Differentially expressed proteins in primary endothelial cells derived from patients with acute myocardial infarction

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Short title: Label free quantitative protein profiling in AMI

1. ABSTRACT:

Endothelial dysfunction is one of the primary factors in the onset and progression of atherothrombosis resulting in acute myocardial infarction (AMI). However, the pathological and cellular mechanisms of endothelial dysfunction in AMI have not been systematically studied. Protein expression profiling in combination with a protein network analysis was employed using a mass spectrometry-based label-free quantification approach. This identified and quantified 2,246 proteins, of which 335 were differentially regulated in coronary arterial endothelial cells from patients with AMI compared to controls. The differentially regulated protein profiles reveal the alteration of a) metabolism of RNA, b) platelet activation, signalling and aggregation, c) neutrophil degranulation, d) metabolism of amino acids and derivatives, e) cellular responses to stress, and f) response to elevated platelet cytosolic Ca^{2+} pathways. Increased production of oxidants and decreased production of antioxidant biomarkers as well as down regulation of proteins with antioxidant properties suggests a role for oxidative stress in mediating endothelial dysfunction during AMI. In conclusion, this is the first quantitative proteomics study to evaluate the cellular mechanisms of endothelial dysfunction in patients with AMI. A better understanding of the endothelial proteome and pathophysiology of AMI may lead to the identification of new drug targets.

Key words:

Endothelial dysfunction, myocardial infarction, protein profiling, high-resolution quantitative mass spectrometry, protein network.

2. Introduction:

The vascular endothelium plays a key role in the maintenance of homeostasis and it also acts as a multi-functional organ for vascular tone regulation. Endothelial dysfunction is more frequently observed in patients with cardiovascular risk factors and contributes to the development of atherogenesis, hypertension, peripheral vascular disease, chronic kidney failure, diabetes, viral infections, coronary artery disease, and myocardial ischemia[1–5]. It is characterized by several features including the association of increased production of reactive oxygen species (ROS), growth factors, adhesive molecules; impaired redox status and fibrinolytic ability; a discrepancy in the functions of endothelium-mediated vasodilation, prothrombic and proinflammatory responses[6–8]. Alterations in endothelial function contribute to the development, progression and clinical manifestations of atherosclerosis[9].

Acute myocardial infarction (AMI) is one of the leading causes of morbidity and mortality throughout the world and the prevalence of this disease is increasing rapidly in developing countries. Worldwide it is estimated that there were 15.9 million cases of AMI in 2015[10]. AMI is the most common manifestation of the acute coronary syndrome, occurring as a consequence of coronary thrombosis and a reduction in myocardial perfusion. The rupture of an atherosclerotic plaque or erosion of the coronary endothelial monolayer exposes blood to thrombogenic lipids, which leads to the activation of clotting factors and platelets. Atherosclerotic plaque with a thin fibrous cap and lipid-rich core are more prone to rupture. Previous studies have shown that circulating microparticles are increased in patients with AMI and endothelial dysfunction occurs in young patients with AMI irrespective of whether they have conventional cardiovascular risk factors[11,12].

Emerging data specifies that pathological blood vessel responses and endothelial dysfunction are associated with metabolic alterations in endothelial cells (ECs)[13]. However, the underlying mechanisms associated with endothelial dysfunction may be multidimensional and have not

been fully defined. Therefore, insights into the cellular mechanisms of endothelial dysfunction may aid our understanding of the acute coronary syndrome and lead to the development of new therapeutic tools. In this study, we aimed to identify the differentially regulated proteins associated with the endothelial dysfunction in patients with AMI. For this purpose, we isolated primary ECs from the thrombotic material aspirated from the coronary arteries of patients undergoing treatment for acute ST-segment elevation myocardial infarction. We applied a mass spectrometry-based label-free quantification approach to evaluate the molecular mechanisms, network and signalling pathways associated with endothelial dysfunction in AMI.

3. Methods:

Data available on request from the authors. Total protein was extracted from control and pathological ECs and subjected to reduction followed by alkylation. In-solution trypsin digested peptides were separated by nano LC-HRMS analysis. The resulting raw files were analysed by using various software for the protein identification, quantification. Statistically significantly differentially regulated proteins were used for gene ontology, network and pathway analysis. See supplemental materials and methods for the detailed procedures.

4. Results:

4.1 Identification and differential proteomic analysis of dysfunctional ECs

The aim of this study was to identify the differentially regulated proteins associated with endothelial dysfunction in AMI. For this purpose, primary ECs were isolated from the atherothrombotic material aspirated from the occluded coronary artery in patients with AMI. A mass spectrometry-based label-free quantitation approach enables the evaluation of the complex network and signalling pathways associated with the endothelial dysfunction. The confluent HCAEC-AMI and normal HCAECs were collected to extract the protein content. Altered protein expression profiling in combination with gene ontology, network and pathway analysis was employed by using the mass spectrometry-based label-free quantification

approach to explore the molecular mechanisms. Multi scatter plot analysis of peptide intensities resulted in Pearson coefficients higher than 0.98, attesting the high grade of reproducibility of technical and biological replicates. 2246 protein and 17653 unique peptides were quantified, consulting the *Uniprot_Homosapiens* database; the settings of searches were 10 ppm tolerance on peptides, 0.8Da on fragments and less than 1% false discovery rate. The data analysis was performed as specified in our publication[14]. To discriminate the differentially expressed proteins a two-sided t test was applied to two category groups: HCAEC-AMI (disease) and HCAEC (control). The log2 of ratios of disease versus control (fold change) were plotted against the -log10 of p-value, resulting in the Volcano plot of Figure 1. All proteins with a fold change higher than 1.5 and p-value less than 0.05 were considered significant because these proteins differed from most quantified proteins (with fold change about 1) whose expression in patient samples did not change related to controls. 335 proteins were differentially regulated, of which 40 and 123 proteins were up- and down-regulated respectively, with a fold change higher than 1.5 in the HCAEC-AMI cells compared to control cells (Table 1a and 1b).

4.2 Endothelial dysfunction and upregulation

The majority of upregulated proteins in HCAEC-AMI cells originated from the proteasome complex, nucleosome, nuclear nucleosome, or large ribosomal subunit and cytosolic ribosome (Table S1). The actin-binding protein TMSB10 was upregulated in dysfunctional ECs with a log2 fold change of 2.68. This protein is involved in actin filament organization and plays an important role in the cytoskeleton. Similarly, we identified vonWilibrand Factor (vWF), an important adhesion protein, was upregulated with a 2.06 log2 fold change. vWF is a key protein in vascular hemostasis, blood coagulation, platelet degranulation and activation, and in extracellular matrix organization. Studies have shown that vWF promotes the formation of a molecular bridge between platelet-surface receptor complex GPIb-IX-V and sub-endothelial collagen matrix, which further increases the binding of platelets to the site of vascular injury.

