	3.29E+05	1.53	2.58E+05	1.47
P02750	LRG1	DLLLPQPD LR	590.34	32.80	1.29	2.38E+05	6.03	3.84E+05	1.08	2.39E+05	1.74
		VAAGAFQGLR	495.28	17.48	4.46	1.69E+06	4.77	3.40E+06	7.39	2.19E+06	0.95
P51884	LUM	FNALQYLR	512.78	29.80	2.19	1.20E+06	6.61	2.59E+06	6.86	1.76E+06	1.31
		NNQIDHIDEK	409.20	15.90	3.47	6.29E+05	4.45	8.50E+05	2.36	4.24E+05	0.72
P54802	NAGLU	GDTVDLAK	409.72	15.94	3.46	5.46E+06	6.94	2.49E+06	13.70	7.60E+06	0.73
		YGVSHPDAGAAWR	693.83	41.58	1.18	2.17E+05	9.30	4.60E+04	2.56	1.21E+05	4.86
P19652	ORM2	SDVMYTDWK	572.75	23.37	1.98	6.57E+06	5.87	2.74E+06	4.62	7.48E+06	0.88
		TEDTIFLR	497.76	22.52	2.43	4.03E+05	5.73	2.26E+05	8.44	4.95E+05	0.40
P0DJ18	SAA1	TLMFGSYLDDEK	709.83	33.04	1.30	3.22E+05	1.18	1.72E+05	4.87	3.88E+05	0.79
		FFGHGAEDSLADQAANEWGR	726.66	30.33	2.76	1.23E+05	15.15	6.08E+04	1.68	1.07E+05	2.85
P35542	SAA4	SFFSFLGEAFD GAR	775.87	48.87	1.31	3.33E+06	5.00	7.04E+06	2.46	3.46E+06	1.61
		EALQGVGDMGR	566.77	17.16	3.21	1.05E+06	6.46	1.36E+06	4.88	1.03E+06	4.03
P01011	SERPINA3	FRPDGLPK	465.26	12.42	4.08	1.89E+06	5.70	4.85E+06	2.50	1.86E+06	1.42
		LYGSEAFATDFQDSAAAK	946.44	31.41	2.04	3.72E+05	7.12	8.25E+05	2.95	5.65E+05	1.55
Q9Y490	TLN1	MEEVEAMLLPETLK	816.92	38.13	1.42	8.19E+04	0.53	3.17E+06	2.46	4.54E+04	1.99
		NCGQMSEIEAK	605.27	37.79	1.86	6.38E+04	5.87	3.04E+06	1.69	2.74E+04	4.63
		SAQPASAEPR	507.25	30.87	2.69	2.34E+04	4.95	3.71E+05	10.27	2.11E+04	4.15
		TLAESALQLLYTAK	507.96	31.04	1.63	4.57E+06	3.06	7.85E+06	2.87	4.06E+06	3.36
<i>House-keeping proteins</i>						2.26E+06	4.66	3.17E+06	6.12	1.50E+06	2.69
P01023	A2M	LSFYLLIMAK	624.84	40.95	1.45	2.94E+06	4.71	7.51E+06	2.14	2.56E+06	3.73
P02768	ALB	VGFYESDVMGR	630.29	25.22	1.84	1.04E+06	4.41	1.72E+06	2.10	6.88E+05	0.47
		AVMDDFAAFVEK	671.82	38.31	1.00	5.50E+05	5.66	1.11E+06	3.25	3.93E+05	0.88
		HPDYSVLLLLR	656.38	30.69	2.12	4.90E+05	3.48	6.10E+05	0.00	2.95E+05	1.87
		LVAASQAALGL	507.30	31.18	1.30	2.09E+06	6.11	1.37E+06	3.61	2.58E+06	1.79
P02787	TF	LVNEVTEFAK	575.31	21.57	2.13	7.45E+05	5.16	5.71E+05	5.73	8.67E+05	3.87
		EGYYGYTGAFR	642.29	24.91	1.77	507.25	30.87	2.69	2.80E+05	4.85	8.91E+05
		YLGEEYVK	500.75	20.15	2.40	9.32E+05	2.86	5.15E+05	2.03	8.25E+05	0.69
						6.43E+06	5.09	6.11E+06	4.62	6.32E+06	1.27
						2.11E+06	4.51	1.58E+06	11.04	1.76E+06	1.70
						3.52E+05	3.86	1.75E+05	2.00	2.79E+05	2.33
						4.11E+06	4.94	4.40E+06	2.67	4.29E+06	2.37
						4.19E+08	3.87	4.17E+08	3.36	4.50E+08	1.22
						6.35E+07	9.52	4.74E+07	5.34	7.55E+07	1.86
						5.47E+06	7.11	5.36E+06	4.66	4.56E+06	1.62
						3.78E+07	7.67	2.24E+07	4.04	4.28E+07	2.21
						3.12E+08	2.28	3.42E+08	3.71	3.26E+08	1.51
						2.70E+07	0.74	2.60E+07	2.40	3.09E+07	1.04
						6.89E+06	1.17	4.91E+06	2.01	6.81E+06	2.26
						9.07E+06	0.50	1.02E+07	1.70	1.05E+07	2.86
						1.10E+07	1.89	1.09E+07	4.00	1.36E+07	4.09

m/z, mass-to-charge ratio; RT, retention time; ^a, values derived from 147 individual LC-MRM runs; ^b, mean total peak intensity derived from triplicate LC-MRM-MS runs

