ORIGINAL ARTICLE

Proteomic Profiles of Young Adults with Acute Myocardial Infarction

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ABSTRACT

Introduction: Proteomic profiling is essential in understanding the pathophysiological process of multifactorial diseases such as acute myocardial infarction (AMI). Despite the increasing incidence of AMI in young adults, proteomic-based study focusing on young AMI remains limited. This study aimed to examine the plasma proteomic profiles of young adults with AMI compared to control subjects. We also hope to identify disease-specific protein biomarkers that contribute to the development of AMI in the young. **Methods:** Pooled plasma protein from 10 AMI patients aged 18 to 45 years and 10 age, gender and race-matched volunteers were separated using two-dimensional electrophoresis (2-DE). The spots proteins were analysed using the PD Quest analysis software. The spots proteins that were found to have been expressed differently between the two groups were identified by Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometry. **Results:** There were three differently expressed proteins namely Apolipoprotein AI (Apo AI), Apolipoprotein AIV (Apo AIV) and Haptoglobin ($p < 0.05$). The expressions of these proteins were found to be increased in young patients with AMI compared to control subjects. **Conclusion:** The up regulation of Apo AI, Apo AIV and Haptoglobin in AMI patients indicate their important roles in the development of atherosclerotic disease. Thus, Apo AI, Apo AIV and Haptoglobin are potential disease biomarkers for young AMI.

KEYWORD: proteomic, young adults, myocardial infarction

INTRODUCTION

Coronary artery diseases (CAD) which most frequently present as acute myocardial infarction (AMI) are the main causes of morbidity and mortality worldwide. It is well-known that advancing age is a risk factor for AMI. However, young age is no longer considered to be protective nowadays since the incidence of AMI in young adults is increasing. In the United States, despite a significant decline in AMI hospitalisations over the past four decades, there has been no concomitant reduction in AMI admissions for patients aged below 55 years.[1-2] As for the United Kingdom, although the prevalence of young AMI is relatively low (3%), the rate is expected to rise in view of various reasons.[3] Comparatively, the occurrence of AMI in young adults is higher in South Asia, with the prevalence being 16.2%, 11.7%, and 10.5% in Pakistan, India, and Bangladesh respectively.[4-5] AMI also manifests a decade earlier in these groups of people, as compared to the Western populations.[6] Meanwhile, 16% of all AMI cases that admitted into tertiary referral hospitals in Malaysia between 2007 and 2009 were aged less than 45 years for male and 55 years for female.[7]

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There is no standard definition of 'young' AMI, but the most commonly-used cut-off age is 45 years.[4,7-13] Some studies have even employed younger cut-off ages like 40 and 35 years.[5,14-16] The diagnosis of AMI at this crucial age; during which family and career establishment occurs, gives rise to significant adverse effects on the well-being and mental states of the patients, their families, as well as the community. Furthermore, the male preponderance of this disease results in a greater socioeconomic impact owing to the loss of vital human capital, increased burden of patient care in the families, and excessive usage of public healthcare facilities.[13] Many studies on AMI in young adults have mainly focused on the epidemiology, risk factors, and clinical presentations variations.[4,5,8,12,15] Evidently, smoking has been found to be the most important risk factor of young AMI, whereas dyslipidaemia, hypertension, and diabetes had a more significant role in the disease among elderly patients.[3,10,14,16,17] Meanwhile for clinical characteristics, single vessel disease was documented to be more frequent among young AMI patients compared to their older counterparts.[16-17] Despite the less extensive CAD in the younger patients, those reports indicated that CAD in young adult is associated with rapid disease development rather than with gradually progressing process.[18]

Other than the differences in regard to the risk factors profiles and clinical presentations, study on the actual molecular changes that contribute to the acceleration of the pathogenesis of young AMI remains lacking. Recently, proteomics has become a valuable tool for the investigation of specific disease processes. The ability of proteomic profiling to characterise proteins that are present in diseased tissues is also essential to facilitate an understanding of the pathophysiological processes of individual diseases.[19] Additionally, this technology enables the identification of proteins that have the potential to become diagnostic/ prognostic biomarkers or therapeutic target proteins. By virtue of the higher prevalence of AMI in elderly patients, previous proteomic analyses of AMI mainly concerned samples from this age group.[20-22] However, elderly patients usually have other comorbidities that require pharmacological interventions, which may in turn affect the protein profiles. At present, proteomic-based researches on AMI in young adults remain limited. We hypothesize that beyond the discrepancies in the risk factor profiles and clinical presentations, the young adults with AMI might be associated with different pathophysiological changes that would be reflected in proteomic profiles. The study of proteomic profiles and the discovery of protein markers that are specific for young AMI will improve current understanding of the disease process and facilitate the formulation of a more age-specific preventive and therapeutic approach. Thus, the main objective of this study was to compare the proteomic profiles of young AMI patients relative to the controls.