The plasminogen activator (PLAT) protein involved in tissue remodelling and degradation was also upregulated in dysfunctional ECs. Along with these proteins, based on our results, a platelet-expressed protein HSD17B12 might play a role in the pathogenesis of coronary artery disease. This protein has 3-ketoacyl-CoA reductase activity, suggesting a role in lipid metabolism and fatty acid biosynthesis. Other differentially regulated proteins and their associated biological processes are reported in Figure 2a.

4.3 Endothelial dysfunction and down-regulation

Cytosolic small and large ribosomal subunits and proteasome complex were the major sources of down-regulated proteins (Table S2). These proteins are involved in ribosome biosynthesis, small and large subunit assembly, the regulation of cardiac muscle contraction by the release of sequestered calcium ions, regulation of oxidative stress-induced cell death, cellular responses to reactive oxygen species, and cellular aldehyde metabolic processes (Figure 2b, Table S3). UBE2I, SYNE1, and MGST1 showed extensive downregulation among all the identified proteins. The MAPEG family (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism) protein MGST1, is involved in the protection of the endoplasmic reticulum and outer mitochondrial membrane from oxidative stress. Therefore, the downregulation of this protein in dysfunctional ECs might indicate increased oxidative stress in endothelial cells during AMI.

4.4 Network and pathway analysis: To better understand the nature of differentially regulated proteins, they were further classified in terms of reactome pathways. Data from this analysis showed the association of a) metabolism of RNA, b) platelet activation, signaling and aggregation, c) neutrophil degranulation, d) metabolism of amino acids and derivatives, e) platelet degranulation, f) cellular responses to stress, and g) response to elevated platelet cytosolic Ca2+ pathways in endothelial dysfunction of AMI (Figure 3).

4.5 Validation of protein by western blot:

To validate the expression of vWF, we used western blot analysis. The peak intensity area of each protein band was calculated using ImageJ software and the data illustrated as histograms after analyzing against the ß- actin as housekeeping protein. As shown in Figure 4, the relative abundance of vWF protein in the dysfunctional ECs was analogous to the results from label-free quantification study.

5. Discussion:

A deep understanding of the molecular mechanisms of disease is crucial in the discovery of novel therapeutic strategies for the treatment of endothelial dysfunction and its associated vascular diseases. Mass spectrometry-based proteomics is one of the advanced tools to identify new biomarkers and therapeutic targets. In the present study, for the first time, we describe the change in proteome profile of coronary arty endothelial cells in patients with AMI.

The endothelium which exists between the circulatory system and surrounding tissues plays an important role in protecting the vascular from injury. Based on local sheer stress and tissue requirements, ECs can create a prothrombotic or antithrombotic environment. Usually, healthy ECs express anticoagulant and antiplatelet proteins that prevent fibrin formation and platelet aggregation. Whereas dysfunctional ECs stimulate platelet aggregation and adhesion, and fibrin formation. ECs are also able to produce pro-fibrinolytic proteins, such as tissue plasminogen activate to enhance the fibrinolysis and promote degradation of local thrombus. In this study TMSB10 and vWF proteins were highly upregulated. TMSB10 has a role in the organization of the cytoskeleton, while the increased expression of vWF would be expected to enhance thrombus formation in the coronary artery. vWF also binds to collagen which plays a key role in the maintenance of the integrity and elasticity of the vasculature as well and when the vessel is injured and the prothrombotic collagen matrix exposed this interaction could precipitate AMI[15]. Interestingly, mass spectrometry based iTRAQ analysis with vascular

smooth muscle cells (VSMCs) isolated from the aortic wall of MI patients also revealed the overexpression of VWF protein[16].

Over expression of vWF, RAP1A, and RAP1B proteins would also promote platelet aggregation via the integrin alpha IIb beta3 signaling and MAP2K and MAPK activation pathways. MAPKs are implicated in multiple important cellular processes such as apoptosis, cellular proliferation, motility, differentiation, stress response, and survival. In this study, MAPK3 was down regulated, suggesting that endothelial cells from patients with AMI may have lower proliferation capability. Recent studies have shown that the interferon-induced GTP-binding protein MX1 is involved in AMI[17,18]. In this study, MX1 was upregulated with a 2.4-fold change, supporting a role for this protein in endothelial dysfunction. Rap1, which controls EC function, is one of the critical mediators of FGF-induced ERK activation in angiogenesis[19]. In this study, RAP1a and RAP1b were upregulated in patient-derived ECs perhaps to facilitate angiogenesis and promote myocardial salvage following AMI. RAP1a and 1b may also play a role in the protection of vascular ECs, based on the results of knockdown studies[20].

The identified differentially regulated proteins in our patient-derived endothelial cells, such as VWF[21], CRYAB[22], MX1[23], CNN2[24,25], PSME2[26], CIAPIN1[27], TAGLN[28], and EDIL3[29] have previously been reported to be implicated in AMI, vascular injury, morphogenesis, and angiogenesis in experimental models of disease. Furthermore, previous studies have shown the increased synthesis of type VIII collagen in the development of atherosclerosis in both humans and animal models[30,31]. The over expressed HSD17B12 protein is a reductase involved in collagen binding activity and long fatty acid chain metabolism. However, in the case of dysfunctional ECs of disease, HSD17B12 was down regulated, suggesting a different role in these diseases. MMRN1, belonging to EMILIN family[32], is usually found in platelets, and has

an adhesive ligand property in synergy with vWF in platelet adhesion to collagen at the sites of vascular injury[33,34].

EC apoptosis has a fundamental role in the angiogenesis process and pathological vascular remodelling and regression[35–37]. In this study proteins related to apoptosis pathway were differentially regulated. In particular, CYCS, DNM1L, HMGB1, LMNB1, PSMA4, PSMA7, PSMB5, PSMC5, PSMD13, PSME1, YWHAB, YWHAE showed decreased expression and PSMA2, PSMB3 (1.08-fold change) and PSME2 showed increased expression, attesting their prominent role during endothelial reorganization in AMI.

Metabolic alterations and oxidative stress are important inducers of endothelial dysfunction in cardiovascular diseases and diabetes mellitus[38–40]. Normal ECs constantly produce endothelial nitric oxide synthase (eNOS). Impairment of the eNOS–NO system causes oxidative stress and endothelial dysfunction that accelerates atherogenesis. In this investigation, a reduced expression of eNOS activation pathway, evidenced by the downregulation of CALM1, CALM2, and CALM3 proteins, and the activation of NF-kappaB were observed. PARK7, PAWR, PSAP, TXN and UBQLN1 proteins, involved in the regulation of oxidative stress-induced cell death, and MGST1 protein, involved in the cell protection against oxidative stress, were also down regulated. Moreover, the overexpression of superoxide dismutase (SOD1) was involved in hypoxia response and superoxide radical degradation pathways in VSMCs of patients with MI[41]. This confirms the involvement of oxidative stress in endothelial dysfunction. The inhibition of vascular oxidative stress and enhancement of endothelial NO production could be a potential therapeutic strategy along with the treatments of established risk factors.

