Plasma-derived Extracellular Vesicles Contain Predictive Biomarkers and Potential Therapeutic Targets for Myocardial Ischemic (MI) Injury*⑤

Esther Sok Hwee Cheow‡, Woo Chin Cheng§, Chuen Neng Lee§¶‖, Dominique de Kleijn§, Vitaly Sorokin§¶, and Siu Kwan Sze‡‡‡**

Myocardial infarction (MI) triggers a potent inflammatory response via the release of circulatory mediators, including extracellular vesicles (EVs) by damaged cardiac cells, necessary for myocardial healing. Timely repression of inflammatory response are critical to prevent and minimize cardiac tissue injuries, nonetheless, progression in this aspect remains challenging. The ability of EVs to trigger a functional response upon delivery of carried bioactive cargos, have made them clinically attractive diagnostic biomarkers and vectors for therapeutic interventions. Using label-free quantitative proteomics approach, we compared the protein cargo of plasma EVs between patients with MI and from patients with stable angina (NMI). We report, for the first time, the proteomics profiling on 252 EV proteins that were modulated with >1.2-fold after MI. We identified six up-regulated biomarkers with potential for clinical applications; these reflected postinfarct pathways of complement activation (Complement C1q subcomponent subunit A (C1QA), 3.23-fold change, $p = 0.012$; Complement C5 (C5), 1.27-fold change, $p = 0.087$), lipoprotein metabolism (Apoliporotein D (APOD), 1.86-fold change, $p = 0.033$; Apolipoprotein C-III (APOCC3), 2.63-fold change, $p = 0.029$) and platelet activation (Platelet glycoprotein Ib alpha chain (GP1BA), 9.18-fold change, $p < 0.0001$; Platelet basic protein (PPBP), 4.72-fold change, $p = 0.027$). The data have been deposited to the ProteomeXchange with identifier PXD002950. This novel biomarker panel was validated in 43 patients using antibody-based assays (C1QA ($p = 0.005$); C5 ($p = 0.0047$), APOD ($p = 0.0267$); APOC3 ($p = 0.0064$); GP1BA ($p = 0.0031$); PPBP ($p = 0.0465$)). We further present that EV-derived fibrinogen components were paradoxically down-regulated in MI, suggesting that a compensatory mechanism may suppress post-infarct coagulation pathways, indicating potential for therapeutic targeting of this mechanism in MI. Taken together, these data demonstrated that plasma EVs contain novel diagnostic biomarkers and therapeutic targets that can be further developed for clinical use to benefit patients with coronary artery diseases (CADs). *Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.055731, 2628–2640, 2016.*

Coronary artery diseases (CADs)[1], the principal cause of cardiovascular mortality is predicted to increase in prevalence in future years because of the aging global population (1, 2). In CAD patients, stenotic narrowing or sudden occlusion of the coronary arteries can restrict blood flow to the heart and result in myocardial infarction (MI) (3). Although interventions that can limit cardiac damage in the early stages of MI have been identified, treatment options are currently limited because of the lack of accessible biomarkers of myocardial inflammation to inform patient care in the clinic.

[1] The abbreviations used are: CV, coefficient variation; APOC3, apolipoprotein C-III; APOD, apoliporotein D; AUC, area under the curve; BCA, bicinchoninic acid; C1Q1A, complement C1Q subcomponent subunit A; C5, complement component C5; CAD, coronary artery disease; CVD, cardiovascular disease; EM, electron microscopy; ERLIC, electrostatic repulsion-hydrophilic interaction chromatography; EV, extracellular vesicles; FDR, false discovery rate; GO, gene ontology; GP1BA, platelet glycoprotein Ib alpha chain; HDL, high density lipoproteins; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LDL, low density lipoprotein; MI, myocardial infarction; NMI, nonmyocardial infarction; PD, Proteome Discoverer™; PPBP, platelet basic protein; PSM, peptide spectrum match; PUC, prolong ultracentrifugation; SD, standard deviation; WAX, weak anion exchange.

MI triggers an orchestrated inflammatory cascade that is actually required for myocardial healing, but excessive and/or prolonged inflammatory responses can induce pathological remodeling of the damaged tissues and even heart failure (4, 5). Accordingly, complement proteins and other inflammatory mediators have already been shown to influence the progression of cardiac tissue damage and repair in both experimental and clinical models of MI. Temporal regulation and eventual suppression of the MI-induced inflammatory response is critical for effective myocardial tissue healing (5). There have been numerous efforts to identify anti-inflammatory strategies for the treatment of MI, including inhibition of complement components or receptors (6–9); administration of free radical scavengers or nonenzymatic antioxidants (10–13); and depletion of tissue-infiltrating neutrophils (14–17). Although numerous studies have achieved promising reductions in experimental infarct size via the application of these and similar anti-inflammatory interventions, so far there has been little success in translating these approaches into genuine clinical applications (partly owing to the complex pathophysiology and heterogeneity of human diseases). Consequently, there remain an unmet clinical need for robust biomarkers that can inform MI patient care, and to identify novel therapeutic strategies with potential to limit myocardial injury.

Extracellular vesicles (EVs), including microvesicles and exosomes, are diverse population of membrane-bound structures secreted by almost all human cell types into the extracellular fluids (18). The growing body of functional studies on the pathophysiological roles of EVs have provided evidence of major roles in immune regulation (19, 20), and cell-cell communication via the transfer of bioactive molecules including proteins (21, 22), lipids (23, 24), and nucleic acids (25, 26). The influence exerted by EVs on a multitude of physiological processes (27, 28) and pathological disorders (29–31) have rendered these structures as highly promising targets for clinical biomarker discovery. Moreover, the ability of EVs to deliver protein cargo and elicit a functional response from distant cell types and tissues also indicates considerable potential for exploitation as vehicles for drug delivery. Indeed, recent studies have demonstrated the therapeutic potential of exosomes derived from transplanted mesenchymal stem cells (MSCs) in reducing myocardial injury and improving cardiac function following experimental MI (32, 33). Although the ability of EVs to exert potent influences on host cell and tissue function have been well established, it remains unclear whether the composition of circulating EVs is modified in MI, or whether these structures act as reservoirs of clinically useful biomarker that can inform patient care.

The objective of this study was to interrogate the plasma EV proteome to determine how this is modulated in MI, with the ultimate aim of identifying candidate biomarkers and drug targets with diagnostic and/or therapeutic potential. To do this, we first used a label-free quantitative proteomics approach to characterize the proteomes of plasma EVs isolated from patients with acute MI (infarcted in the preceding 5 days) and control patients with noninfarcted stable angina (NMI). To our knowledge, this report presents the first comparative proteomic profiling of dysregulated plasma EV proteins in MI patients *versus* NMI controls, and we confirm the active participation of EVs in myocardial damage and healing processes following infarction in human patients. Intriguingly, all fibrinogen components encapsulated in plasma EVs were downregulated in MI, suggesting a decrease in coagulation activity that could reflect a compensatory response to cardiac damage. Furthermore, we propose that a panel of six EV-encapsulated biomarkers may facilitate clinically useful assessment of myocardial injury; these reflected post-infarct pathways of complement activation (C1Q1A, $\sim$3.23 fold-change, $p = 0.012$; C5, $\sim$1.27 fold-change, $p = 0.087$), lipoprotein metabolism (APOD, $\sim$1.86-fold change, $p = 0.033$; APOC3, $\sim$2.63-fold change, $p = 0.029$) and platelet activation (GP1BA, $\sim$9.18-fold change, $p < 0.0001$; PPBP, $\sim$4.72-fold change, $p = 0.027$). With the application of antibody-based assays, we successfully validated this biomarker panel by confirming the MI modulation of C1QA ($p = 0.005$); C5 ($p = 0.0047$), APOD ($p = 0.0267$); APOC3 ($p = 0.0064$); GP1BA ($p = 0.0031$) and PPBP ($p = 0.0465$), in a separate cohort of 43 individual angina patients. Together, these data underscore the potential of EV-based diagnostic and therapeutic strategies to exert a substantial impact on the clinical care of patients with CAD.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless stated otherwise. Water and acetonitrile (ACN) were HPLC grade (Thermo Scientific, Waltham, MA). All buffers used in proteomic sample preparation were supplemented with protease inhibitors (1:50 v/v) and phosphatase inhibitors (1:10 v/v) (Roche Diagnostics, Mannheim, DE).