MATERIALS AND METHODS

Subject Recruitment and Sample Collection

Ten patients aged 18 to 45 years who were admitted to the Emergency Department of Hospital Tengku Ampuan Afzan (HTAA) following the diagnosis of ST-elevated myocardial infarction (STEMI) and non ST-elevated myocardial infarction (NSTEMI) were recruited. The diagnosis was confirmed by the presence of prolonged chest pain, typical changes in a 12-lead electrocardiogram (ECG) and/ or elevated serum creatine kinase (CK). Patients presented with unstable angina were excluded. Ten healthy volunteers who were matched for age, gender, and race were recruited as control subjects during health screening programs. AMI patients who had chronic illnesses like diabetes, neoplasms, infections, autoimmune diseases, or previous episode(s) of AMI were excluded from the study. None of the patients were on lipid-lowering agents or antihypertensive drugs prior to admission. Informed consent was obtained from all participants. Blood samples from AMI patients were collected in the Emergency Department prior to the administration of Streptokinase or percutaneous coronary intervention (PCI) procedure to ensure the proteomic profiles accurately reflect the recent AMI instead of other invasive interventions. Some 10 ml of blood sample was collected from each patient and transferred into tubes coated with ethylenediaminetetra-acetic acid (EDTA). The blood samples were centrifuged at 2 500 revolutions per minute (rpm) for 10 minutes. Subsequently, the plasma was pipetted into 1.5 ml-micro centrifuge tubes and stored at -80 ^{o}C freezer pending further proteomic laboratory work. This research was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by

the Ministry of Health's Medical Research and Ethics Committee (MREC), ID NMRR-16-2572-32869.

Two-Dimensional Electrophoresis

Some 20 µL of plasma from ten individual samples in each group were pooled. Highly-abundant proteins like albumin were removed from the aforementioned sample using a ProteoExtract Albumin Removal Kit (Merck, Darmstadt, Germany). The pooled samples from each group were prepared in triplicates to be run on three immobilised pH gradient (IPG) strips. A total of 500 µg of protein was precipitated with 300 µL of precipitating agent 1 and 300 µL of precipitating agent 2. Following centrifugation at 15 000 rpm, the mixture was washed and air-dried as per the ReadyPrep 2-D Cleanup Kit (Bio-Rad) protocol.

The protein pellet was resuspended in 125 µL of rehydration buffer [7 mol/L, 4% CHAPS, 0.2% Bio-Lyte, 3/10 ampholyte (pH 4–7), and 0.002% bromophenol blue (w/v)]. The protein samples were loaded on 7 cm-IPG strips (Bio-Rad, ReadyStrip; pH 4-7) and allowed to passively rehydrate for 12 hours. Isoelectric focus was performed at a maximum current of 50 µA per strip at 20 ^{o}C with reference to the protocol of PROTEAN IEF Cell System (Bio-Rad). Linear and rapid voltage-ramping were conducted at 250 V for Step 1 and 4 000 V for Step 2 and 3. Prior to second dimension electrophoresis, the IPG strips were equilibrated for 10 minutes with equilibrium solution I [6 M urea, 0.375 M Tris-HCl, 2% sodium dodecyl sulphate (SDS), 20% glycerol, 2% dithiothreitol (DDT); pH [8.8] followed by equilibrium solution II (Equilibirum solution I, in which 2% DTT was substituted with 2.5% iodoacetamide) for another 10 minutes. The strips were then carefully placed on the top of the 2-dimension gels (12% SDS-polyacylamide) with 0.5% agarose. Vertical 2-dimensional electrophoresis (2-DE) was performed at a constant current of 120 V until the blue dye line reached the bottom of the gel. The gels were stained overnight with Coomassie Brilliant Blue R-250 stain and then de-stained with 10% acetic acid plus 40% methanol until the background was acceptable clear. The stained gels were scanned using UMAX POWERLOOK 1000 and the images analysed by the PD Quest 7.2.0 2-D image analysis software (Bio-Rad). The protein spots that were significantly different between the case and control gels were manually excised from the gels using a biopsy punch for identification purposes.