Ribosome (including RNA and proteins) is an essential component in the machinery of protein synthesis and ribosomal proteins (RPs) plays a critical role in the cell proliferation, differentiation, apoptosis, DNA repair, and other cellular processes[42]. Studies have shown that

the dysfunction of ribosomal biosynthesis may result in abnormal cell proliferation in metabolic disorders and cancer[43,44]. Moreover, various RPs have been reported to be associated with the progression of cardiovascular diseases[45,46]. However, we observed the downregulation of several RPs in dysfunctional ECs which are corresponding to the ribosome biogenesis, small and large subunit assembly processes.

6. Perspectives:

In this study, the main involved pathways and related proteins have been extensively described and they will be further investigated in clinical prospective. This protein atlas in underlying mechanism of endothelial dysfunction can provide new perspectives in drug discovery for the treatment of cardiovascular diseases.

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8. Disclosures:

None.

9. References:

1 Celermajer DS. Endothelial Dysfunction: Does It Matter? Is It Reversible? *J Am Coll Cardiol* 1997; 30:325–333.

2 Vilahur G, Padró T, Casaní L, Mendieta G, López JA, Streitenberger S, *et al.* Polyphenol-enriched Diet Prevents Coronary Endothelial Dysfunction by Activating the Akt/eNOS Pathway. *Rev Española Cardiol (English Ed* 2015; 68:216–225.

3 Hasdai D, Gibbons RJ, Holmes DR, Higano ST, Lerman A. Coronary endothelial dysfunction in humans is associated with myocardial perfusion defects. *Circulation* 1997; 96:3390–3395.

4 Zeiher AM, Krause T, Schächinger V, Minners J, Moser E. Impaired endothelium-dependent vasodilation of coronary resistance vessels is associated with exercise-induced myocardial ischemia. *Circulation* 1995; 91:2345–52.

5 Zeiher AM, Drexler H, Wollschläger H, Just H. Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation* 1991; 83:391–401.

6 Sena CM, Pereira AM, Seiça R. Endothelial dysfunction — A major mediator of diabetic vascular disease. *Biochim Biophys Acta - Mol Basis Dis* 2013; 1832:2216–2231.

7 Addabbo F, Montagnani M, Goligorsky MS. Mitochondria and reactive oxygen species. *Hypertens (Dallas, Tex 1979)* 2009; 53:885–92.

8 Taddei S, Ghiadoni L, Virdis A, Versari D, Salvetti A. Mechanisms of Endothelial Dysfunction: Clinical Significance and Preventive Non-Pharmacological Therapeutic Strategies. *Curr Pharm Des* 2003; 9:2385–2402.

9 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362:801–809.

10 GBD 2015 Disease and Injury Incidence and Prevalence Collaborators T, Allen C,

Arora M, Barber RM, Bhutta ZA, Brown A, *et al.* Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet (London, England)* 2016; 388:1545–1602.

11 Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A, *et al.* Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. *Circulation* 2001; 104:2649–52.

12 Chen S-M, Tsai T-H, Hang C-L, Yip H-K, Fang C-Y, Wu C-J, *et al.* Endothelial dysfunction in young patients with acute ST-elevation myocardial infarction. *Heart Vessels* 2011; 26:2–9.

13 Goveia J, Stapor P, Carmeliet P. Principles of targeting endothelial cell metabolism to treat angiogenesis and endothelial cell dysfunction in disease. *EMBO Mol Med* 2014; 6:1105–20.

14 Nukala SB, Baron G, Aldini G, Carini M, D'Amato A. Mass Spectrometry-based Label-free Quantitative Proteomics To Study the Effect of 3PO Drug at Cellular Level. *ACS Med Chem Lett* 2019; :acsmedchemlett.8b00593.

15 Peuhkurinen K, Risteli L, Jounela A, Risteli J. Changes in interstitial collagen metabolism during acute myocardial infarction treated with streptokinase or tissue plasminogen activator. *Am Heart J* 1996; 131:7–13.

16 Wongsurawat T, Woo CC, Giannakakis A, Lin XY, Cheow ESH, Lee CN, *et al.* Transcriptome alterations of vascular smooth muscle cells in aortic wall of myocardial infarction patients. *Data Br* 2018; 17:1112–1135.

17 Chen Y-F, Pottala J V., Weltman NY, Ge X, Savinova O V., Gerdes AM. Regulation of Gene Expression with Thyroid Hormone in Rats with Myocardial Infarction. *PLoS One* 2012; 7:e40161.

18 Zhao Q, Wu K, Li N, Li Z, Jin F. Identification of potentially relevant genes for myocardial infarction using RNA sequencing data analysis. *Exp Ther Med* 2018; 15:1456–1464.

19 Yan J, Li F, Ingram DA, Quilliam LA. Rap1a is a key regulator of fibroblast growth factor 2-induced angiogenesis and together with Rap1b controls human endothelial cell functions. *Mol Cell Biol* 2008; 28:5803–10.

20 Birukova AA, Meng F, Tian Y, Meliton A, Sarich N, Quilliam LA, *et al.* Prostacyclin post-treatment improves LPS-induced acute lung injury and endothelial barrier recovery via Rap1. *Biochim Biophys Acta* 2015; 1852:778–91.

21 Wang X, Zhao J, Zhang Y, Xue X, Yin J, Liao L, *et al.* Kinetics of plasma von Willebrand factor in acute myocardial infarction patients: a meta-analysis. *Oncotarget* 2017; 8:90371–90379.

22 Mitra A, Basak T, Datta K, Naskar S, Sengupta S, Sarkar S. Role of α-crystallin B as a regulatory switch in modulating cardiomyocyte apoptosis by mitochondria or endoplasmic reticulum during cardiac hypertrophy and myocardial infarction. *Cell Death Dis* 2013; 4:e582–e582.

23 Zhao Q, Wu K, Li N, Li Z, Jin F. Identification of potentially relevant genes for myocardial infarction using RNA sequencing data analysis. *Exp Ther Med* 2018; 15:1456–1464.

24 Chen J, Yu L, Zhang S, Chen X. Network Analysis-Based Approach for Exploring the Potential Diagnostic Biomarkers of Acute Myocardial Infarction. *Front Physiol* 2016; 7:615.

25 Ahammad I. Identification of Key Proteins Associated with Myocardial Infarction using Bioinformatics and Systems Biology. doi:10.1101/308544

26 Wang Y, Huang Y, Zhang M, Zhang X, Tang X, Kang Y. Bioinformatic Analysis of

the Possible Regulative Network of miR-30a/e in Cardiomyocytes 2 Days Post Myocardial Infarction. *Acta Cardiol Sin* 2018; 34:175–188.

27 Zhang Y, Fang J, Ma H. Inhibition of miR-182-5p protects cardiomyocytes from hypoxia-induced apoptosis by targeting CIAPIN1. *Biochem Cell Biol* 2018; 96:646–654.

28 Derda AA, Woo CC, Wongsurawat T, Richards M, Lee CN, Kofidis T, *et al.* Gene expression profile analysis of aortic vascular smooth muscle cells reveals upregulation of cadherin genes in myocardial infarction patients. *Physiol Genomics* 2018; 50:648–657.