Patient Recruitment—This study was approved by the National Healthcare Group Domain Specific Review Board of Singapore (NHG-DSRB). All participants provided written informed consent prior to inclusion. Patients with coronary artery disease (CAD; $n = 35$) were undergoing coronary artery bypass graft surgery (CABG) and exhibited either unstable disease (MI, myocardial infarction within the preceding 5 days; $n = 15$), or stable angina pectoris for ≥ 1 month (NMI; $n = 20$). CAD was confirmed by coronary angiogram and myocardial infarction was confirmed by ECG and cardiac troponin test (cTn). Patient demographics and clinical history are displayed in Table I.

Plasma Sampling and EV Isolation—Before commencing CABG, patient blood samples were collected into lithium-heparin vacutainers and processed immediately. Plasma aliquots were cryopreserved at -80 °C until proteomic processing. In order to normalize biological variation, plasma from multiple individuals within each patient group were combined in equal proportions to obtain a total sample volume of 5 ml for analysis. Two biological replicates were performed in this study. Enrichment of plasma extracellular vesicles was performed accordingly to a previously described method (34), except for minor modifications. Briefly, pooled plasma samples were diluted to 30 ml with cold 1$\times$ phosphate buffer saline (PBS) and differentially centrifuged at 200 $\times$ g (30 min), 2000 $\times$ g (30 min), and 12,000 $\times$ g (60 min) to remove intact cells and cellular debris. Plasma EVs were then pelleted by ultracentrifugation at 200,000 $\times$ g (18 h) using a Beckman L100-XP Ultracentrifuge (Beckman Coulter, Brea, CA). The EV-en-

TABLE I
Clinical-demographic characteristics of recruited patients

	Myocardial infarction (MI) (n = 15)	Stable angina (NMI) (n = 20)
Age [Mean ± SD)]	63 (± 11)	62 (± 7)
Female [n (%)]	5 (33.33)	5 (25)
Male [n (%)]	10 (66.67)	15 (75)
Chinese [n (%)]	8 (53.33)	10 (50)
Malay [n (%)]	4 (26.67)	5 (25)
Indian [n (%)]	2 (13.33)	5 (25)
Others [n (%)]	1 (6.67)	0 (0)
Hypertension [n (%)]	14 (93.33)	19 (95)
Renal impairment [n (%)]	2 (13.33)	0 (0)
Hyperlipidemia [n (%)]	15 (100)	20 (100)
Diabetes Mellitus [n (%)]	9 (30)	14 (70)
Smoking [n (%)]	5 (33.33)	10 (50)

n, number of individuals; SD, standard deviation.

riched pellets were washed in 1× PBS and pelleted again at 200,000 × *g* (18 h) to remove contaminants.

Western Blotting—EV proteins (50 μg) were mixed with 4× laemmli protein sample buffer (BioRad, Hercules, CA), separated on 12% polyacrylamide gels, and transferred onto 0.45 μM nitrocellulose membranes (BioRad, Hercules, CA). Nitrocellulose membranes were blocked and probed overnight at 4 °C with the following primary antibodies: anti-CD9 (1:1000; sc-13118; Santa Cruz Biotechnology) and anti-CD81 (1:1000; sc-9158; Santa Cruz Biotechnology), followed by incubation with horseradish-peroxidase-conjugated secondary antibody (1:4000, sc-2371, sc-2370; Santa Cruz Biotechnology). Proteins were detected by enhanced chemiluminescence assay (Millipore Corporation, Billerica, MA).

Cryo-electron Microscopy (Cryo-EM)—Concentrated suspensions of EVs (4 μl total volume) were applied onto holey carbon filmed (R2/2 Quantifoil) EM grids at 99% humidity. Filter paper was used to blot excess sample from the grids, which were then rapidly plunged into liquid ethane (Vitrobot, FEI Company, Hillsboro, OR). Cryo grids were examined using a field emission gun transmission electron microscope operated at 80 kV (Arctica, FEI Company) and equipped with a direct electron detector (Falcon II, FEI Company).

In-solution Tryptic Digestion and Peptide Fractionation—Proteomic sample preparation was conducted according to a previously described method that minimizes experimentally induced deamidation (35, 36), with some minor modifications. Briefly, the EV-enriched pellet was solubilized in 8 M urea and 50 mM ammonium acetate (pH 6.0) lysis buffer. Plasma protein concentration was determined using the bicinchoninic acid (BCA) assay according to the manufacturer's instructions. Approximately 200 μg of proteins were reduced using 20 mM dithiothreitol (DTT) for 3 h at 30 °C before being alkylated in the dark with 55 mM iodoacetamide (IAA) for 1h at room temperature. The concentration of urea was diluted to less than 1 M using 50 mM ammonium acetate buffer (pH 6.0) prior to tryptic digestion in order to ensure adequate enzyme activity. Proteins were enzymatically digested overnight at 37 °C using sequencing-grade trypsin (1:100 w/w, trypsin to protein; Promega, Madison, WI). The tryptic peptides were then desalted using a Sep-Pak C18 cartridge (Waters, Milford, MA) and dried in a vacuum concentrator. Vacuum-dried desalted peptides were reconstituted in 200 μl mobile phase A (90% ACN containing 0.1% HAc) and fractionated using a PolyWAX LP weak anion-exchange column (4.6 × 200 mm, 5 μm, 300Å; PolyLC, Columbia, MD) on a Prominence UFLC system (Shimadzu, Kyoto, Japan). The UV spectra of the peptides were collected at 280 nm. Mobile phase A and mobile phase B (30% ACN, 0.1% FA) were used to establish the 60 min gradient; starting with 3 min of 100% A, 17 min of 0–8% B, 25 min of 8–45% B, 10 min of 45–100% B, followed by

5 min at 100% B (constant flow rate 1 ml/min). Forty separate fractions were collected, combined into 15 pooled fractions, and then vacuum-dried. Dried peptides were reconstituted in 3% ACN, 0.1% FA for LC-MS/MS analysis.