Mass Spectrometry and Protein Identification

The protein samples were digested with trypsin. Their respective peptides were extracted according to the standard techniques and analysed using a Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometer with a 5800 Proteomic Analyser (AB Sciex).[23] The proteins of interest were identified via comparisons with those in the MSPnr100 database which consisted of *Homo sapiens* as taxonomy using the Mascot (Matrix Science) sequence matching software.

Statistical Analysis

The baseline characteristics of the subjects were expressed as means ± standard deviations (SDs). As for the spots protein analysis, the intensity of each spot was measured in a form of optical density (OD) unit. Data from both groups were analysed using Independent Student's T-test and the level of statistical significance was set at 0.05.

RESULTS

Baseline Characteristics

Table I demonstrates the baseline characteristics of controls and AMI patients in the study. All subjects were Malay males who have been carefully matched for age and several risk factors. All participants were active smokers with insignificant difference of body mass index (BMI), waist circumference and total cholesterol levels. Young adults with AMI presented with significantly higher number of smoking pack-year, fasting glucose levels and blood pressure. As for the family history, half of the AMI patients and 40% of the controls had at least one first-degree relative who was previously diagnosed with CAD.

There were more than 100 spots protein were detected in each two-dimensional gel using an IPG strip pH range of 4 to 7. The coefficients of correlation of the three sample gels in the same group were more than 0.8, hence denoting the good consistency of the experimental system. Figure 1 shows the representative two-dimensional gel plasma map of AMI patients and the three spots tht were noted to be differently expressed in AMI

patients vis-à-vis the controls ($p < 0.05$). These proteins were expressed higher in AMI patients than control subjects.

Table I: Baseline characteristics of all participants
Data expressed as means (standard deviations) and analysed using Independent Student's T-test. *indicates a significant difference ($p < 0.05$). AMI: acute myocardial infarction, BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure.

Variables	Control (n=10)		AMI (n = 10)		P value
Age, years	38.2	(3.7)	39.7	(3.7)	0.380
BMI, kg/m^2	24.9	(5.3)	25.7	(3.6)	0.712
Waist circumference, cm	84.6	(9.3)	88.4	(9.3)	0.384
Current smokers, %	100		100		-
Smoking pack years	5	(8)	20	(9)	*0.003
Total cholesterol, mmol/L	6.3	(1.1)	5.7	(1.23)	0.146
Blood glucose, mmol/L	5.0	(0.5)	6.2	(0.5)	*0.000
SBP, mmHg	114	(3)	127	(19)	*0.043
DBP, mmHg	71	(4)	84	(13)	*0.010
Family history of CAD, %	40		50		-

Meanwhile, Table II summarises the charactheristics of the three proteins as analysed by MALDI-TOF mass spectrometry. The proteins were identified as Apolipoprotein AI (Apo AI), Apolipoprotein AIV (Apo AIV), and Haptoglobin.

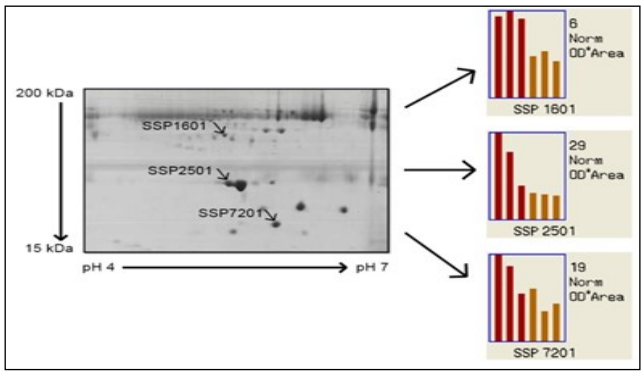

Figure 1: A representative two-dimensional gel containing plasma from acute myocardial infarction (AMI) patients. The spots in the 2-dimentional electrophoresis gels of pH 4 - 7 were examined and analysed using the PD Quest 7.2.0 software. Arrows denote the three spots that were differently expressed in young AMI patients ($p < 0.05$) as compared to the controls, recognized as SSP 1601, SSP 2501 and SSP 7201. The graph corresponds with the intensity of the protein in terms of optical density (OD). Red bars denote data from the AMI group while orange bars denote data from control group.