29 Jiang S-H, Wang Y, Yang J-Y, Li J, Feng M-X, Wang Y-H, *et al.* Overexpressed EDIL3 predicts poor prognosis and promotes anchorage-independent tumor growth in human pancreatic cancer. *Oncotarget* 2016; 7:4226–40.

30 Sibinga NE, Foster LC, Hsieh CM, Perrella MA, Lee WS, Endege WO, *et al.* Collagen VIII is expressed by vascular smooth muscle cells in response to vascular injury. *Circ Res* 1997; 80:532–41.

31 Sinha S, Kielty CM, Heagerty AM, Canfield AE, Shuttleworth CA. Upregulation of collagen VIII following porcine coronary artery angioplasty is related to smooth muscle cell migration not angiogenesis. *Int J Exp Pathol* 2001; 82:295–302.

32 Jeimy SB, Tasneem S, Cramer EM, Hayward CPM. Multimerin 1. *Platelets* 2008; 19:83–95.

33 Reheman A, Tasneem S, Ni H, Hayward CPM. Mice with deleted multimerin 1 and alpha-synuclein genes have impaired platelet adhesion and impaired thrombus formation that is corrected by multimerin 1. *Thromb Res* 2010; 125:e177-83.

34 TASNEEM S, ADAM F, MINULLINA I, PAWLIKOWSKA M, HUI SK, ZHENG S, *et al.* Platelet adhesion to multimerin 1 *in vitro* : influences of platelet membrane

receptors, von Willebrand factor and shear. *J Thromb Haemost* 2009; 7:685–692.

35 Chavakis E, Dimmeler S. Regulation of endothelial cell survival and apoptosis during angiogenesis. *Arterioscler Thromb Vasc Biol* 2002; 22:887–93.

36 Folkman J. Angiogenesis and apoptosis. *Semin Cancer Biol* 2003; 13:159–167.

37 Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003; 9:653–660.

38 Goveia J, Stapor P, Carmeliet P. Principles of targeting endothelial cell metabolism to treat angiogenesis and endothelial cell dysfunction in disease. *EMBO Mol Med* 2014; 6:1105–20.

39 Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000; 87:840–4.

40 Ceriello A, Assaloni R, Da Ros R, Maier A, Piconi L, Quagliaro L, *et al.* Effect of atorvastatin and irbesartan, alone and in combination, on postprandial endothelial dysfunction, oxidative stress, and inflammation in type 2 diabetic patients. *Circulation* 2005; 111:2518–24.

41 Wongsurawat T, Woo CC, Giannakakis A, Lin XY, Cheow ESH, Lee CN, *et al.* Distinctive molecular signature and activated signaling pathways in aortic smooth muscle cells of patients with myocardial infarction. *Atherosclerosis* 2018; 271:237–244.

42 Wang W, Nag S, Zhang X, Wang M-H, Wang H, Zhou J, *et al.* Ribosomal proteins and human diseases: pathogenesis, molecular mechanisms, and therapeutic implications. *Med Res Rev* 2015; 35:225–85.

43 Ruggero D, Pandolfi PP. Does the ribosome translate cancer? *Nat Rev Cancer* 2003; 3:179–192.

44 Wang W, Nag S, Zhang X, Wang M-H, Wang H, Zhou J, *et al.* Ribosomal proteins and human diseases: pathogenesis, molecular mechanisms, and therapeutic

implications. *Med Res Rev* 2015; 35:225–85.

45 Di R, Wu X, Chang Z, Zhao X, Feng Q, Lu S, *et al.* S6K inhibition renders cardiac protection against myocardial infarction through PDK1 phosphorylation of Akt. *Biochem J* 2012; 441:199–207.

46 Casad ME, Abraham D, Kim I-M, Frangakis S, Dong B, Lin N, *et al.* Cardiomyopathy is associated with ribosomal protein gene haplo-insufficiency in Drosophila melanogaster. *Genetics* 2011; 189:861–70.

10. Novelty and significance of the work:

What is new?

In this study we define the proteomic signature of endothelial cells derived from patients with acute myocardial infarction and identify plausible pathophysiological mechanisms of endothelial dysfunction.

What is relevant?

Quantitative proteomic profiling in endothelial dysfunction revealed the biomarker signature of thrombosis, metabolism and oxidative stress. The overexpression of VWF strongly associated with thrombus formation, platelet aggregation and angiogenesis that might play a key role endothelial dysfunction of AMI. Increased production of oxidants and decreased production of antioxidant biomarkers, as well as down regulation of proteins which had an antioxidant property, strongly suggest a role for oxidative stress in the endothelial dysfunction of AMI.

Summary

Our mass spectrometry-based label-free quantitative proteomics approach revealed several cardiovascular related biomarkers in AMI. Thrombus formation, collagen deposition, vascular remodeling, platelet aggregation, metabolic alterations, oxidative stress related events have a potential unfavorable impact on the endothelial dysfunction.

11. Figure and Table Legends:

Figure 1: Distribution of differentially regulated proteins in HCAEC-AMI cells. Volcano Plot obtained by two-sided t test of the groups: HCAEC-AMI (disease) versus HCAEC (control). Dots on the left indicates the down-regulated proteins, dots on the right represents the upregulated ones.

Figure 2: Biological processes associated with up (a) and down (b) regulated proteins found in dysfunctional HCAEC-AMI cells. The significance of the clustering is shown by the size of nodes.

Figure 3: Main pathways and involved genes found in dysfunctional HACEC-AMI cells. ClueGo analyses enriched and clustered Reactome pathway terms. The significance of the clustering is shown by the size of nodes.

Figure 4: : Protein expression levels of selected proteins. Bar graph was plotted with mean ± SEM, the x-axis represents the HCAEC-AMI and HCAEC groups and the y-axis represents the relative expression of a respective protein to control. Differences were considered significant when $p < 0.05$ (*), $0.001 < p < 0.01$ (**), $p < 0.001$ (***). β-actin was used as a housekeeping protein.

Table 1a: List of upregulated proteins associated with endothelial dysfunction in AMI.

Table 1b: List of down-regulated proteins associated with endothelial dysfunctionality in AMI.