LC-MS/MS—The fractionated peptides were separated and analyzed on a LC-MS/MS system equipped with an Ultimate 3000 RSLC nano-HPLC system (Dionex, Amsterdam, NL) and coupled to an online LTQ-FT Ultra linear ion trap mass spectrometer (Thermo Scientific Inc., Bremen, Germany). Approximately 2 μg of peptides from each fraction were injected via the Dionex autosampler, concentrated into a Zorbax peptide trap column (Agilent Technologies, Santa Clara, CA), and subsequently separated in a capillary column (75 μm × 10 cm) packed with C18 AQ (5 μm, 300Å; Bruker Michrom Billerica, MA) at a flow rate of 300 nL/min. Mobile phase A (0.1% FA in HPLC water) and mobile phase B (0.1% FA in ACN) were used to establish the 60 min gradient; starting with 1 min of 5–8% B, 44 min of 8–32% B, 7 min of 32–55% B, 1 min of 55–90% B and 2 min of 90% B, followed by re-equilibration in 5% B for 5 min. The samples were ionized using an electrospray potential of 1.5 kV in an ADVANCE™ CaptiveSpray™ Source (Bruker-Michrom). The LTQ-FT Ultra was set to perform data acquisition in the positive ion mode. A full MS scan (350–1600 *m/z* range) was acquired in the FT-ICR cell at a resolution of 100,000 and a maximum ion accumulation time of 1000 ms. The automatic gain control (AGC) target for FT was set at 1×10^6, and precursor ion charge state screening was activated. The linear ion trap was used to collect peptides and measure the fragments generated by collision induced dissociation (CID). The default AGC setting was used in the linear ion trap (full MS target 3.0×10^4, MSn 1×10^4). The 10 most intense ions above a 500 count threshold were selected for MS^2 fragmentation by CAD, which was performed concurrently with a maximum ion accumulation time of 200 ms. Dynamic exclusion was activated for this process (60 s exclusion duration, repeat count 1). For CID, the activation Q was set at 0.25, activation time was 30 ms, isolation width (*m/z*) was 2.0, and normalized collision energy was 35%.

Mass Spectrometric Data Analysis—MS data analysis was performed using Thermo Scientific™ Proteome Discoverer™ (PD) 1.4 software, connected to an in-house Mascot server (V 2.4.1, Matrix Science, Boston, MA). Protein identification was performed by querying the MS/MS spectra against the Uniprot Human database (Released on 11/29/2013, 88, 421 sequences, 35,070,517 residues). An automatic target-decoy search strategy was used in combination with Percolator to score peptide spectral matches for estimation of false discovery rate (FDR). Only peptides identified with strict spectral FDR of < 1% (q-value < 0.01) were considered in this study. The search was restricted to a maximum of 2 missed trypsin cleavages; peptide precursor mass tolerances of 10 ppm; and 0.8 Da mass tolerance for fragment ions. Carbamidomethylation (+57.021 Da) of cysteine residues was fixed as a static peptide modification, oxidation (+15.995 Da) of methionine residues and deamidation (+0.984 Da) of asparagine and glutamine residues were set as dynamic peptide modifications. Calculation of area under the curve (AUC) for each precursor ion peak was conducted using the event detector and precursor ion area detector algorithm (embedded in PD 1.4) with a mass precision setting of 2 ppm. To maximize accuracy, protein and peptide relative quantities were calculated based on the average area of the three most abundant unique peptides per protein. Searched results were exported to a tab-delimited file for further analyses. The reported MI/NMI ratio for each protein was computed by dividing the MI protein area by the NMI protein area (both calculated in PD). Modulated protein expression was defined as ≥ 1.2-fold change in expression level (cut-off was set and a fold change of 1.2 and 0.83 indicates up-regulated and down-regulated proteins respectively). Relative expression ratios for proteins of interest were extracted and *p* values

were determined using unpaired Student's t-tests ($p < 0.05$ was considered significant). The standalone open access tool FunRich V2.1.2 (37) was used to assess the functional enrichment and to determine the network interactions in the plasma EV proteome. The mass spectrometry proteomics data have been deposited to the Proteome Xchange Consortium via the PRIDE partner repository (38) with the data set identifier PXD002950 and the following submission details.

ProteomeXchange title: Plasma-derived extracellular vesicles contain predictive biomarkers and potential therapeutic targets for myocardial ischemic injury

ProteomeXchange accession: PXD002950

PubMed ID: 27234505

Publication DOI: Not applicable

Project Webpage: http://www.ebi.ac.uk/pride/archive/projects/PXD002950

FTP Download: ftp://ftp.pride.ebi.ac.uk/pride/data/archive/2016/05/PXD002950

Antibody-based Assay—Plasma EVs from 26 NMI patients and 17 MI patients were individually isolated using ExoQuick™ (System Biosciences, Mountain View, CA) as per manufacturer's protocol, but with minor modifications. Briefly, 250 μl plasma was mixed with 63 μl ExoQuick™ solution and incubated overnight at 4 °C. The recovered pellet containing EVs was lysed in complete lysis-M buffer (Roche Diagnostics, Mannheim, DE). Plasma EV levels of C5 and C1Q were measured using a single-plex bead assay (Luminex Corporation, Austin, TX) according to the manufacturer's instructions. The resulting Luminex raw data, reported as fluorescent intensity, were acquired using Bio-Plex Manager™ software (BioRad, Richmond, CA) to obtain protein concentration values. Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the concentration of EV-derived APOD (CSB-EL001935HU, Cusabio Biotech, Wuhan, CN); GP1BA (CSB-EL12101h, Cusabio Biotech, Wuhan, CN); APOC3 (EHAPOC3, Thermo Scientific, Frederick, MD) and PPBP (EHPPBP, Thermo Scientific, Frederick, MD) in plasma, as accordingly to the respective manufacturer's instructions.

Experimental Design and Statistical Rationale—In the discovery phase, sample pooling design was used to reduce between-patient biological variation. Two biological replicates were performed to increase the reliability and achieve adequate predictive power for global label-free quantitative proteomics profiling. Scatter dot plots were generated and analyzed using GraphPad Prism V 6.0 (Graphpad Software, San Diego, CA). Variance was assessed by F test. Statistically significant differences between numeric variables between MI-cases and NMI-cases were determined using parametric analyses and unpaired student's *t*-tests with Welch's correction, to minimize type I statistical error in unequal sample sizes. *p values* < 0.05 were considered as statistically significant, and data are presented as mean ± standard deviation (S.D.), to show the variation among the duplicate experiments. In the validation phase, EVs were individually isolated from an independent cohort of 43 angina patients, to cross-validate the expression profiles of targeted biomarkers with the discovery EV proteomics data set, and to value-add the predictive value and robustness of the proposed multimarker panel in this study.

RESULTS AND DISCUSSION

Analysis of EVs in Human Plasma by Quantitative Label-free Proteomics—EVs released by host cells into the circulation have potential to inform clinicians about virtually any pathophysiological process that occurs in the human body (39, 40), and the relative ease with which these structures can be sampled from peripheral blood renders them highly attractive targets for prognostic testing. To obtain EVs from human
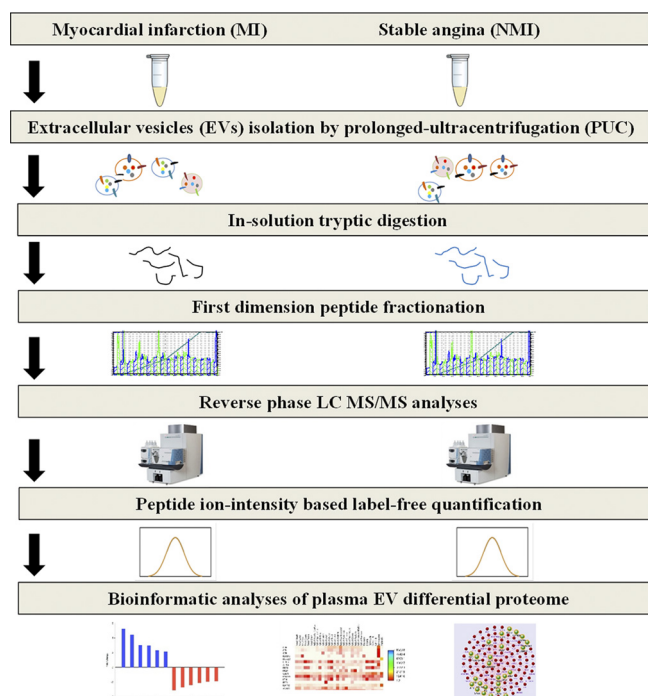

FIG. 1. **Schematic workflow showing the label-free quantitative proteomic analysis of extracellular vesicles (EVs) isolated from the pooled plasma of patients with stable angina (NMI) or myocardial infarction (MI).**

blood samples, we employed a prolonged ultracentrifugation (PUC) (34) approach previously reported to effectively simplify plasma complexity prior to EV isolation (workflow illustrated in Fig. 1). To limit the influence of sample variability on our analyses, we performed biological duplicate experiments in this study. Briefly, EVs harvested from the plasma of patients with known medical conditions (either MI or NMI) were enzymatically digested into peptides using trypsin, followed by first dimensional fractionation using weak anion-exchange chromatography. Finally, the fractionated EV peptides were analyzed by reverse phase LC-MS/MS, and EV proteins that were differentially expressed between patient groups were determined based on ion intensity/AUC label-free quantification.

