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Aortic Wall Extracellular Matrix Proteins Correlate with Syntax Score in Patients Undergoing Coronary Artery Bypass Surgery

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Supplementary Material

Liquid Chromatography Mass Spectrometry (LC-MS/MS)

Protein digestion, peptide extraction, and peptide labeling 50 µL of plasma were pooled from each disease group. Plasmas from the same groups were pooled to reduce individual biological variations and to increase the analytical throughput. Plasma proteins were precipitated using 80% acetone and reconstituted in 8 M urea, 200 mM Tris-HCl pH 6.8. Protein quantification was performed using bicinchoninic acid (BCA) (Sigma-Aldrich, St Louis, MO) assay. Approximately 200 µg of proteins from each group were reduced with 5 mM tris-(2-carboxyethyl) phosphine (TECP) and alkylated with 10 mM methyl methanethiosulfonate (MMTS) for subsequent iTRAQ experiment. Digestion was carried out with sequencing grade modified trypsin (Promega Corporation, Madison, WI) at a 1:100 (w/w, trypsin to protein) ratio. The tryptic peptides were desalted using a Sep-Pak C18 cartridge (Waters, Milford, MA) and dried in a vacuum concentrator (Thermo Electron, Waltham, MA). The dried peptides were labeled with the 8-plex isobaric tags (Applied Biosystems, Foster City, CA) as follows: MI, 115; NMI, 116; LS (NDM), 117; HS (NDM), 118; LS (DM), 119; HS (DM), 121; Control, 113, 114. Labeled peptides were combined and dried using a vacuum concentrator.

Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) iTRAQ-labeled peptides (~1600 µg) were reconstituted in 200 µL of Mobile phase A (85% ACN, 0.1% HAc, 5 mM CH3COONH4) and fractionated using a PolyWAX LP anion-exchange column (4.6 × 200 mm, 5 µm, 300 Å, PolyLC, Columbia, MD) on a Shimadzu Prominence UFLC system (Kyoto, Japan). UV spectra of the peptides were collected at 280 nm. Mobile phase A and Mobile phase B (30% ACN, 0.2% FA) were used with a 60 min gradient elution of 0 - 36% B over 30 min and 36 − 100% B over the next 20 min, followed by 10 min at 100% B, at a flow rate of 1 mL/min. 30 fractions were collected and vacuum dried. Dried peptides in each fraction were then reconstituted in 3% ACN, 0.1% FA for subsequent LC-MS/MS analysis.

LC-MS/MS Analysis Using Q-Exactive Mass Spectrometer In order to achieve better coverage of the target proteome, minimize technical variations, increase statistical power and to ensure reproducibility and reliability of the quantitative dataset, three technical replicates of iTRAQ-labeled peptides were separated and analyzed. We used a LC-MS/MS system comprising of a Dionex Ultimate 3000 RSLC nano-HPLC system, coupled to an online Q-Exactive (QE) hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Hudson, NH, USA). Dried peptides in each fraction were injected onto a Acclaim® PepMap100 trap column (75 µm × 2 cm) packed with C18 (3 µm, 100 Å)

(Thermo Scientific, Hudson, NH, USA) via the auto-sampler of the Dionex system with a flow rate of 1 µL/min. Peptides were subsequently resolved in an EASY-spray column (75 µm × 15 cm) packed with C18 (3 µm, 100 Å; Bruker-Michrom, Auburn, CA, USA) at a flow rate of 300 nl/min. Mobile phase A (0.1% FA) and B (80% ACN,0.1% FA) were used to established the 60-min gradient; starting with 1 min of 3 - 6% B,46 min of 6 -35 % B, 6 min of 35 - 60% B, 1 min of 60 - 70 % B and 1 min of 70% B followed by re-equilibration at 3% B for 5 min. The samples were ionized in an EASY-Spray™ Source (Thermo Scientific, Hudson, NH, USA) with a nano- electrospray potential of 1.5 kV. The QE MS instrument was set to perform data acquisition in the positive ion mode. A full MS scan (350–1600 *m/z* range) was acquired at a resolution of 70,000 in the orbitrap when the maximum automatic gain control (AGC) target of 3×10^6 or a maximum ion accumulation time of 100 ms was reached. The 10 most intense peptide ions with dynamic exclusion duration of 30s and charge states of 2 -5 were sequentially fragmented in HCD collision cell and isolated to a maximum AGC target of 2×10^5 or a maximum ion accumulation time of 120 ms, MS/MS scan was acquired at a resolution of 35,000 in the orbitrap. For HCD, the normalized collision energy (NCE) was 28%, isolation width (*m/z*) was 2.0 and under fill ratio was defined as 0.5%.