12. Tables:

Table 1a:

Protein ID	Protein names	Gene names	Log2 (HCAEC-AMI vs HCAEC)	-LOG(P-value)
P63313	Thymosin beta-10	TMSB10	2.68	4.97
P04275	von Willebrand factor;von Willebrand antigen 2	VWF	2.06	1.41

B4DWS6	Very-long-chain 3-oxoacyl-CoA reductase	HSD17B 12	1.47	5.70
E9PR44	Alpha-crystallin B chain	CRYAB	1.43	3.29
B4DRD3	Tissue-type plasminogen activator;Tissue-type plasminogen activator chain A;Tissue-type plasminogen activator chain B	PLAT	1.37	13.16
A0A140VJL6	Isoamyl acetate-hydrolyzing esterase 1 homolog	IAH1	1.33	5.87
P20591	Interferon-induced GTP-binding protein Mx1;Interferon-induced GTP-binding protein Mx1, N-terminally processed	MX1	1.25	3.67
O95433	Activator of 90 kDa heat shock protein ATPase homolog 1	AHSA1	1.20	3.35
B4DDF4	Calponin;Calponin-2	CNN2	1.06	4.70
Q86SZ7	Proteasome activator complex subunit 2	PSME2	1.03	2.36
B4E2S7	Lysosome-associated membrane glycoprotein 2	LAMP2	1.03	3.60
Q6FI81	Anamorsin	CIAPIN1	0.99	6.26
A0A0S2Z4G7	Nucleophosmin	NPM1	0.93	3.75
A0A024R6I3	Transmembrane emp24 domain-containing protein 10	TMED10	0.91	5.50

Q6IBN4	Enoyl-CoA delta isomerase 2, mitochondrial	PECI	0.86	5.85
O94874	E3 UFM1-protein ligase 1	UFL1	0.85	3.59
O15511	Actin-related protein 2/3 complex subunit 5	ARPC5	0.84	2.69
A0A024R3C4	KDEL motif-containing protein 2	KDELC2	0.84	3.28
Q5RLJ0	UPF0568 protein C14orf166	C14orf166	0.83	8.21
Q9UPN1	Serine/threonine-protein phosphatase;Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	PPP1CC	0.83	3.05
A0A024RA52	Proteasome subunit alpha type;Proteasome subunit alpha type-2	PSMA2	0.81	2.55
X5D2T3	60S ribosomal protein L10	RPL10	0.80	4.07
P61026	Ras-related protein Rab-10	RAB10	0.79	2.57
B7ZAY2	Ras-related protein Rap-1b;Ras-related protein Rap-1b-like protein;Ras-related protein Rap-1A	RAP1B	0.77	4.39
Q5U0D2	Transgelin	TAGLN	0.77	2.63
Q6IPH7		RPL14	0.76	4.33
O15231	Zinc finger protein 185	ZNF185	0.76	4.13
P51571	Translocon-associated protein subunit delta	SSR4	0.75	5.64
J3KQ32	Obg-like ATPase 1	OLA1	0.74	4.78

A0A024RB14	40S ribosomal protein S26;Putative 40S ribosomal protein S26-like 1	RPS26	0.74	3.59
Q13509	Tubulin beta-3 chain	TUBB3	0.74	4.60
A0A024R7B7	Hsp90 co-chaperone Cdc37;Hsp90 co-chaperone Cdc37, N-terminally processed	CDC37	0.72	5.11
B7Z3K3	Inositol-3-phosphate synthase 1	ISYNA1	0.71	3.68
B3KT06	Tubulin alpha-1B chain	TUBA1B	0.71	6.93
A0A024R1M8	Apolipoprotein L2	APOL2	0.71	1.95
O43854	EGF-like repeat and discoidin I-like domain-containing protein 3	EDIL3	0.69	1.98
P37802	Transgelin-2	TAGLN2	0.63	4.79
P63172	Dynein light chain Tctex-type 1	DYNLT1	0.63	2.39
B4DFL1	Dihydrolipoyl dehydrogenase;Dihydrolipoyl dehydrogenase, mitochondrial	DLD	0.61	2.36
V9HW44	Platelet-activating factor acetylhydrolase IB subunit beta	HEL-S-303	0.60	3.84

Table 1b:

Protein ID	**Protein names**	**Gene names**	**Log2 (HCAEC-AMI vs HCAEC)**	**-LOG(P-**

				valu e)
H3BPC4	SUMO-conjugating enzyme UBC9	UBE2I	-1.68	1.38
E7ENN3	Nesprin-1	SYNE1	-1.45	6.12
F5H7F6	Microsomal glutathione S-transferase 1	MGST1	-1.45	5.54
A0A1W2PRF6	Lysosome membrane protein 2	SCARB2	-1.43	4.16
B3KPC7	Actin-related protein 2/3 complex subunit 5;Actin-related protein 2/3 complex subunit 5-like protein	ARPC5L	-1.43	3.29
A8K651	Complement component 1 Q subcomponent-binding protein, mitochondrial	C1QBP	-1.39	5.53
Q9BQQ5	60S ribosomal protein L27a	L27a	-1.39	8.30
P62851	40S ribosomal protein S25	RPS25	-1.38	6.19
Q13201	Multimerin-1;Platelet glycoprotein Ia*;155 kDa platelet multimerin	MMRN1	-1.33	3.14
Q9Y5B9	FACT complex subunit SPT16	SUPT16H	-1.31	4.57
Q96FQ6	Protein S100-A16	S100A16	-1.29	6.83
Q6IBA2	Activated RNA polymerase II transcriptional coactivator p15	PC4	-1.25	7.05
Q13151	Heterogeneous nuclear ribonucleoprotein A0	HNRNPA0	-1.21	4.49
B4DWN1	Vesicular integral-membrane protein VIP36	LMAN2	-1.20	2.20

C9J3L8	Translocon-associated protein subunit alpha	SSR1	-1.20	5.57
Q2TAM5	Transcription factor p65	RELA	-1.17	6.14
P29966	Myristoylated alanine-rich C-kinase substrate	MARCKS	-1.15	6.06
H0YN26	Acidic leucine-rich nuclear phosphoprotein 32 family member A	ANP32A	-1.12	6.03
Q53FJ5	Prosaposin;Saposin-A;Saposin-B-Val;Saposin-B;Saposin-C;Saposin-D	PSAP	-1.09	8.79
Q13404	Ubiquitin-conjugating enzyme E2 variant 1	UBE2V1	-1.09	3.47
A0A140VJK1	Glutaredoxin-3	GLRX3	-1.06	4.43
F8W6I7	Heterogeneous nuclear ribonucleoprotein A1;Heterogeneous nuclear ribonucleoprotein A1, N-terminally processed;Heterogeneous nuclear ribonucleoprotein A1-like 2	HNRNPA1	-1.05	5.46
A0A024R3J7	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	hCG_2032701	-1.03	5.00
Q9Y3U8	60S ribosomal protein L36	RPL36	-1.01	5.47
P51858	Hepatoma-derived growth factor	HDGF	-1.00	4.74