Verification of Plasma EV Isolation—The presence of EVs in patient plasma-derived vesicle preparations was confirmed via the routinely used methods of electron microscopy and immunoblotting. Electron micrographs of harvested EVs (Figs. 2A and 2B) revealed circular membrane-bound structures with sizes varying from ~50 to 100 nm in diameter. Western blot analyses of the harvested EVs detected the commonly reported exosomal tetraspanin markers CD9 and CD81 (41) in both biological replicates for both patient groups (MI and NMI patients; Fig. 2C). Further verification of successful EV isolation was performed via proteomic detection of exosome-specific surface markers including CD9 and ALIX/PDCD6IP. Thus, the efficient isolation of plasma EVs by PUC separation was verified using multiple different techniques, and the har-
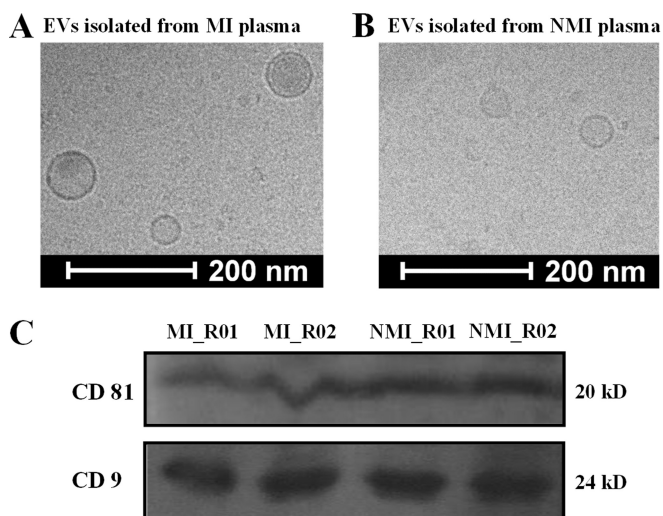
FIG. 2. **Verification of the identity of extracellular vesicles (EVs; size 50–100 nm) isolated from human plasma.** *A* and *B*, Electron microscopy images of EVs harvested from the pooled plasma of patients with myocardial infarction (MI; *A*) or stable angina (NMI; *B*). Immunoblot analyses confirmed the presence of EV markers CD9 (24 kDa) and CD81 (20 kDa) among the harvested proteins (*C*). MI_R01 and MI_R02; NMI_R01 and NMI_R02 are biological replicates.

vested EVs exhibited morphology, characteristics and proteomic characteristics consistent with that of exosomes.

Plasma EV Protein Identification and Determination of Relative Abundance—By searching the combined LC-MS/MS database for both the MI and NMI patient groups, we achieved high-confidence identification of a total 165,223 peptide-spectrum matches (PSMs), corresponding to 4363 unique peptides and 573 EV proteins (FDR < 1%; q-value < 0.01). The detailed PD1.4 searched information are available in supplemental Data S1 (worksheets CAD2DPro and CAD2DPep). We identified 422 proteins in plasma EVs from patients with myocardial infarction (MI) and 453 proteins in patients with stable angina (NMI), with 302 of the total 573 unique proteins identified (~53%) being common to both patient groups.

Label-free peptide ion-intensity/AUC based quantification has been shown to correlate well with protein abundance in complex samples (42). In this study, reproducibility was evaluated using the LC-MS/MS data sets obtained from two independent biological replicates performed in parallel for each patient group. Using a strict FDR < 1%, the number of proteins, peptides and PSMs identified in each biological replicate run were plotted against the calculated mean and S.D. for repeatability assessment (Fig. 3*A*). The detailed PD1.4 searched information are available in supplemental Data S2 (worksheet BioRepPro and BioRepPep). The corresponding percentage coefficients of variation (%CV) in the number of proteins, peptides and PSMs identified between biological replicates were low for both patient groups (2.35%, 7.13% and 5.95% respectively for NMI; 0.00%, 6.84% and 6.60% for MI). The statistical analyses were performed in GraphPad

Prism V6.0 and are shown in supplemental Data S2 (worksheet BioRepStat). The low variability observed in each patient group indicated that this study achieved high levels of repeatability at the levels of protein, peptide, and spectrum.

We next compared protein relative abundance between biological replicates in each patient group in order to assess the efficacy of our standardized protocol for sample preparation and instrument optimization. Pearson's correlation coefficient (r) confirmed strong concordance of protein ion intensities (scatter $\log_{10}$ values) between biological replicates in both the MI group ($r = 0.8348$, $p < 0.0001$) and NMI group ($r = 0.9096$, $p < 0.0001$; Fig. 3*B* and 3*C*), indicating that our label-free quantitative data sets were highly reproducible (details in supplemental Data S2; worksheet BioRepStat). Taken together with the highly consistent number of protein/peptide/PSM identifications, this strong correlation between extracted ion intensities indicated that we achieved reliable relative quantitation of plasma EV proteins across the biological replicates of independent LC-MS/MS runs for both patient groups.

In order to assess changes in the plasma EV proteome in both stable angina (NMI) and myocardial infarction (MI), the average area of the three most abundant unique peptides per protein were used to accurately determine protein relative abundance in each patient group. The differential proteome data set was generated by dividing the AUC values of the MI group by the AUC values of the NMI group (MI/NMI ratio) and applying a 1.2-fold change cut-off to identify altered protein expression. As a result, 125 proteins with ratios ≥ 1.2 were classified as being up-regulated in MI, whereas 127 proteins with ratios ≤ 0.83 (down-regulated by 1.2 fold) were classified as down-regulated in MI. The detailed quantification data are available in supplemental Data S1 (worksheet CAD2DReg). These data suggest that MI modulates multiple biological processes that can be successfully probed via proteomic analysis of EVs isolated from human blood plasma.