Mass Spectrometric Raw Data Analysis Spectral data analysis was performed using Thermo Scientific™ Proteome Discoverer™ (PD) 1.4 software, connected to SEQUEST®HT search engine, a high-performance implementation of the SEQUEST™ algorithm. The spectra were searched against the Uniprot Human database (Released on 20130405, 121862 sequences; 43940360 residues) for protein identification. 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 less than 1% (*q-value ≤0.01*) were considered. The search was constrained with maximum 2 missed trypsin cleavages; peptide precursor mass tolerances of 10 ppm; and 0.02 Da mass tolerance for HCD fragment ions. Carbamidomethylation (+57.021 Da) of cysteine residues and iTRAQ8plex isobaric labeling (+304.205 Da) for lysine and N-terminus residues were fixed as static peptide modifications, while iTRAQ8plex isobaric labeling (+304.205 Da) of tyrosine residues, oxidation of methionine residues (+15.995 Da) and deamidation of asparagine and glutamine (+0.984 Da) residues were set as dynamic peptide modifications. The iTRAQ8plex quantification method within PD 1.4 software was used for the calculation of reporter ratios with mass tolerance set at 10 ppm. The average ratio, percentage variability and only unique peptides were employed for protein quantitative changes in protein composition and expression. Normalized protein ratios were expressed as a median value of overall measured iTRAQ reported ratios values by PD1.4 software. The reported ratio of High Score/Low Score (118+121)/ (117+119) in this study was calculated using the ratio reporting function within the iTRAQ 8plex quantification method in PD1.4. A fold change cut-off was set 1.2 and 0.83 to indicate up-regulated and down-regulated proteins respectively. Expression ratios of target proteins of interest were extracted and *p*-value s were determined by unpaired Student's *t*-test . A *p*-value <0.05 was used to indicate statistical significance.

Immunohistochemistry Results

Table 1. Independent significant predictors of composite Extended Major Cardiovascular and Cerebrovascular Events using univariate and multivariate Cox regression analysis.

	Univariate Cox regression			**Multivariate Cox regression (Final fitted model)**		
Variables	HR(unadjusted)	95% CI	P value	HR(unadjusted)	95% CI	P value
Age at the time of Surgery	0.98	0.93 -1.03	0.449			
Gender (Female) (reference) - Male	1.0 2.25	- 0.31-16.60	- 0.425			
Low Syntax score (reference) High syntax score	1.0 4.40	- 1.01 - 19.23	- 0.049	1 1.91	- 0.16 – 5.02	- 0.914
Syntax Score	1.03	1.00 - 1.06	0.066			
EuroSCORE	0.94	0.87 - 1.01	0.106			
Pre-op EF (mild & moderate) (Reference) Pre-op EF (severe)	1.0 3.41	- 1.15 - 10.15	- 0.028	5.59	1.20 - 26.06	0.028
Post - op EF (mild & moderate) (Reference) Post - op EF (severe)	1.0 6.12	- 1.37 - 27.38	- 0.018			
Graft Number	1.41	1.15 - 1.72	0.001	0.78	0.32 - 1.92	0.588
Biomarkers:						
Collagen 1	1.14	1.03 - 1.26	0.009	1.12	1.01 - 1.23	0.024
Collagen 3	0.007	0.001 - 1570.018	0.426			

(Table 1) contd.....

	Univariate Cox regression			**Multivariate Cox regression (Final fitted model)**		
Elastin	1.15	0.95 - 1.40	0.144			
Collagen 3/Collagen 1 ratio	0.043	0.001 - 16.274	0.299			
Elastin/Collagen 1 ratio	0.75	0.39 - 1.44	0.384			
Elastin/Collagen 3 ratio (10: 100)	10.006	10.003 – 10.008	<0.0001	10.003	10.005 – 10.006	0.021