F5GXX5	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	DAD1	-0.99	2.82
Q5T7C4	High mobility group protein B1;Putative high mobility group protein B1-like 1	HMGB1	-0.99	7.17
B4DTT0	N-acetylglucosamine-6-sulfatase	DKFZp686E12166	-0.97	8.94
O14907	Tax1-binding protein 3	TAX1BP3	-0.97	3.69
A0A024R4X0	NADH-cytochrome b5 reductase;NADH-cytochrome b5 reductase 3;NADH-cytochrome b5 reductase 3 membrane-bound form;NADH-cytochrome b5 reductase 3 soluble form	CYB5R3	-0.97	7.22
H9ZYJ2	Thioredoxin	TXN	-0.96	5.01
B5BU25	Splicing factor U2AF 65 kDa subunit	U2AF2	-0.96	8.00
Q86U79	Adenosine kinase	ADK	-0.95	2.58
L7RT18	Adapter molecule crk	CRK	-0.94	4.18
V9HW48	SH3 domain-binding glutamic acid-rich-like protein	HEL-S-115	-0.93	2.73
A0A140VJF4	Biliverdin reductase A	BLVRA	-0.93	5.15
E9PI87	Oxidoreductase HTATIP2	HTATIP2	-0.92	2.13
J3KNF4	Copper chaperone for superoxide dismutase	CCS	-0.92	4.48
Q6FGV9	Phosphomevalonate kinase	PMVK	-0.92	2.92

P20700	Lamin-B1	LMNB1	-0.91	9.48
Q6FGY1	Hippocalcin-like protein 1	HPCAL1	-0.91	3.83
A0A024R258	Ubiquilin-1;Ubiquilin-4	UBQLN1	-0.90	5.55
Q53XX5	Cold-inducible RNA-binding protein	CIRBP	-0.90	3.80
Q59H57	RNA-binding protein FUS	FUS	-0.90	3.77
B4E324	Carboxypeptidase;Lysosomal protective protein;Lysosomal protective protein 32 kDa chain;Lysosomal protective protein 20 kDa chain	CTSA	-0.89	4.30
K7ESE8	Bleomycin hydrolase	BLMH	-0.89	3.32
P46926	Glucosamine-6-phosphate isomerase 1;Glucosamine-6-phosphate isomerase	GNPDA1	-0.88	4.45
B7Z4K8	Basic leucine zipper and W2 domain-containing protein 2	BZW2	-0.87	4.26
B7Z2Z1	Scaffold attachment factor B1	SAFB	-0.87	5.93
Q6IRT1	S-(hydroxymethyl)glutathione dehydrogenase;Alcohol dehydrogenase class-3	ADH5	-0.87	4.62
P15531	Nucleoside diphosphate kinase A	NME1	-0.86	1.85
A2A2D0	Stathmin	STMN1	-0.86	8.87
C9JFR7	Cytochrome c	CYCS	-0.84	6.18
A8K2W3	Serum deprivation-response protein	SDPR	-0.83	11.43
F5GYN4	Ubiquitin thioesterase OTUB1	OTUB1	-0.81	5.20

O75436	Vacuolar protein sorting-associated protein 26A	VPS26A	-0.81	2.38
V9HW74	Ubiquitin carboxyl-terminal hydrolase;Ubiquitin carboxyl-terminal hydrolase isozyme L1	HEL-117	-0.79	1.94
A0A0S2Z410	3-hydroxyacyl-CoA dehydrogenase type-2	HSD17B10	-0.79	4.64
H3BPQ3	Protein NDRG4	NDRG4	-0.78	3.46
Q5T9B9	Endoglin	ENG	-0.77	3.90
A0A087X296	Prostaglandin G/H synthase 1	PTGS1	-0.77	4.28
J3K000	Xaa-Pro dipeptidase	PEPD	-0.76	3.38
A0A024RAV2	ATP-dependent DNA helicase Q1	RECQL	-0.76	5.90
A0A024R3Z6	Basic leucine zipper and W2 domain-containing protein 1	BZW1	-0.75	5.44
P18077	60S ribosomal protein L35a	RPL35A	-0.75	2.68
Q6FH49	Nicotinamide N-methyltransferase	NNMT	-0.74	4.02
V9HWI0	Alcohol dehydrogenase [NADP(+)]	HEL-S-165mP	-0.74	7.43
B3KMZ6	SUMO-activating enzyme subunit 2	UBA2	-0.74	3.60
E9PJ81	UBX domain-containing protein 1	UBXN1	-0.74	2.45
B4DIZ2	Ubiquitin-conjugating enzyme E2 K	UBE2K	-0.74	3.24
A5PLK7	Protein RCC2	RCC2	-0.73	4.02
B0YJ88	Radixin	RDX	-0.73	3.67

A0A024R883	V-type proton ATPase subunit G 1	ATP6V1G1	-0.73	3.30
A0A024R3W7	Elongation factor 1-beta	EEF1B2	-0.73	3.44
Q5IST1	Serine/arginine-rich splicing factor 5	SFRS5	-0.71	2.49
A0A024R8W0	Eukaryotic initiation factor 4A-III;Eukaryotic initiation factor 4A-III, N-terminally processed	DDX48	-0.71	4.98
Q6FHX6	Flap endonuclease 1	FEN1	-0.70	2.56
Q53HS0	Glutamine--tRNA ligase	QARS	-0.70	5.15
O95831	Apoptosis-inducing factor 1, mitochondrial	AIFM1	-0.70	2.02
O15400	Syntaxin-7	STX7	-0.70	4.56
Q5T123	SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	-0.69	4.48
Q9HDC9	Adipocyte plasma membrane-associated protein	APMAP	-0.69	2.58
Q05BM8	Polypeptide N-acetylgalactosaminyltransferase;Polypeptide N-acetylgalactosaminyltransferase 1;Polypeptide N-acetylgalactosaminyltransferase 1 soluble form	GALNT1	-0.69	3.82
Q9BRL5		CALM3	-0.69	2.73
J3QRT5	Intercellular adhesion molecule 2	ICAM2	-0.69	2.31