DISCUSSION

All twenty subjects in the present study were Malay male and active smokers with insignificant differences of the mean age, BMI, waist circumference and total cholesterol levels. In this study, the participants were purposely matched for gender, race, age and several risk factors.

This measure was taken to minimize the effects of confounding factors in the proteomic profiling. The exclusion of participants who were on anti-diabetic, anti-hypertensive and/or anti-hypercholesterolemia agents also to confirm the protein expression modifications observed in the profiling accurately reflect the pathophysiological changes in AMI instead of other medical conditions and drugs interaction. Evidently, the intake of simvastatin treatment of hypercholesterolemia patients modified the plasma expression of proteins such as fibrinogen, Apo AI and Haptoglobin.[24]

The main finding of the present study was that the three proteins – Apo AI, Apo AIV, and Haptoglobin - were expressed differently in young AMI patients relative to healthy individuals. Apo AI is a major component of high-density lipoprotein (HDL); the former accounts for approximately 65% of the mass of the latter's proteins.[25-26] The up regulation of Apo AI in AMI patients in this study was an unexpected outcome as Apo AI is a known anti-atherogenic protein.

In fact, the cardio protective effect of HDL is mainly attributed to the atheroprotective role of Apo AI, the latter of which removes excess cholesterol from peripheral tissues by promoting reverse cholesterol transport (RCT).[25] Accordingly, Basak et al. (2016) have found that the plasma expression of Apo AI was reduced in stable coronary artery disease (CAD) patients.[21] In contrast, our study observed that Apo AI expression was increased in young AMI patients as compared to the controls. These contradictory outcomes suggested that Apo AI might have other actions instead of lipid regulation alone.

The increase in Apo AI expression could have been due to an inflammatory response secondary to the recent myocyte injury. Evidently, Shah et al. (1998) has demonstrated the anti-inflammatory property of Apo AI via an ApoE-null mice model.[27] The administration of reconstituted Apo

Table II: List of proteins that were differently expressed in AMI patients (relative to controls), as identified by MALDI-TOF MS

Spot No	Protein name	Account no	Nominal mass	Calculated pI	Sequence coverage	Fragment
SSP 2501	Apolipoprotein A-I	P012647	30759	5.56	48 %	DLATVYVDVLK
SSP 1601	Apolipoprotein A-IV	NP_000473.2	35344	5.28	31 %	KLVPFATELHER
SSP 7201	Haptoglobin	P00738	45177	6.13	8 %	LRTEGDGVYTLNNEK

MALDI-TOF MS = Matrix-assisted laser desorption/ionization of time of flight mass spectrometry. pI-isoelectric point.

$AI_{(Milano)}$ particles prevented the progression of aortic atherosclerosis and reduced macrophage infiltration.[27] As per another animal study, injections of reconstituted HDL which contained plasma-derived Apo AI led to a reduction in the expression of vascular cell adhesion molecule-1 (VCAM-1) after the first week of arterial injury.[28] The current finding also consistent with Shao et al. that found the level of Apo AI chlorinated and oxidized with myeloperoxidase (MPO) were elevated in coronary artery disease (CAD) and acute coronary syndrome (ACS) patients.[29] MPO is a heme protein that is expressed at high level by inflammatory macrophage in human atherosclerotic tissue. The study also reported that the elevation of Apo AI in CAD and ACS patients was inversely correlated with HDL's cholesterol efflux capacity.[29]

Apparently, acute inflammation may lead to conversion of Apo AI from anti-inflammatory particles to pro inflammatory particles. Consistently, Ogasawara et al. have observed that the level of serum Apo AI bonded to oxidized low density lipoprotein (LDL) was higher in CAD patients than in the control group.[30] Apo AI-LDL that was produced through an oxidizing reaction that occurs during CAD has been proposed to be a more sensitive inflammatory protein marker of CAD than C-reactive protein (CRP).[30] Therefore, the elevation of Apo AI protein as observed in the present study was likely exhibiting its inflammatory protein function in response to the recent cardiac injury.

Meanwhile, Apolipoprotein AIV (Apo AIV) is a 46 kDa glycoprotein that circulates in the plasma. It is also a part of a small, lipid-poor HDL-like particle that does not contain Apo AI.[31] Apo AIV is also known to exhibit an anti-atherogenic property since acts as an activator of lecithin cholesterol acyl transferase (LCAT) during RCT.[32] Apo AIV inhibits the oxidation of lipoprotein oxidation in the plasma, thereby protecting the vessel walls from radical-induced damage.[33] Apo AIV has been reported to be significantly associated with stable CAD in which it was less expressed in CAD patients relative to the normal controls (7.6 ± 3.5 mg/dL vs 10.4 ± 4.1 mg/dL; $p < 0.001$).[34]

However, the current study has found that expression of Apo AIV in AMI patients was higher than that in the controls. The current finding suggested that Apo AIV might serve functions other than lipid metabolism regulation during the acute-phase response. The overexpression of Apo AIV could have been a form of response to the acute inflammatory process. This was consistent with the outcome of a study whereby Apo AIV was deemed to be a positive acute phase protein since there was an increase in the level of Apo AIV hepatic mRNA secondary to endotoxin injections.[35] Consequently, the plasma expression of Apo AIV increased during acute inflammation, as observed in our study.

Another likely cause of the contradictory finding could have been the difference in the timing of sample collection. Instead of obtaining samples from stable CAD patients as in the former study, blood samples for this study were collected when the patients presented to the Emergency Department following acute clinical onset of AMI. Therefore, our results were more likely to reflect the Apo AIV changes during the acute phase reaction than that during the development of the disease. Another explanation to the contradicted finding in the trend of expression for Apo AIV is the difference in the age group of study participants being analysed.

Participants in previous studies were much older with the mean age above 60 years.[34,36] Thus, the present study might reflect the proteomic changes during AMI in younger populations that possibly associated with more significant inflammation reaction.

In the current study, Haptoglobin was also observed to be up regulated in AMI patients. It is known as an acute-phase reactant of non-cardiac origin that acts as a high-affinity haemoglobin-binding protein and an antioxidant. The Apolipoprotein Mortality Risk Study (AMORIS) Study – a large prospective trial on 342 125 subjects – has established that Haptoglobin was a significant risk factor of AMI.[37] In fact, the protein was almost as predictive of AMI as total cholesterol since there was a 4.2-fold increase in the risk of the disease in the individuals whose Haptoglobin levels were in the upper quartile.[37] Consistent with its role as an inflammatory marker, the hepatic mRNA of Haptoglobin attained a peak concentration 24-48 hours post-inflammation, after which it reassumed its basal levels within 2-7 days. The principal inducer of the gene expression of Haptoglobin was found to be a cytokine called IL-6, which is the main mediator of the acute-phase response.[38]

The increase in Haptoglobin expression following an inflammatory reaction has also led to the hypothesis that Haptoglobin might have anti-inflammatory properties. This is in light of the fact that Haptoglobin binds to highly-toxic free haemoglobin, hence facilitates the removal of the latter and subsequently eliminating the trigger of oxidative tissue damage. Haas et al. (2011), who conducted proteomic analysis of the plasma samples of AMI patients, have followed-up the patients for one year to assess the occurrence of heart failure as per the New York Association (NYHA) classification.[39] According to the study, high levels of Haptoglobin during AMI were associated with lower NYHA grades. Therefore, while a high level of Haptoglobin could be a risk marker for AMI during the development of the disease, it might be beneficial post AMI owing to the potentially protective function of its peroxidase.

LIMITATIONS OF THE STUDY

The 2-DE proteomic analysis in this study may only serve as a screening for the potential proteins to be candidate biomarkers due to two factors; the use of pooled sample and the small sample size. Consequently, the present study did not provide information on the biological variations of the individual sample. Therefore, this primary finding requires further studies with larger and more heterogeneous sample to capture the environmental, genetic, and risk factors variations in the studied population. In addition, the expression of the candidate biomarkers need to be verified using more established proteins detection methods such as enzyme-linked immunosorbent assay (ELISA). Importantly to note that the current study was limited to the proteomic analysis of young adult with AMI in comparison to the healthy control in the same age group. Therefore, differential proteomic analysis of samples collected from young and elderly patients during the same timeline may result in better proteomic profiles comparisons between young AMI and older counterparts.

CONCLUSION

In conclusion, the up regulation of Apo AI, Apo AIV, and Haptoglobin in young AMI reflected their significant roles in the inflammatory reaction of the disease. These proteins could be potential protein biomarkers and are likely to be useful for the understanding of the molecular mechanisms of young AMI.

CONFLICT OF INTEREST

There are no conflicts of interest.

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