Expression Profiling of the Plasma EV Proteome in Patients with Myocardial Infarction—Using the combined search results detailed above, we next assessed whether EV protein expression levels could be used to elucidate the pathophysiological processes that determine the clinical course and severity of myocardial ischemic injury. Gene ontology (GO)-based functional enrichment and network analyses were performed using all EV proteins previously identified as being modulated in MI. The list of up-regulated EV proteins was compared with the list of down-regulated EV proteins to determine enrichment for specific molecular functions (MF) and biological processes (BPs) in each patient group (ranked by the Benjamini-Hochberg adjusted p value; $p < 0.05$ indicates high enrichment), followed by interrogation using FunRich V 2.1.2 to identify the cellular pathways that were most perturbed in MI (details provided in supplemental Data S3; worksheets GO and Path).
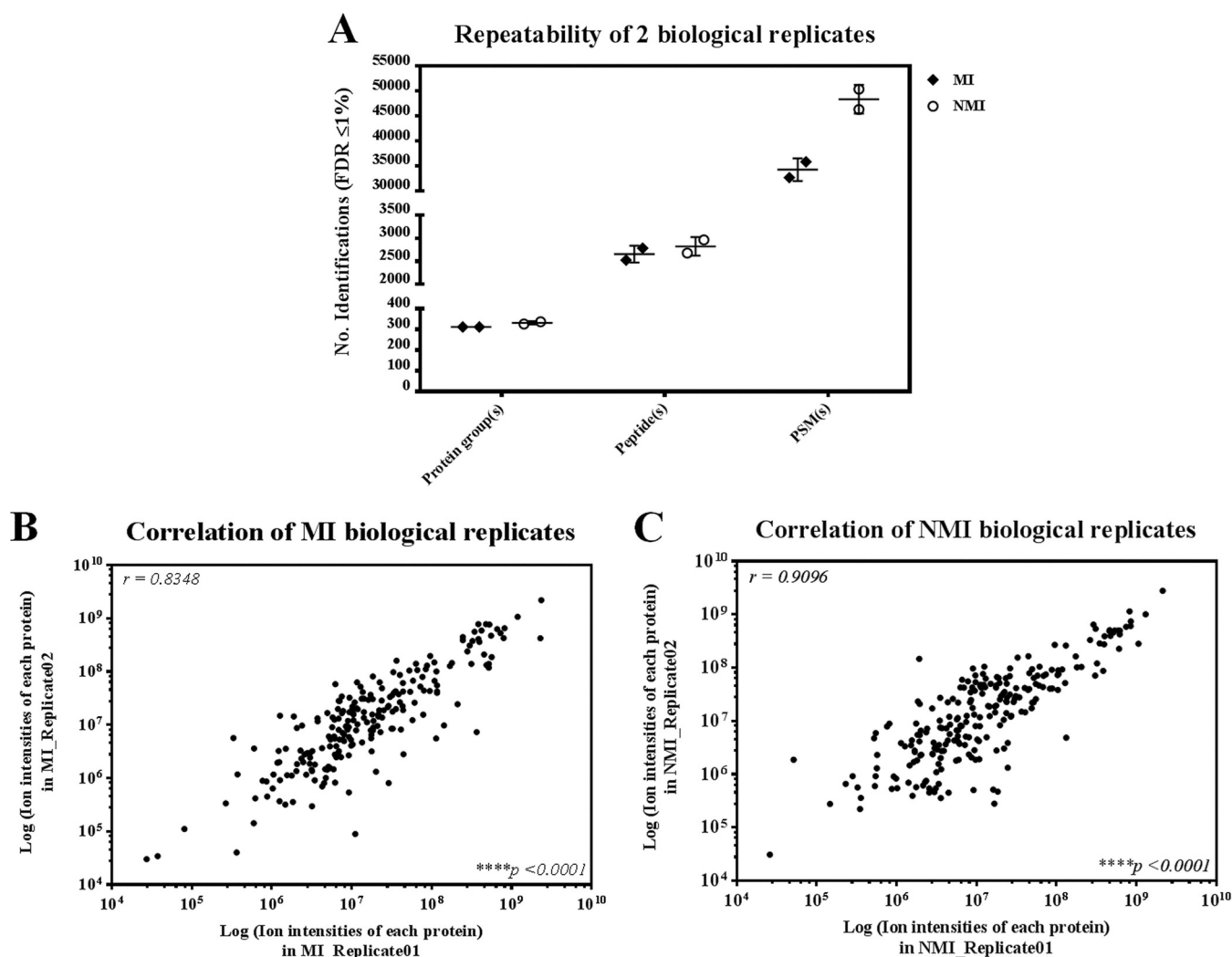
FIG. 3. **Repeatability and reproducibility assessment of two independent biological replicates in patients with myocardial infarction (MI) or stable angina (NMI).** *A*, Linear scatter plot of the number of protein group/s, peptide/s and PSM/s identified with FDR < 1% in each biological replicate in each patient group. Dots were plotted with mean and S.D. *B*, *C*, Scatter plots showing protein ion intensities and plotting biological replicates on opposing axes to determine the extent of correlation between MI replicates (*B*) and NMI replicates (*C*). r, pearson correlation coefficient.

EV proteins that were differentially expressed after MI were highly enriched in putative molecular functions (MF) associated with complement activities (~26%) and transporter activities (~24%) (Fig. 4*A*). Down-regulated proteins were instead enriched in mediators of protease inhibitor activities (~11%) and serine-type peptidase activities (~9%). Proteins associated with cytoskeletal binding were up-regulated, whereas mediators of host defense mechanisms appeared largely down-regulated. GO-annotated mediators of the immune response (~43.8%) and protein metabolism (34.5%) constituted the most prominently modulated categories according to BP classification (Fig. 4*B*). Cellular communication (~ 13.8%) and transport processes (~ 14.9%) were overrepresented in the up-regulated proteins. Intriguingly, we observed that processes involving energy expenditure such as

protein metabolism, cell growth and/or maintenance were more likely to be down-regulated after MI.

Together, these data indicated that the host functions most strongly modulated in MI were the complement cascade and innate immune response, which represent primary modes of host defense against infection. EVs produced by both immune cells and nonimmune cell types are thought to play important roles in complement immunoregulation (43, 44). It has been previously shown that that EVs produced by polymorphonuclear neutrophils (PMNs) down-regulates the inflammatory activity of macrophages (45). In addition, reports have indicated that EVs secreted from endothelial cells and monocytes elicits and enhances thrombosis (46, 47). Complement activation can be mediated via the classical pathway (CP), lectin pathway (LP), or alternative pathway (AP) (48), and has been
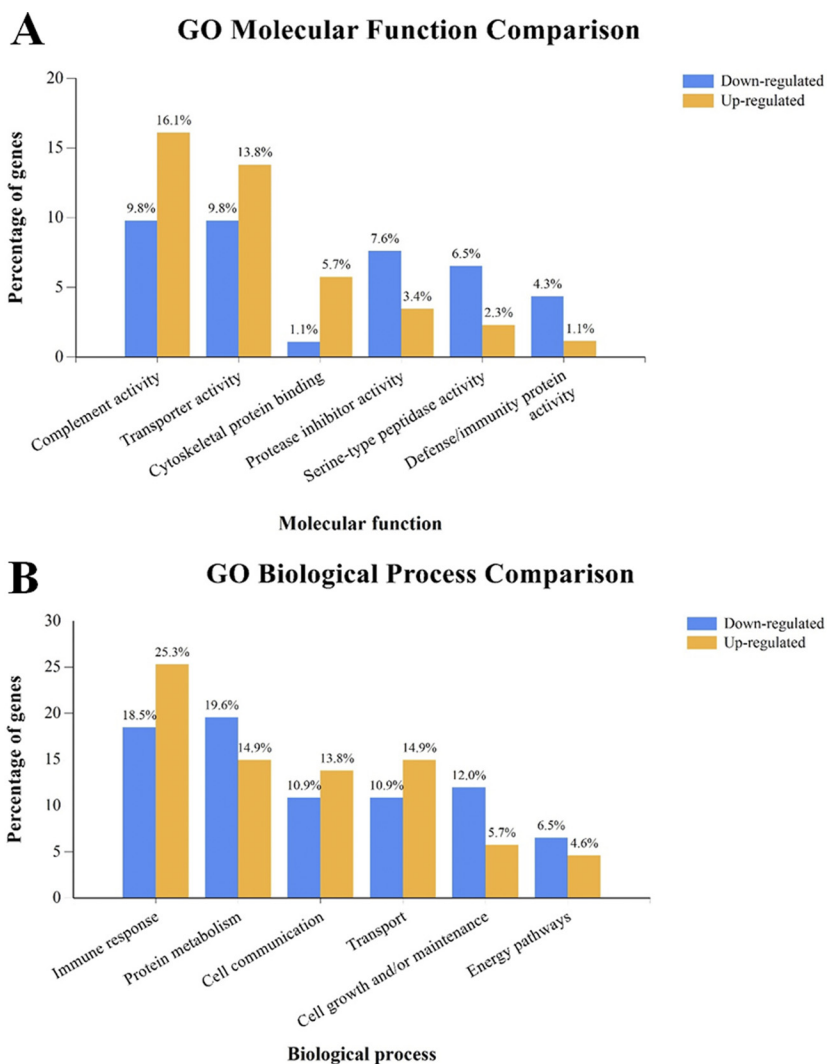
FIG. 4. **Gene ontology (GO)-based functional enrichment analysis of modulated extracellular vesicle (EV) proteins.** *A* and *B*, Distribution of modulated EV proteins according to associated molecular functions (*A*) and biological processes (*B*). Predictions were generated in FunRich V2.1.2.