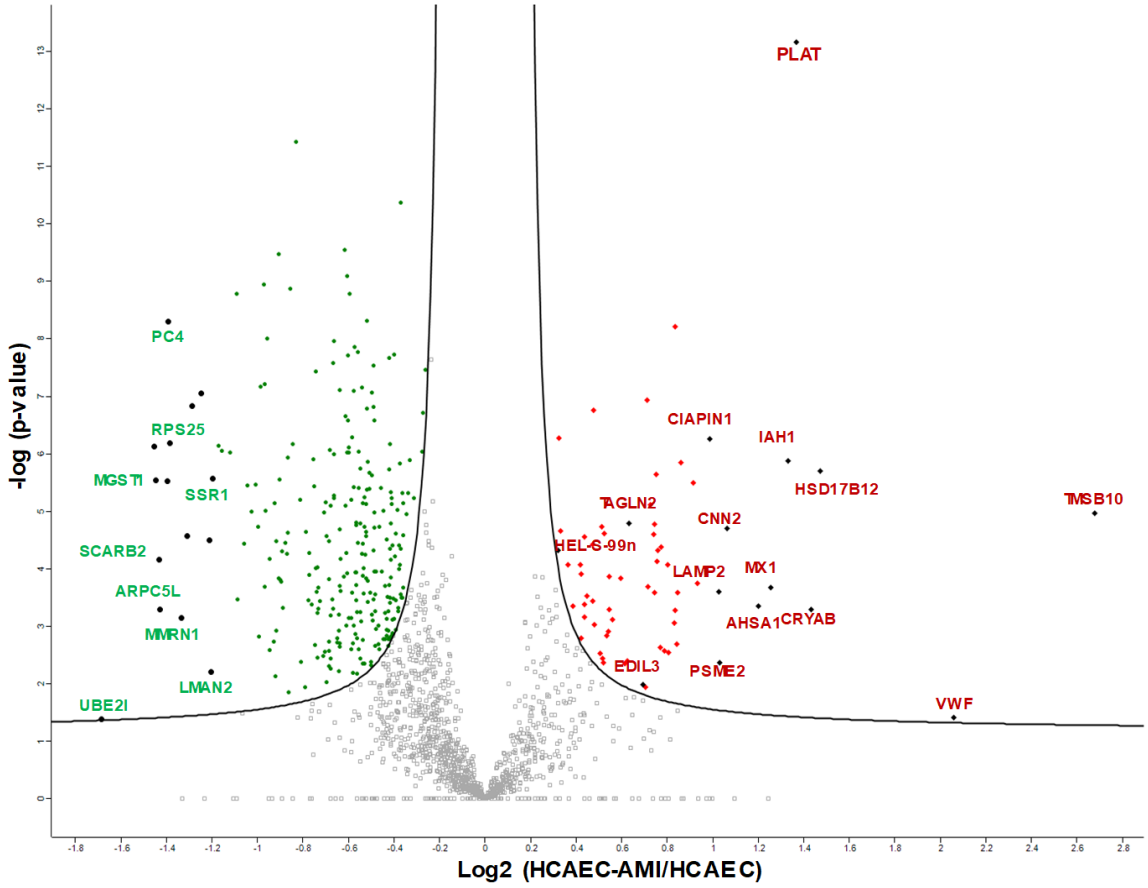
F8WCF6	Actin-related protein 2/3 complex subunit 4	ARPC4-TTLL3	-0.68	6.06
I3L2L5	Protein FAM195B	FAM195B	-0.68	3.19
B3KN57	Sorting nexin-2	SNX2	-0.68	5.10
F8VQE1	LIM domain and actin-binding protein 1	LIMA1	-0.68	2.45
D3DP46	Signal peptidase complex subunit 3	SPCS3	-0.68	2.27
A0A024R9D2	Protein LYRIC	MTDH	-0.67	3.46
P35580	Myosin-10	MYH10	-0.67	7.58
K7ES31	Eukaryotic translation initiation factor 3 subunit K	EIF3K	-0.67	3.30
J3KT73	60S ribosomal protein L38	RPL38	-0.66	6.00
P62263	40S ribosomal protein S14	RPS14	-0.66	7.96
P62495	Eukaryotic peptide chain release factor subunit 1	ETF1	-0.66	5.27
Q53XC0	Eukaryotic translation initiation factor 2 subunit 1	EIF2S1	-0.66	5.24
Q96C90	Protein phosphatase 1 regulatory subunit 14B	PPP1R14B	-0.65	2.71
F5H2R5	Rho GDP-dissociation inhibitor 2	ARHGDIB	-0.65	2.80
A4D275	Actin-related protein 2/3 complex subunit 1B	ARPC1B	-0.64	2.92

Q14240	Eukaryotic initiation factor 4A-II;Eukaryotic initiation factor 4A-II, N-terminally processed	EIF4A2	-0.64	3.21
B4E3A8	Leukocyte elastase inhibitor	HEL57	-0.64	2.22
A0A024R1K8	Splicing factor 3A subunit 1	SF3A1	-0.64	*2.65*
H7BY10	60S ribosomal protein L23a	RPL23A	-0.64	3.71
Q96IZ0	PRKC apoptosis WT1 regulator protein	PAWR	-0.64	4.69
A8K719	Core-binding factor subunit beta	CBFB	-0.64	2.71
A0A024RDB0	Ubiquitin-like modifier-activating enzyme 6	UBE1L2	-0.64	7.12
A0A0A6YYJ8	Putative RNA-binding protein Luc7-like 2	LUC7L2	-0.64	3.62
A0A140VJP5	S-adenosylmethionine synthase isoform type-2;S-adenosylmethionine synthase	MAT2A	-0.64	4.18
Q53R19	Actin-related protein 2/3 complex subunit 2	ARPC2	-0.62	3.86
A0A1X7SBZ2	Probable ATP-dependent RNA helicase DDX17	DDX17	-0.62	9.54
A9UK01	Rho GTPase-activating protein 18	ARHGAP18	-0.61	5.11
A0A024RB85	Proliferation-associated protein 2G4	PA2G4	-0.61	6.66
V9HW41	Ubiquitin-conjugating enzyme E2 N	HEL-S-71	-0.61	3.01
Q5VVD0	60S ribosomal protein L11	RPL11	-0.61	4.27

P51991	Heterogeneous nuclear ribonucleoprotein A3	HNRNPA3	-0.61	9.09
A0A024R5Q7	Adenylosuccinate synthetase isozyme 2	ADSS	-0.61	6.02
Q59GU6	Sorting nexin-1	SNX1	-0.61	3.93
P13010	X-ray repair cross-complementing protein 5	XRCC5	-0.60	6.59
C9J9K3	40S ribosomal protein SA	RPSA	-0.60	7.71
A0A0A0MRM8	Unconventional myosin-VI	MYO6	-0.60	3.14
L7RXH5	Mitogen-activated protein kinase;Mitogen-activated protein kinase 3	MAPK3	-0.60	6.02
V9HWC2	Protein deglycase DJ-1	HEL-S-67p	-0.60	6.11