Table 5. Comparison of differential expression results obtained by MRM or iTRAQ.

Protein Accession	Gene Symbol	Protein Description	MRM			iTRAQ		
			MI: Ctrl	NMI: Ctrl	MI:NMI	MI: Ctrl	NMI: Ctrl	MI:NMI
<i>Shortlisted biomarkers of atherosclerosis</i>								
P26927	MST1	Hepatocyte growth factor-like protein	22.89	12.62	1.81	2.81	2.63	1.12
P04004	VTN	Vitronectin	6.18	4.01	1.54	5.92	3.50	1.82
P00742	F10	Coagulation factor X	4.43	3.99	1.11	9.38	9.73	0.98
<i>Shortlisted biomarkers of myocardial injury</i>								
P0DJ18	SAA1	Serum amyloid A protein	68.35	0.79	86.57	1.39	0.29	4.61
P02741	CRP	C-reactive protein	13.94	0.94	14.78	10.86	0.39	21.88
P63261	ACTG1	Actin, cytoplasmic 2	1.73	0.39	4.41	0.55	0.12	4.92
P01011	SERPINA3	Alpha-1-antichymotrypsin	1.70	0.63	2.69	3.63	0.46	7.45
P22792	CPN2	Carboxypeptidase N subunit 2	2.10	0.96	2.18	1.63	0.39	3.91
P19652	ORM2	Alpha-1-acid glycoprotein 2	4.09	1.92	2.13	1.47	0.39	3.66
Q9Y490	TLN1	Talin-1	0.25	1.25	0.20	0.60	0.22	2.81
P54802	NAGLU	Alpha-N-acetylglucosaminidase	0.31	1.18	0.26	1.22	3.05	0.40
P06727	APOA4	Apolipoprotein A-IV	0.63	3.14	0.20	0.15	1.60	0.10
P51884	LUM	Lumican	0.25	1.25	0.20	0.20	0.64	0.28

MI, Myocardial infarction; NMI, Stable angina; Ctrl, Control; MRM, multiple reaction monitoring, iTRAQ, isobaric tags for relative and absolute quantification.

Figure 1

Figure 1. Schematic representation of the experimental design from initial discovery-phase to verification by quantitative proteomic analysis of non-depleted human plasma using an 8-plex iTRAQ/MRM-based approach.

Figure 2

Figure 2. Correlation between iTRAQ ratios obtained for each protein (FDR<1%) across three iTRAQ technical replicates (R01, R02 and R03). The differentially labelled plasma protein samples (isobaric tags; 113_{Ctrl} , 114_{MI} , 115_{NMI}) were compared in a pairwise fashion using scatter $\log_2$ plots and calculation of Pearson's correlation coefficient (r). These analyses confirmed that quantification was highly consistent across triplicate LC-MS/MS runs ($****p < 0.0001$).

Figure 3

Figure 3. Gene ontology (GO)-based functional enrichment analysis of all plasma proteins identified as being significantly modulated in either atherosclerosis (**A**, associated processes; **B**, associated pathways) or myocardial injury only (**C**, associated processes; **D**, associated pathways). P-values < 0.05 indicate strong enrichment (generated in FunRich v2.1.2).

Figure 4

Figure 4. Relative expression levels of targeted proteins in 49 individual plasma samples. Donors were patients with myocardial infarction (NSTEMI/MI; n=15), stable angina (NMI; n=20) or controls with no prior history of coronary atherosclerosis (Ctrl; n=14). The data are displayed as linear box-and-whiskers plots (line indicating median, box representing 25th to 75th percentiles, whiskers showing maximum and minimum ranges, dots denote individual values). Expression levels of the housekeeping proteins A2M (A) ALB (B) and TF (C) were comparable between study populations ($p > 0.05$). In contrast, with control samples as reference, when comparing MI plasma samples with NMI plasma samples we detected significant differential expression ($p < 0.05$) of the candidate biomarkers F10 (D), VTN (E), MST1 (F), SERPINA3 (G), CRP (H), SAA1 (I), ORM2 (J), CPN2 (K), ACTG1 (L), APOA4 (M), LUM (N), NAGLU (O), and TLN1 (P)

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