strongly implicated in post-infarct inflammation, tissue damage, and healing (8, 49). Under physiological conditions, activation of the complement cascade is controlled by soluble and cell-bound complement regulatory proteins (50), and in the current study we detected that MI samples were enriched in the AP inhibitory complement factors H and I (~1.44- and ~1.27-fold change respectively), but depleted of the AP positive regulatory complement factors B (~0.77-fold) and properdin (~0.18-fold). In addition, the LP-associated mannan-binding lectin serine protease 1 (MASP1) and mannan-binding lectin serine protease 2 (MASP2) were down-regulated (MASP1, ~0.369-fold; MASP2, ~0.378-fold) in post-infarct plasma EVs despite the simultaneous down-regulation of the major MASP2 inhibitor antithrombin-III (SERPINC1; ~0.67-fold) (51). Further evidence of LP suppression following MI was provided by the decreased detection of LP activators such as ficolin-2 (~0.36-fold change) and ficolin-3 (~0.52-fold

change). These data are consistent with recent reports that LP inhibition using inhibitors of MASP2 or mannan-binding lectin can confer protection against myocardial ischemic injury in mice/rats models of post-ischemic reperfusion injury (52, 53). Although the role of MASP2 in LP activation has been well documented, MASP1 function and therapeutic potential in myocardial ischemia remain largely unknown. Our data showing modulation of soluble AP/LP complement regulators may reflect efforts to limit the extent of inflammation in MI patients, and implicate the involvement of EVs in complement regulation following ischemic insult. Although we did also observe down-regulation of the complement inhibitory regulators such as clusterin (CLU) and C4b-binding protein alpha and beta chains (C4BPA and C4BPB).

In our differential proteomics data set, post-infarct plasma EVs exhibited up-regulation of a repertoire of complement components (C1QA, C1QB, C1QC, C1R, C2, C3, C5, C6,

C8A, C8B, C8G, C9, CFH, and CFI) when compared with plasma EVs from patients with stable angina. In particular, the complement subunit C1QA that initiates the classical pathway displayed the highest fold change detected by our analyses (~3.23-fold). Similarly, we observed a 2.69-fold increase in levels of C-reactive protein (CRP), an antibody-independent C1q recognition molecule (54), which is routinely used as a diagnostic marker of inflammation in clinical settings. In addition to its known role in complement activation, CRP levels in serum have been shown to correlate with disease severity and can predict adverse outcomes in CAD (55, 56). In our differential proteomic data set, post-infarct EVs also displayed ~1.27-fold up-regulation of the complement component C5, which can be cleaved into anaphylatoxin C5a and fragment C5b to triggers pro-inflammatory signaling events and initiate assembly of the lytic membrane attack complex. Increased levels of C5a have shown to promote pro-inflammatory activities leading to local tissue damage secondary to ischemia and reperfusion (50, 57, 58). C5a mediates a broad range of biological activities such as promoting PMN chemotaxis and activation, mast cells degranulation, smooth muscle cell contraction and increased vascular permeability (59, 60). The administration of C5a into coronary arteries of healthy pigs have produced an up to 40% reduced in regional coronary blood flow and decreased in local myocardial function, attributed by the aggregation of circulatory granulocytes in the microcirculation (61). Inhibition of complement with novel drugs such as the anti-C5 antibody Pexelizumab have yielded promising results in animal models of cardiovascular disease (6–9), but so far this promise has yet to be translated into successful clinical applications for human patients with MI. In our study, the massive up-regulation of complement proteins detected in post-infarct EVs, particularly the CP activation initiator C1QA and downstream terminal complement effector C5, strongly suggested a pathological role for complement activation in the development of post-infarct inflammation. Intriguingly, our data further suggest that EVs can play conflicting roles in complement activation and suppression after myocardial injury. Although up-regulation of CP activators appears to be intricately involved in the development of post-infarct inflammation, plasma EVs also contained elevated levels of AP and LP regulators, suggesting complex effects of MI on complement function.

MF-transporter activity and BP-transport processes that were closely related to metabolism of lipids and lipoproteins pathway and chylomicron-mediated lipid transport were over-represented among the pathways associated with the MI plasma EV proteome. It is well established that CAD risk is increased by high levels of circulating low density lipoproteins (LDLs) and very low density lipoproteins (vLDLs), alongside with low levels of high density lipoproteins (HDLs) (62). Accordingly, we observed marked depletion of anti-atherogenic HDLs, including ~0.47-fold change in apolipoprotein A-II (APOA2) and ~0.41-fold change in apolipoprotein A-VI

(APOA4) (63). As might be predicted from the pro-atherogenic role of LDLs in CAD (64), we also detected up-regulation of apolipoprotein B (APOB-100), apolipoprotein C-III (APOC3), apolipoprotein E (APOE), and apolipoprotein (a) (LPA). The major pro-atherogenic protein component APOB displayed ~1.24-fold change, but the most pronounced change observed among LDLs was ~2.63-fold change in APOC3, which increases plasma triglyceride (TG) levels by inhibiting lipoprotein lipase and blocking hepatic uptake (65). Overexpression of APOC3 is indicative of hypertriglyceridemia, and high plasma TG levels are associated with increased risk of CAD, hence our data are consistent with previous reports that loss of APOC3 confers atheroprotection and reduces risk of adverse cardiovascular events (66).

Although established CAD is typically associated with low levels of HDL in the circulation, we observed a paradoxical up-regulation of the HDL species apoliporotein D (APOD; ~1.86-fold change) in MI patients. APOD is ubiquitously present in mammalian tissues, but is most highly expressed in the spleen, testes and brain, where it mediates transport of lipids and other small hydrophobic ligands for cell metabolism, maintenance, and tissue repair (67–69). Elevated levels of APOD expression have previously been linked with neurodegenerative diseases, as well as various cancers, psychiatric disorders, and physiological aging (70). Intriguingly, fruit flies transfected with human APOD exhibit increased life expectancy associated with enhanced anti-stress and anti-oxidation functions (71). Despite being a component of HDL, APOD has received considerably less attention in cardiovascular research because of its relative low abundance (68). However, an earlier report has shown that APOD was highly enriched in HDL isolated from CAD patients (72), consistent with our own findings in blood plasma EVs. Although APOD deposition has previously been identified in atherosclerotic plaques (68), it remains unclear whether APOD contributes to plaque formation or instead protects against atherosclerosis. Thus, our observation that APOD levels are increased in post-infarct EVs could reflect either a pathological process or a compensatory response to dyslipidemia following myocardial injury. Imbalanced cholesterol metabolism leading to an altered ratio of LDLs to HDLs has already been shown to induce endothelial dysfunction and oxidative stress, which could potentially drive the development of post-infarct inflammation (73). However, the complex roles that different lipoproteins play in MI pathology remain only partially characterized, hence a better understanding of how APOD function influences CAD pathogenesis may lead to new diagnostic and therapeutic approaches for patients with myocardial injury.

Our analyses detected a number of functions that were enriched in the proteome of post-infarct EVs, including protein metabolism, protease inhibitor activity, cytoskeletal protein binding, and cell communication/growth/maintenance, as well as pathways of platelet degranulation, hemostasis, and

TABLE II
Candidate biomarkers of myocardial injury: known functions and potential clinical relevance in myocardial ischemia

Accession No.	Protein description	Proteomics fold change	P value	Mean AUC (MI)	Mean AUC (NMI)	SE of difference	Clinical relevance
P02656	Apolipoprotein C-III	2.633	0.029	1.013E+08	4.037E+07	1.819E+07	Promotes development of hyperglyceridemia; atherogenic
P05090	Apolipoprotein D	1.860	0.033	6.877E+06	3.397E+06	1.088E+06	Compensatory response to dyslipidemia after myocardial injury
P02745	Complement C1q subcomponent subunit A	3.229	0.012	1.710E+07	5.457E+06	2.637E+06	Promotes post-infarct inflammation; aggravates myocardial injury
P01031	Complement C5	1.269	0.087	2.307E+07	1.713E+07	2.625E+06	Promotes post-infarct inflammation; aggravates myocardial injury
P07359	Platelet glycoprotein Ib alpha chain	9.181	< 0.0001	6.050E+06	5.587E+05	1.003E+05	Possible indicator of plaque rupture and/or thrombosis
P02775	Platelet basic protein	4.725	0.027	5.680E+06	1.460E+06	1.240E+06	Immune activation; possible indicator of vascular inflammation

AUC; Area under curve; CAD, Coronary artery disease; LDL, Low-density lipoprotein, HDL, High-density lipoprotein; SE: Standard error; MI, Myocardial infarction; NMI, Stable angina. Parametric student's t test analyses were performed using ion intensities obtained from MIbiological replicate 01, MIbiological replicate 02, MIcombined replicates search, NMIbiological replicate 01, NMIbiological replicate 02 and NMIcombined replicates search ($p < 0.05$ was considered statistically significant).

blood clotting. In addition to their critical role in hemostasis and thrombosis, platelets can also release pro-inflammatory mediators and form platelet-leukocyte complexes that promote atherosclerosis (74). It has previously been shown that platelets are among the first populations of inflammatory cells to accumulate within the infarcted myocardium (75). Accordingly, in our MI plasma EVs we observed marked up-regulation of the major platelet integrin alpha-IIb (ITGA2B; ~1.41-fold change) and its interacting partners such as von Willebrand factor (VWF; ~3.29-fold change) and components of the VWF receptor complex (~9.18-fold increase in platelet glycoprotein Ib alpha chain (GP1BA); ~1.31-fold increase in platelet glycoprotein Ib beta chain (GP1BB); and ~1.22 increase in platelet glycoprotein IX (GP9)). Interaction between these pro-thrombotic mediators after vascular injury results in platelet adhesion and aggregation, leading to rapid hemostasis and eventual wound repair (76, 77). However, platelet activation under inappropriate conditions can also trigger pathological events such as arterial thrombosis, restenosis, and even MI. At the site of plaque rupture, GP1BA binding to VWF initiates platelet rolling contact with the exposed subendothelial collagen (76), hence our observation that GP1BA expression is increased in plasma EVs from MI patients could represent a clinical indicator of recent plaque rupture and thrombosis.

In addition to up-regulation of pro-thrombotic platelet proteins, post-infarct plasma EVs were also enriched in platelet-derived chemokines known to mediate the recruitment of circulating neutrophils and monocytes (74). We observed ~2.36-fold enrichment of platelet factor-4 (PF4) and ~4.72-fold enrichment of platelet basic protein (PPBP), which are both chemotactic and stimulatory for blood neutrophils (78). Up-regulation of PPBP in post-infarct plasma EVs may represent an important event in amplification of the immune response to myocardial injury. Levels of the acute-phase protein fibrinogen are typically raised in inflammatory and hypercoaguable states such as stroke and MI (79), but in our differential EV proteome data set, we observed that all components of fibrinogen were unexpectedly down-regulated (see for example ~0.28-fold change in plasma EV levels of fibrinogen alpha chain). To the best of our knowledge, no previous report has identified diminished levels of fibrinogen in MI-related disease. We therefore propose that the reduced levels of fibrinogen detected post-MI could reflect an EV-mediated compensatory response to hypercoaguability after myocardial injury.

Because CAD is a multifactorial disease, the clinical utility of any single biomarker is likely to be limited. Instead, the identification of a panel of markers that reflect the initiation, progression, and resolution of key disease-associated events would be likely to offer better prognostic value. By categorizing our patients into groups with MI and stable angina, we aimed to increase the probability of detecting stage-specific EV biomarkers that delineate atherosclerotic progression and plaque instability. Using this type of approach, it may be possible to determine which biomarkers can successfully stratify patients according to risk of adverse events, and to enable the timely administration of novel drugs that can prevent major cardiovascular complications. By capitalizing on existing knowledge of EV biology in MI, we have identified a panel of six biomarkers of myocardial injury with potential utility for clinical applications (Table II); these reflect post-infarct pathways of complement activation (C1QA and C5), lipoprotein metabolism (APOD and APOC3) and platelet activation (GP1BA and PPBP). Our findings demonstrate the potential benefits of comparative profiling of the perturbed EV proteome in patients with MI and stable angina, as presented here for the first time, and they indicate that EVs have a significant role to play in the progression of myocardial damage and eventual healing that can be successfully sampled and analyzed via the peripheral circulation.

Validation of Differential Expression—In effort to cross-validate the expression levels of EVs obtained from PUC, Exo-Quick™ (System Biosciences, Mountain View, CA), an alter-
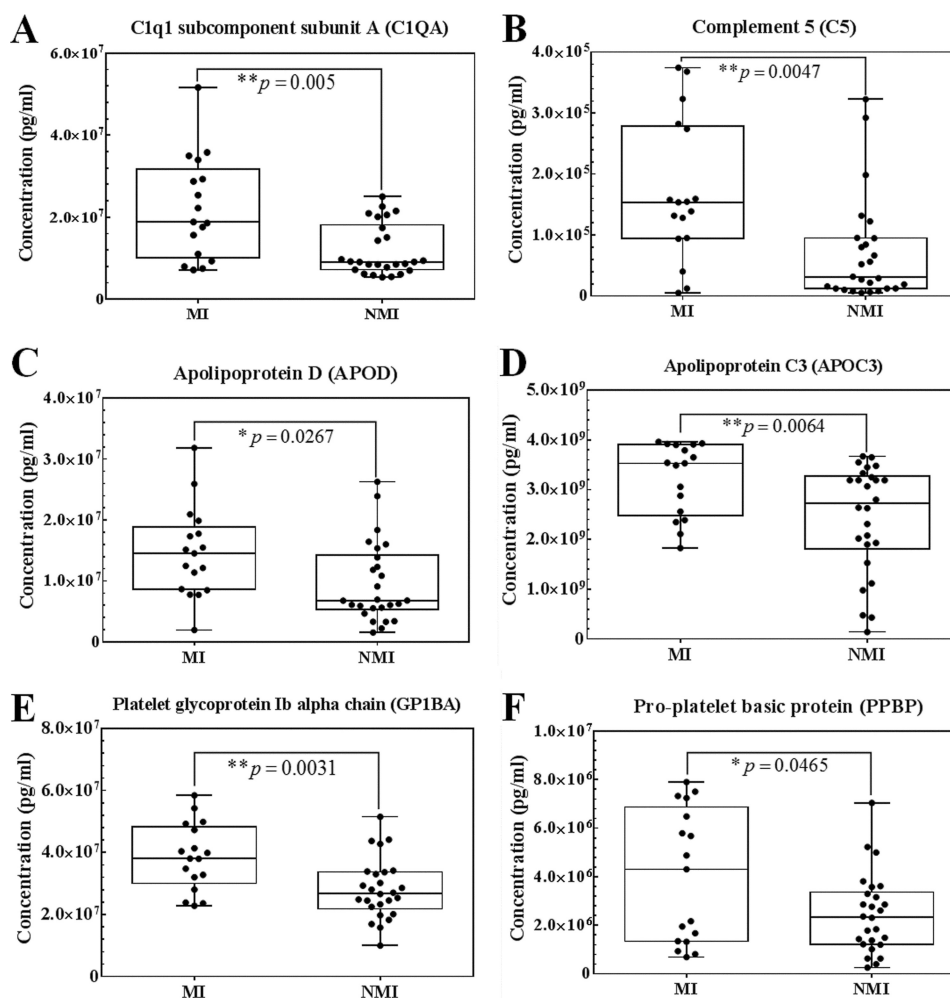
FIG. 5. **Expression levels of plasma extracellular vesicular (EV) proteins detected in 43 individual human plasma samples and displayed as linear box-and-whiskers plots (*n* = 17 patients with myocardial infarction (MI); *n* = 26 patients with stable angina (NMI)).** Line represents median, box represents 25th to 75th percentiles, whiskers represent range maximum and minimum, and dots represent individual values. *A*, Significant (*p* = 0.005) differential protein expression of complement C1q1 subcomponent subunit A (C1QA) measured by Luminex assay between MI and NMI patient groups. *B*, Significant (*p* = 0.0047) differential 34 protein expression of complement 5 (C5) measured by Luminex assay between MI and NMI patient groups. *C*, Significant (*p* = 0.0267) differential protein expression of apolipoprotein D (APOD) measured by enzyme-linked immunosorbent assay (ELISA) between MI and NMI patient groups. *D*, Significant (*p* = 0.0064) differential protein expression of apolipoprotein C3 (APOC3) measured by ELISA between MI and NMI patient groups. *E*, Significant (*p* = 0.0031) differential protein expression of platelet glycoprotein Ib alpha chain (GP1BA) measured by ELISA between MI and NMI patient groups. *F*, Significant (*p* = 0.0465) differential expression data of pro-platelet basic protein (PPBP) measured by ELISA between MI and NMI patient groups. *, significant; **, very significant.

native method for EV precipitation, (80, 81), was used to isolate plasma EVs from 17 MI patients and 26 NMI patients. In order to ensure the accuracy of label-free quantitation from the discovery study, established luminex assay and ELISA was used to validate the expression levels of the prospective candidate complement proteins (C1QA and C5), lipoproteins (APOD and APOC3) and platelet-associated proteins (GP1BA and PPBP), in 43 individual patient plasma samples (detailed concentration readings available in supplemental Data S4; worksheet LumCon). The median expression data are presented as a linear box-and-whiskers plot (Fig. 5) and tabular results of the corresponding statistical analyses are provided

in supplemental Data S4 (worksheet LumStat). With reference from Fig. 5, plasma EV levels of C1QA (Fig. 5*A*; $2.210E^{+07} \pm 1.246E^{+07}$ pg/ml in MI; $1.189E^{+07} \pm 6.256E^{+06}$ pg/ml in NMI; p = 0.005), C5 (Fig. 5*B*; $1.702E^{+05} \pm 1.154E^{+05}$ pg/ml in MI; $6.994E^{+04} \pm 8.498E^{+04}$ pg/ml in NMI; p = 0.0047), APOD (Fig. 5*C*; $1.468E^{+07} \pm 7.328E^{+06}$ pg/ml in MI; $9.572E^{+06} \pm 6.572E^{+06}$ pg/ml in NMI; p = 0.0267), APOC3 (Fig. 5*D*; $3.224E^{+09} \pm 7.266E^{+08}$ pg/ml in MI; $2.431E^{+09} \pm 1.083E^{+09}$ pg/ml in NMI; p = 0.0064), GP1BA (Fig. 5*E*; $3.852E^{+07} \pm 1.079E^{+07}$ pg/ml in MI; $2.819E^{+07} \pm 9.653E^{+06}$ pg/ml in NMI; p = 0.0031) and PPBP (Fig. 5*F*; $4.002E^{+06} \pm 2.737E^{+06}$ pg/ml in MI; $2.455E^{+06} \pm 1.626E^{+06}$ pg/ml in NMI; p = 0.0465) were

all observed to be significantly elevated in post-MI patients than in NMI patients, in accordance with label-free quantified expression. Collectively, the validation by antibody-based assay confirmed that EV proteins C1QA, C5, APOD, APOC3, GP1BA, and PPBP expression levels were altered following MI, with expressions levels up-regulated in MI patients, thus confirmed the confidence of the reported label-free quantitative proteomics data. In addition, the generalization of the independent quantitative data set obtained from an alternate EV isolation method into our relative EV proteomics data set obtained from PUC further strengthen the robustness of our label-free expression profiling approach.

CONCLUSIONS

As the global population continues to age, the total burden of MI-related morbidity and mortality is predicted to increase, hence there is an urgent need for efficacious clinical markers that can inform patient care and uncover novel therapies that can minimize myocardial tissue injury. EVs are attractive targets in both respects because they can be sampled from blood plasma in a minimally invasive manner and provide useful information as to the status of the damaged heart muscle, which is essential for the development of new approaches in theranostics. Despite this significant untapped potential, proteomic analyses of circulatory EVs in the context of MI-related disease are extremely limited, and it has yet to be determined whether EVs play a significant role in human myocardial ischemic injury. Herein, we have performed a global ion intensity label-free differential proteome analyses of plasma EVs obtained from MI patients and from NMI patient as control. The confidence and reliability of our quantitative data set was demonstrated by the consistency in the number of identification (FDR <1%) on protein, peptide and spectral levels and by good correlation of extracted ion intensities between biological replicates. We presented, for the first time, the comparative proteomics profiling on 252 perturbed EV proteins with 1.2-fold change cut-off in clinical samples, and showed the possible involvement of EVs mediation in myocardial damage and myocardial healing processes following MI. We have identified a panel of six novel EV markers of myocardial injury from three pathways, namely complement activation (C1Q1A, ~3.23-fold-change, $p = 0.012$; C5, ~1.27-fold-change, $p = 0.087$), lipid metabolism (APOD, ~1.86-fold change, $p = 0.033$; APOC3, ~2.63-fold change, $p = 0.029$), and platelet activation (GP1BA, ~9.18-fold change, $p < 0.0001$; PPBP, ~4.72-fold change, $p = 0.027$) that showed significant association with post-infarction response. Subsequent antibody-based validation of C1QA ($p = 0.005$); C5 ($p = 0.0047$), APOD ($p = 0.0267$); APOC3 ($p = 0.0064$); GP1BA ($p = 0.0031$) and PPBP ($p = 0.0465$) performed in an independent cohort of 43 individual patients, were aligned with the discovery proteomics data set. Larger studies will now be required to assess whether the novel biomarkers we identified can be used to inform clinical deci-

sion making in a wider range of CAD phenotypes in human patients. Our findings have provided new insights into EV involvement after MI, the knowledge gained should encourage further studies that drives development of efficacious EV-based diagnostic and therapeutic strategies, benefiting patients with MI, improving their survival and quality of life.

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