13. Figures:

Figure 1: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HSD17B12
TAGLN2
CNN2
HEL-S-99n
LAMP2
MX1
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EDIL3
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VWF
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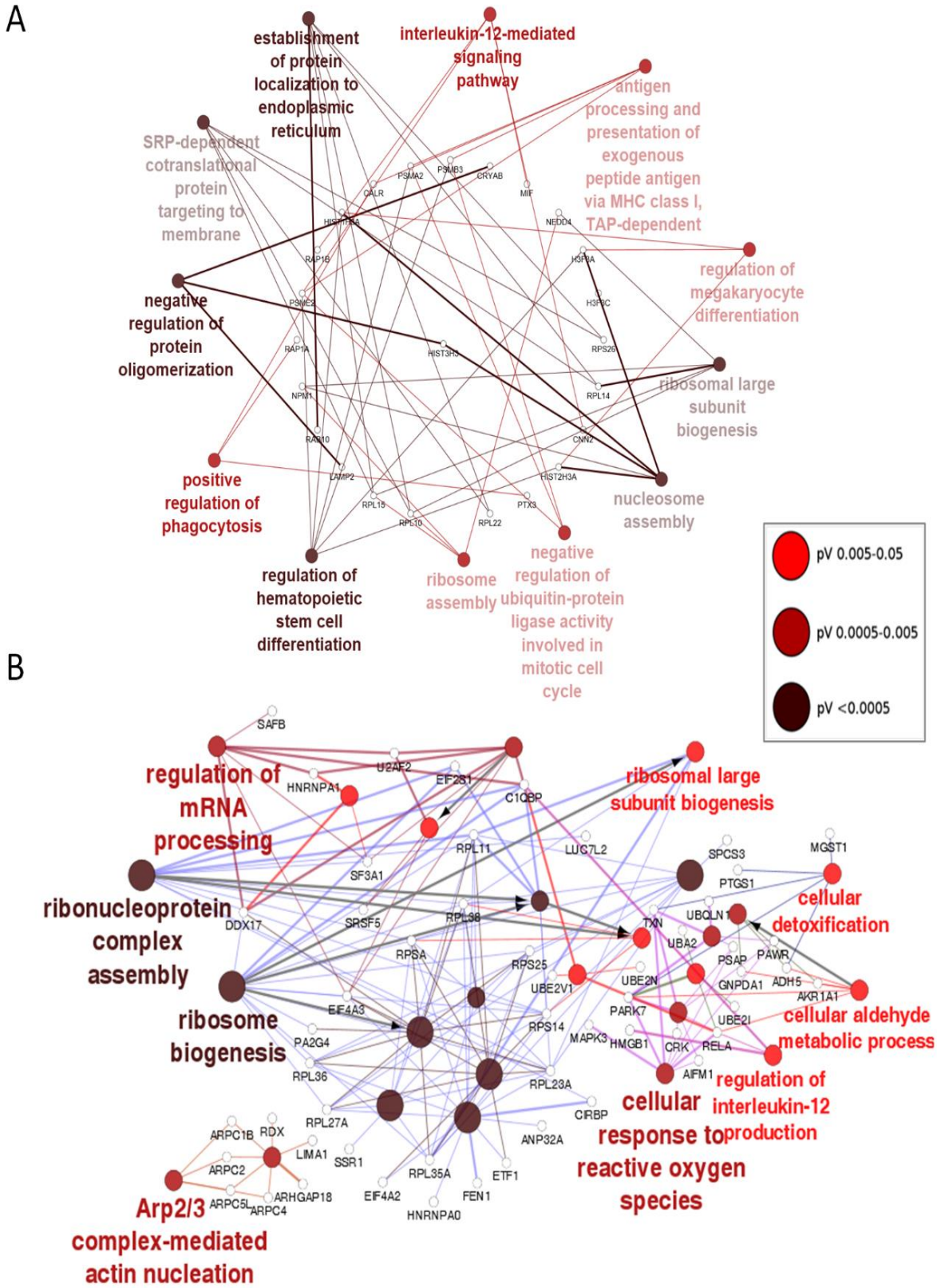
-log (p-value)
Log2 (HCAEC-AMI/HCAEC)

Figure 2: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establishment of protein localization to endoplasmic reticulum
interleukin-12-mediated signaling pathway
antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent
SRP-dependent cotranslational protein targeting to membrane
regulation of megakaryocyte differentiation
negative regulation of protein oligomerization
ribosomal large subunit biogenesis
positive regulation of phagocytosis
nucleosome assembly
regulation of hematopoietic stem cell differentiation
ribosome assembly
negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle
PSMA2
PSMB3
CRYAB
CALR
MIF
HIST1H4A
NEDD4
RAP1B
H3F3A
PSME2
H3F3C
RAP1A
HIST3H3
RPS25
NPM1
RPL14
RAB10
CNN2
LAMP2
HIST2H3A
RPL15
PTX3
RPL10
RPL22
pV 0.005-0.05
pV 0.0005-0.005
pV <0.0005
B
SAFB
regulation of mRNA processing
U2AF2
HNRNPA1
EIF2S1
C1QBP
ribosomal large subunit biogenesis
RPL11
LUC7L2
SPCS3
MGST1
SF3A1
PTGS1
ribonucleoprotein complex assembly
DDX17
SRSF5
RPL38
cellular detoxification
UBQLN1
TXN
UBA2
RPSA
PSAP
PAWR
RPS25
UBE2V1
UBE2N
GNPDA1
ADH5
AKR1A1
EIF4A3
PARK7
UBE2I
cellular aldehyde metabolic process
ribosome biogenesis
RPS14
MAPK3
HMGB1
CRK
RELA
PA2G4
AIFM1
RPL36
RPL23A
regulation of interleukin-12 production
CIRBP
cellular response to reactive oxygen species
RPL27A
ARPC1B
RDX
ANP32A
LIMA1
SSR1
ARPC2
RPL35A
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Figure 3:

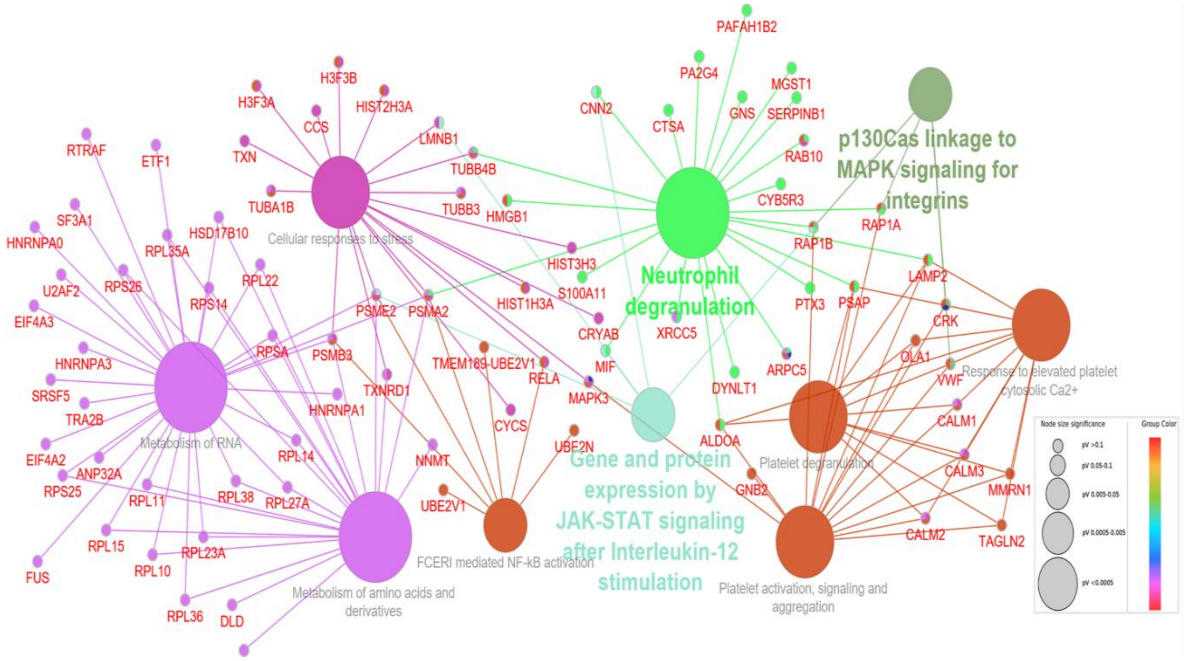

Figure 4: