

# Potential biomarkers found by protein profiling may provide insight for the macrovascular pathogenesis of diabetes mellitus

William C.S. Cho<sup>a,\*</sup>, Tai-Tung Yip<sup>b</sup>, Wai-Shing Chung<sup>a</sup>, Albert W.N. Leung<sup>c</sup>,  
Christopher H.K. Cheng<sup>d</sup> and Kevin K.M. Yue<sup>a,\*</sup>

<sup>a</sup>*School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China*

<sup>b</sup>*Ciphergen Biosystems Inc., Fremont, CA, USA*

<sup>c</sup>*School of Chinese Medicine and Health Care, The Chinese University – Tung Wah Community College, Hong Kong, China*

<sup>d</sup>*Department of Biochemistry, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China*

**Abstract.** Diabetes mellitus (DM) is an alarming threat to health of mankind, yet its pathogenesis is unclear. The purpose of this study was to find potential biomarkers to serve as indicators for the pathogenesis of DM in a time course manner. Based on our previous findings that oxidative stress occurred at week 8, aorta lysate and sera of 102 streptozotocin (STZ)-induced diabetic and 85 control male Sprague-Dawley rats were obtained at the 4th, 8th and 12th week after STZ injection. The protein profiles were studied employing surface-enhanced laser desorption/ionization time-of-flight mass spectrometry technology in attomole sensitivity range. In the aorta, a multiple biomarker panel was discovered at the 4th week. At the 8th week, 4 biomarkers were found, while at the 12th week, 3 biomarkers were identified. In the sera, a triplet of 3 peaks and 2 biomarkers were all discovered to have 100% classification accuracy rate to differentiate the DM and control groups at all time intervals. Besides, 2 biomarkers were also found to have high classification value at week 12. Comparing the aorta and sera from DM and non-DM rats, a bundle of potential biomarkers with significant changes in peak intensities and high classification values were found. Two of the serum biomarkers matched with islet amyloid polypeptide and resistin in the SWISS-PROT knowledgebase. Validation has been conducted using immunoassay kits. These potential biomarkers may provide valuable insight on the pathogenesis of DM and macrovascular complications.

**Keywords:** Diabetes mellitus, protein profiling, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, macrovascular complications

## 1. Introduction

Diabetes mellitus (DM) is a chronic progressive disease that leads to the development of microvascular and macrovascular complications. Recent hypotheses on the pathogenesis of diabetic complications included

activation of protein kinase C, polyol pathway, non-enzymatic glycation and oxidative stress [1–3]. There is now increasing evidence that the generation of reactive oxygen species (ROS) and subsequent oxidative stress may be the common factor in all these pathways [4]. The increased polyol (sorbitol/aldose reductase) pathway flux, production of advanced glycation end-products, generation of ROS, and activation of diacylglycerol and protein kinase C isoforms are all be-

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\*Corresponding author. Tel.: +852 2958 5441; Fax: +852 2715 6139; E-mail: williamcscho@gmail.com or kkmyue@hkbu.edu.hk.

lieved to be biochemical pathways for microvascular complications of DM. These pathways may contribute to endothelial damage and dysfunction, and may alter gene functioning as well [5]. Nevertheless, the exact relationships among hyperglycemia, the diabetic state, and oxidative stress remain undetermined.

Automated proteomic technology, supplementary with advance bioinformatics, could be useful for the identification of molecular alterations implicated in DM. Based on our previous findings of the onset of redox changes in the eye and aorta of DM rats 8 weeks after streptozotocin (STZ) injection [6], as well as the activities of different antioxidant enzymes during the development of DM [7], a diabetic rat model was established for the quantitative study of the overall change in protein expression using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS).

The STZ-induced diabetic rat is a good model of the diabetic human for the development of diabetic neuropathy, retinopathy and ocular cataracts and some cardiovascular changes, especially electrophysiological and functional changes, even though atherosclerosis and hypertension are not produced. Application of SELDI-TOF MS technology was to make use of the tremendous power of proteomics to track the protein expression profiles in the aorta lysate and sera from rats with DM and normal controls at different time course in order to identify potential biomarkers that will provide valuable insights on the early detection and pathogenesis of diabetic complications associating macrovasculature. As early diagnosis is important for the future prognosis and control of DM, advances in understanding the pathogenic mechanisms leading to diabetes and macrovascular complications may facilitate the discovery of new therapeutic targets and reducing the risk of developing DM and its complications.

## 2. Materials and methods

### 2.1. Animal models

Wild-type non-diabetic male Sprague Dawley (SD) rats weighed 250–300 g were used for the experiments. There were 3 cohorts of rats for the 3 time points (4th, 8th and 12th week) study. Each cohort was randomly divided into 2 groups, in which one group rats were injected with STZ to induce DM (N = 32, 38 and 32 respectively) and the rest were the normal controls (N = 30, 30 and 25 respectively). All SD rats were kept

on a standard pellet diet and tap water. The animals were fasted overnight (8–10 h) before the day of STZ injection.

Double dose of 40 mg/kg STZ in 0.1 M sodium citrate at pH 4.5 was injected intraperitoneally using G25 syringe in the diabetic group within 2 days while the control group was injected with the corresponding amount of 0.1 M sodium citrate instead [8]. After STZ administration, all rats were accommodated in laboratory room with 12 h dark/light cycle, and allowed food and water *ad libitum* throughout the experiment.

At the first day of the 1st, 2nd, 4th, 8th and 12th week after STZ injection, venous blood samples were collected from the tails for both groups of rats in order to measure their corresponding serum glucose level. All rats were fasted 8–10 h before blood samples taking. Those rats with serum glucose level >300 mg/dL were regarded as DM.

Descending aorta from all DM rats (N = 21, 26 and 22 at week 4, 8 and 12 respectively) and normal control rats (N = 29, 29 and 25 at week 4, 8 and 12 respectively) were collected on the day of sacrifice at the 3 time points (the 4th, 8th and 12th week). Tissues from the aorta were excised from the rats, rinsed with ice-cold phosphate-buffered saline and blotted dry. They were frozen immediately in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for the elucidation of protein profiling. Descending aorta from all DM rats were evaluated by proteinchip array profiling in parallel with the normal controls.

Apart from collecting and examining tissue samples, blood samples from the DM and control rats were also collected. From the DM group, 15, 11 and 20 rats were randomly chosen at the 3 time points respectively, while 15, 11 and 15 rats were randomly chosen from the control group accordingly. Each blood sample was allowed to clot and centrifuged at  $1,500 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Sera were collected, aliquoted and kept frozen at  $-80^{\circ}\text{C}$  until use.

### 2.2. Tissue samples preparation

After homogenization of tissue samples, the mixture was centrifuged at  $12,000 g$  for 15 min at  $4^{\circ}\text{C}$ . The resulting protein was then washed with 0.3 M guanidine hydrochloride/95% ethanol (2 mL per 50 mg of tissue). After washing, 2 mL 100% ethanol was added and centrifuged. Finally 150  $\mu\text{L}$  6 M guanidine thiocyanate, 1% octyl- $\beta$ -D-glucopyranoside (OGP) and 350  $\mu\text{L}$  9 M urea, 2% 3-[3-(Cholamidopropyl) dimethylammonio]-1-proanesulfonate (CHAPS) in 50 mM Tris-HCl pH 9

were added to the protein pellet for dissolution. The protein lysate was then frozen at  $-80^{\circ}\text{C}$  until use.

Lysate samples were thawed and 3 times of  $120\ \mu\text{L}$  of each lysate was fractionated in a  $120\ \mu\text{L}$  anion exchange Q HyperD F 96-well filter plate (Ciphergen Biosystems Inc., Fremont, CA, USA).

### 2.3. Serum samples preparation

Serum samples were thawed and  $20\ \mu\text{L}$  of each serum was denatured by adding  $30\ \mu\text{L}$  of  $50\ \text{mM}$  Tris-HCl buffer containing  $9\ \text{M}$  urea and  $2\%$  CHAPS at pH 9. The proteins were fractionated in a  $180\ \mu\text{L}$  anion exchange Q HyperD F 96-well filter plate (Ciphergen Biosystems Inc., Fremont, CA, USA).

### 2.4. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry

The proteinchip arrays (Ciphergen Biosystems Inc., Fremont, CA, USA) used in this study included the weak cation exchange (WCX) and immobilized metal affinity capture (IMAC) copper surfaces. The small biochemical surfaces ( $1\text{--}2\ \text{mm}^2$ ) on the chips allowed differential capture of proteins based on the intrinsic properties of the proteins themselves. The reactive surfaces of the WCX proteinchip array are negatively charged with carboxylate groups and they capture positively charged proteins. The reactive surfaces of the IMAC proteinchip array have nitriloacetic acid functional group to capture proteins and peptides which have affinity for metals; proteins with exposed histidine, tryptophan and/or cysteine typically bind to metals immobilized on this chip surfaces. Biological samples of lysate and serum were directly applied onto the surfaces of these 2 kinds of proteinchips, where proteins with affinities to the bait surface would bind. After subsequent washing by appropriate binding buffer to remove non-specifically or weakly bound proteins, only proteins that interacted with the array chemical surface were retained for further analysis. The bound proteins were then laser desorbed and ionized for mass spectrometry (MS) analysis. The instruments only provide a profile of the relative abundance of proteins in a given sample, yet they do not determine protein concentrations directly. Total ion current normalization was routinely performed to account for differences in total protein concentration amongst the samples. Previous analysis has revealed a dynamic range of  $1\text{--}2,000\ \text{fmol}/\mu\text{L}$  with very good linear correlation between signal inten-

sities, demonstrating equally good signal quality over the entire protein concentration [9].

To increase the number of protein peaks to be visualized, an anion exchange fractionation procedure was performed in which the serum was separated into 6 different fractions. The flow-through fraction including pH 9 (wash with  $50\ \text{mM}$  Tris-HCl buffer, containing  $0.1\%$  OGP), pH 7 (wash with  $100\ \text{mM}$  sodium phosphate buffer, containing  $0.1\%$  OGP), pH 5 (wash with  $100\ \text{mM}$  sodium acetate buffer, containing  $0.1\%$  OGP), pH 4 (wash with  $100\ \text{mM}$  sodium acetate buffer, containing  $0.1\%$  OGP), pH 3 (wash with  $50\ \text{mM}$  sodium citrate buffer, containing  $0.1\%$  OGP) were collected by stepwise decrease in pH, and an organic eluent (wash with buffer containing  $33\%$  isopropanol,  $16.7\%$  acetonitrile and  $0.1\%$  trifluoroacetic acid) was finally collected. This fractionation procedure significantly increased the number of peaks detectable from each individual sample [10].

The fractions were then diluted and profiled on an IMAC type 3 or type 30 proteinchip array and a WCX type 2 proteinchip array. The IMAC3, IMAC30 and WCX2 arrays were chosen to increase the proportion of the lysate and serum proteome represented on the arrays for mass spectrometric analysis. All the proteinchip arrays were pretreated according to the manufacturer's protocols. For IMAC3/IMAC30,  $80\ \mu\text{L}$  of binding buffer ( $100\ \text{mM}$  sodium phosphate containing  $0.5\ \text{M}$  NaCl at pH 7) and  $20\ \mu\text{L}$  of each fractionated sample were applied to each well of bioprocessor (Ciphergen Biosystems Inc., Fremont, CA, USA) and incubated on a shaker for 30 min at room temperature. For WCX2,  $90\ \mu\text{L}$  of binding buffer ( $100\ \text{mM}$  sodium acetate at pH 4) and  $10\ \mu\text{L}$  of each fractionated sample were applied to each well of bioprocessor (Ciphergen Biosystems Inc., Fremont, CA, USA) and incubated on a shaker for 30 min at room temperature. The chips were washed twice with the binding buffer on the bioprocessor, followed by 2 rinses in distilled water and then air dried. Each fraction was then applied to both the IMAC and WCX2 proteinchips [11].

Sinapinic acid (Ciphergen Biosystems Inc., Fremont, CA, USA) served as energy absorbing molecule, prepared per the manufacturer's protocols, was applied onto each proteinchip array before SELDI analysis to facilitate desorption and ionization of proteins.

### 2.5. Data acquisition

The chip was air-dried and read in a Protein Biological System IIC Proteinchip Reader (Ciphergen Biosys-

tems Inc., Fremont, CA, USA) and the TOF spectra were generated by 352 laser-shots average per sample. The process involved surface-enhanced laser desorption and ionization of proteins from the array surfaces, and detection by time-of-flight mass spectrometry to determine the precise molecular weight (MW) of multiple proteins from the samples. Mass accuracy was calibrated (from 1–7 kDa) externally to < 0.1% using the All-in-1 Peptide and Protein Standard (Ciphergen Biosystems Inc., Fremont, CA, USA).

A protein retentate map was generated in which individual proteins were displayed as unique peaks based on their mass to charge ratios ( $m/z$ ). Selective protein retention combined with the ability to assess a protein's MW made it possible to identify hundreds of unique proteins from a single sample [12]. Lysate and serum samples from the comparable groups were run concurrently, intermingling on multiple chips to minimize experimental variations.

## 2.6. Bioinformatic analysis and statistics

The proteinchip profiling spectra from all the lysate and serum samples were collected and analyzed by ProteinChip Software 3.2.1 (Ciphergen Biosystems Inc., Fremont, CA, USA) and Biomarker Wizard (Ciphergen Biosystems Inc., Fremont, CA, USA). ProteinChip Software analyzed and compared multiple mass spectra to identify potential biomarkers. Peaks between 500 and 200,000  $m/z$  values were autodetected with a signal to noise ratio (S/N) of > 5 and the peaks clustered using second-pass peak selection with  $S/N > 2$  and a 0.3% mass window. The  $m/z$  values that were within 0.3% mass accuracy window were considered to be identical between replicates. The resulting peak intensity values were logarithmically transformed to reduce the variance of the data over multiple samples [13]. All of the spectra were compiled, normalized to the total ion current of  $m/z$  between 2 and 100 kDa, and the baselines subtracted. Biomarker Wizard presented data sets of normalized peak intensities with statistical value ( $P$  value) for further analysis. Data was viewed in a variety of formats including spectra view and gel view.

Biomarker Patterns Software (BPS) 4.0.1 (Ciphergen Biosystems Inc., Fremont, CA, USA) was then applied to reveal hierarchy of specific proteins. Using the classification and regression trees algorithm to discover specific patterns, the results were presented in tree models [14]. The software identified and ranked the potential importance of individual biomarkers for further protein characterization analysis. It was also

used to determine multiple biomarkers correlation with phenotype in translational and clinical studies to improve sensitivity and specificity over single biomarker methods. The software built hierarchy trees of protein multi-markers and provided their predictive accuracies. It built up classification model with a training data set consisted of samples. A classification tree was set up to divide the data set into 2 bins based on the intensities of peaks. At each bin, a peak intensity threshold was set. If the peak intensity of a sample was lower than or equal to the threshold, this sample would go to the left-side bin. Otherwise, the sample would go to the right-side bin. The process would go on until a blind sample entered a final bin, either labeled as DM sample or control sample. Peaks selected by the process to form the model were the ones that yielded the least classification error when they were combined to use. The software built a large number of small trees, each designed to help correcting the errors of its predecessors. The pre-selecting variables allowed a more focused analysis and improved predictive accuracy.

The diagnostic test was then performed to determine the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and classification accuracy rate of top ranking potential single biomarkers [15].

Finally, potential single biomarkers with high classification values from different fractions and time points were compared with one another to determine the top potential biomarkers. In case if no significant single biomarker was found, multiple biomarkers panel identified by BPS was presented.

Unless otherwise specified, results are reported as arithmetic mean  $\pm$  S.D. A  $P$  value of < 0.05 determined by the non-parametric 2 samples' Mann-Whitney  $U$  test was considered to indicate a statistically significant difference [16].

## 2.7. Islet amyloid polypeptide measurement

The quantitative measurement (mean  $\pm$  S.E.) of islet amyloid polypeptide (amylin) in sera was performed using the Amylin (Rat) Enzyme Immunoassay (EIA) Kit (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) with intra-assay variability < 5%. Briefly, the immunoplate in the kit was pre-coated with secondary antibody and the nonspecific binding sites were blocked. The secondary antibody could bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment would be competitively bound by both biotinylated peptide and peptide standard or tar-

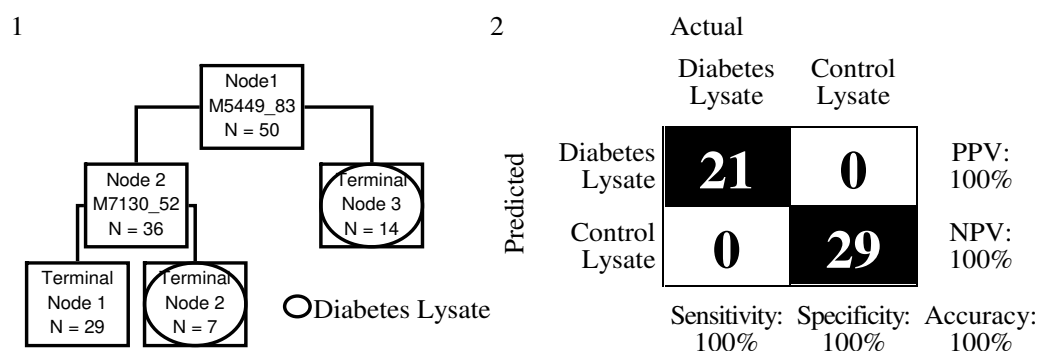


Fig. 1. Tree model and classification values of  $m/z$  5,450 and 7,131 Da in the descending aorta at week 4. The results were expressed as tree model (1) and diagnostic test (2). Diabetic group ( $N = 21$ ) was compared with the control lysate ( $N = 29$ ). PPV, positive predictive value; NPV, negative predictive value.

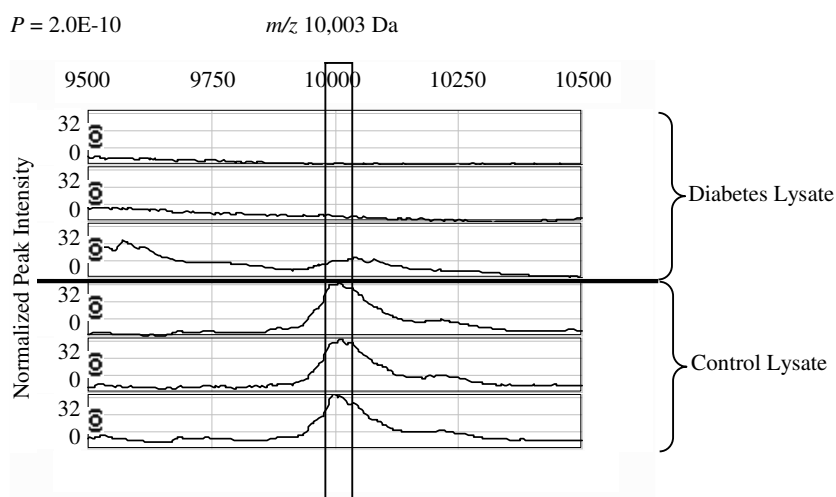


Fig. 2a. Biomarker levels and views of selected spectra in the descending aorta at  $m/z$  10,003 Da at week 8. Statistical value:  $P = 2.0E-10$ . Diabetic group was compared to with the control lysate.

geted peptide in samples. The biotinylated peptide was able to interact with streptavidin-horseradish peroxidase (SA-HRP) which catalyzed the substrate solution composed of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide to produce a blue colored solution. The enzyme-substrate reaction was stopped by hydrogen chloride and the solution turned to yellow. The absorbance of the resulting yellow product was measured at 450 nm. The intensity of the yellow was directly proportional to the amount of biotinylated peptide-SA-HRP complex but was inversely proportional to the amount of the peptide in standard solutions or samples. This was due to the competitive binding of the biotinylated peptide and the peptide in standard solutions or samples to the peptide antibody (primary antibody). A standard curve of a peptide with known concentration

could be established accordingly. The peptide with unknown concentration in samples could be determined by extrapolation to this standard curve.

## 2.8. Resistin measurement

Fasting serum resistin level (mean  $\pm$  S.E.) was determined by Resistin (Rat) Enzyme-linked Immunosorbent Assay (ELISA) Kit (BioVender Laboratory Medicine, Brno, Czech Republic) with an intra-assay variation of 4.3% and an inter-assay variation of 7.2%. In brief, calibrators or samples were incubated with a rabbit polyclonal anti-rat resistin antibody coated in microtiter wells. After 1 h incubation and washing, biotin-labelled rabbit polyclonal anti-rat resistin antibody was added and incubated with captured

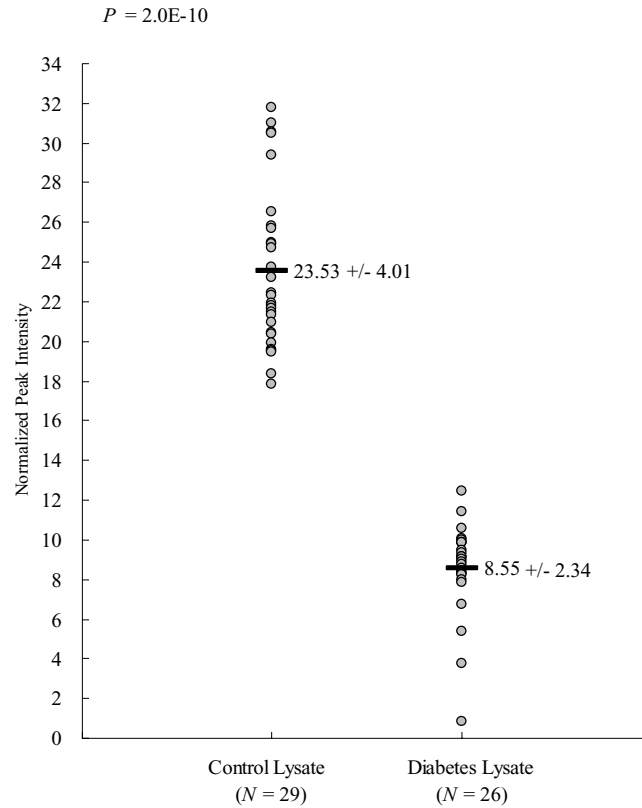


Fig. 2b. Biomarker levels in the descending aorta at  $m/z$  10,003 Da at week 8. The result was expressed as scatter plot. Statistical value:  $P = 2.0E-10$ . Diabetic group ( $N = 26$ ) was compared with the control lysate ( $N = 29$ ).

resistin. Following a thorough wash, SA-HRP conjugate was added. After 1 h incubation and the last washing step, the remaining conjugate was allowed to react with the substrate  $H_2O_2$ -tetramethylbenzidine. The reaction was stopped by addition of acidic solution and absorbance of the resulting yellow product was measured at 450 nm. A standard curve was constructed by plotting absorbance values versus resistin concentrations of calibrators, and concentrations of unknown samples were determined using this standard curve.

### 3. Results

#### 3.1. Differential protein expression in the descending aorta

At week 4, no single biomarker was detected to be statistically different in the descending aorta between the DM group ( $N = 21$ ) and the control group ( $N = 29$ ). Alternatively, using the multiple biomarkers panel of  $m/z$  5,450 and 7,131 Da, a high classification

value (100%) was achieved. This multiple biomarkers panel was identified by BPS. The tree model is shown in Fig. 1(1) and the diagnostic test showing accuracy rate, sensitivity, specificity, PPV and NPV is shown in Fig. 1(2).

At the 8th week, 4 biomarkers at  $m/z$  10,003 ( $8.55 \pm 2.34$  vs  $23.53 \pm 4.00$ ;  $P = 2.0E-10$ ), 10,040 ( $9.95 \pm 2.90$  vs  $20.14 \pm 3.84$ ;  $P = 2.0E-10$ ), 11,110 ( $4.25 \pm 1.61$  vs  $17.43 \pm 4.45$ ;  $P = 2.0E-10$ ) and 11,275 ( $5.23 \pm 1.88$  vs  $12.21 \pm 2.66$ ;  $P = 2.0E-10$ ) Da were found to have high classification values (all of accuracy rate 100%) in the descending aorta to differentiate the DM group ( $N = 26$ ) and the control group ( $N = 29$ ). There were significant decreases (64%, 51%, 76% and 57% respectively) in the descending aorta of the DM group as compared with the corresponding control group.

As shown in Fig. 2(1–2), normalized peak intensities in the descending aorta of the control group at  $m/z$  10,003 Da at week 8 were recorded from 17.2 to 31.7, significant decreases (normalized peak intensities from 0.8 to 13.1) were found in the DM group. As shown in Fig. 2(3), the mean of biomarker level at  $m/z$  10,003 Da

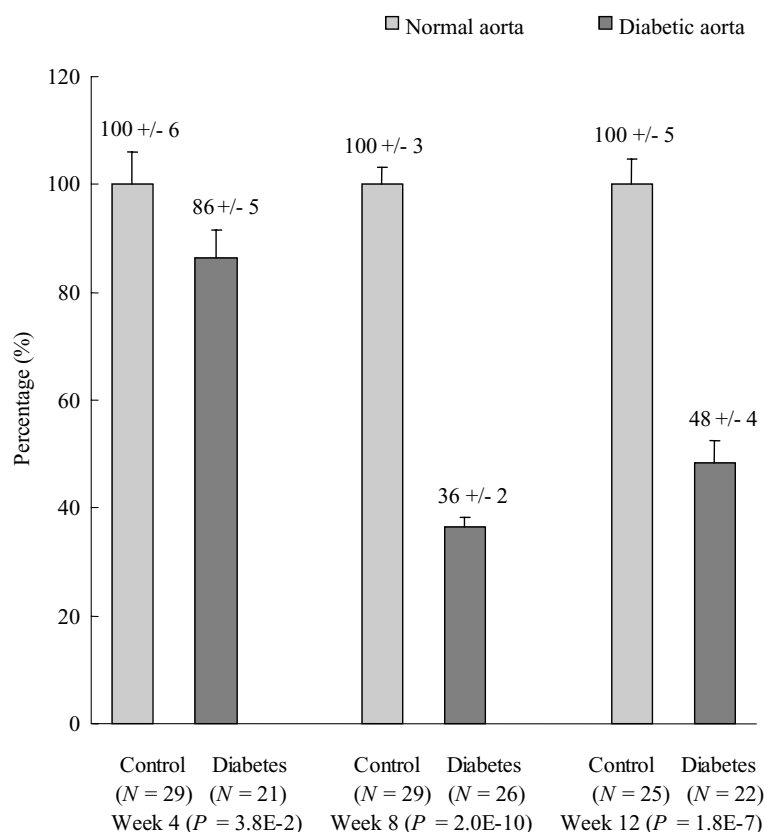


Fig. 2c. Differential expressions (mean  $\pm$  S.E.) in the descending aorta at  $m/z$  10,003 Da at all time intervals. The results were expressed as bar chart. Statistical value:  $P = 3.8E-2$  at week 4,  $P = 2.0E-10$  at week 8,  $P = 1.8E-7$  at week 12. Diabetic group ( $N = 21, 26$  and  $22$  respectively) was compared with the control lysate ( $N = 29, 29$  and  $25$  respectively).

was found to be slightly decreased (14%) in the lysate derived from DM rats when compared with the control lysate at week 4, but was found to be significantly decreased at week 8 and week 12 (64% and 52% respectively). This potential biomarker only had a low classification value (accuracy rate 68%) at week 4, but had achieved its highest classification value (accuracy rate 100%) at week 8, and then slightly dropped (accuracy rate 87%) at week 12.

At the 12th week, 3 biomarkers at  $m/z$  6,552 ( $3.63 \pm 1.01$  vs  $1.49 \pm 0.41$ ;  $P = 1.1E-7$ ), 8,050 ( $1.82 \pm 0.41$  vs  $0.35 \pm 0.24$ ;  $P = 4.5E-9$ ) and 8,193 ( $0.92 \pm 0.28$  vs  $3.34 \pm 1.40$ ;  $P = 2.9E-8$ ) Da were found to have high classification values (accuracy rate 96%, 100% and 98% respectively) in the descending aorta to differentiate the DM group ( $N = 22$ ) from the control group ( $N = 25$ ). The means of biomarker levels at  $m/z$  6,552 and 8,050 Da were found to be significantly increased (144% and 414% respectively) in the lysate derived from DM rats, whereas the mean of biomarker levels at  $m/z$  8,193 Da were found to be significantly decreased

(73%) in the lysate derived from DM rats. Among them, biomarker at  $m/z$  8,193 Da had a progressive pattern at the 3 time points.

As shown in Fig. 3(1–2), 24 out of 25 normalized peak intensities in the descending aorta of the control group at  $m/z$  8,193 Da at week 12 were recorded from 1.7 to 6.4, significant decreases (normalized peak intensities from 0.4 to 1.6) were found in the DM group. Mean of biomarker level was only 12% decreased in the DM group at week 4 at  $m/z$  8,193 Da as compared with the control group, 46% decreased at week 8, and significantly decreased by 73% at week 12. As shown in Fig. 3 (3),  $m/z$  8,193 Da only had a low classification value (accuracy rate 60%) at week 4, with a gradual improvement (accuracy rate 62%) at week 8, and finally achieved its highest classification value (accuracy rate 98%) at week 12.

### 3.2. Differential protein expression in the sera

A triplet of peaks at  $m/z$  5,125, 5,143 and 5,161 Da and 2 other biomarkers at  $m/z$  6,146 and 13,930 Da

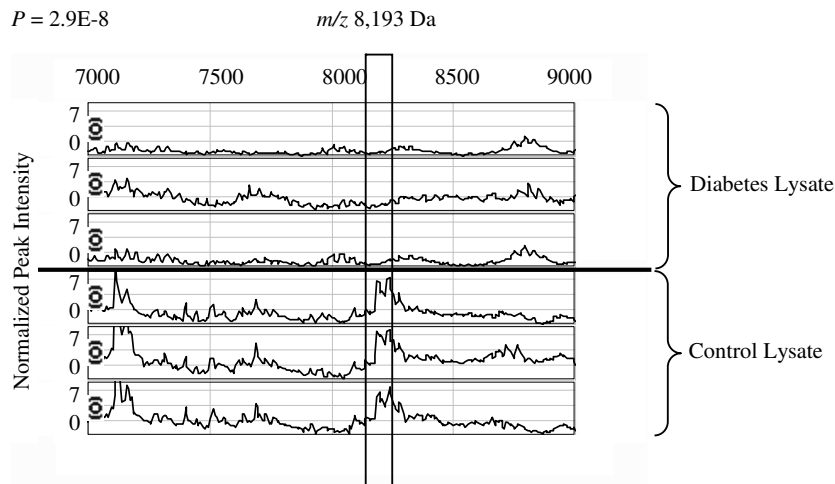


Fig. 3a. Peak view of selected spectra in the descending aorta at  $m/z$  8,193 Da at week 12. Statistical value:  $P=2.9E-8$ . Diabetic group was compared with the control lysate.

were found to have high classification values (accuracy rate all 100%) in the sera to differentiate the DM group and the control group at all time intervals. Besides, 2 biomarkers at  $m/z$  10,018 and 12,074 Da were also found to have high classification values (accuracy rate 83% and 89% respectively) at week 12. Means of biomarker levels at  $m/z$  5,143 ( $29.52 \pm 10.17$  vs  $4.28 \pm 1.32$  at week 4;  $P = 3.1E-6$ ), 6,146 ( $0.14 \pm 0.05$  vs  $0.04 \pm 0.03$  at week 8;  $P = 7.1E-5$ ) and 10,018 ( $2.24 \pm 0.84$  vs  $1.31 \pm 0.59$  at week 12;  $P = 3.6E-4$ ) Da were found to be significantly increased (589%, 250% and 71% respectively) in the sera of the DM rats, while means of the biomarker levels at  $m/z$  12,074 ( $3.08 \pm 1.27$  vs  $6.10 \pm 2.02$  at week 12;  $P = 6.3E-5$ ) and 13,930 ( $14.16 \pm 2.61$  vs  $25.64 \pm 3.72$  at week 4;  $P = 5.7E-7$ ) Da were significantly decreased (50% and 45% respectively) in the sera of DM rats.

As shown in Fig. 4(1–2), normalized peak intensities at  $m/z$  5,143 Da in the sera of the control group ( $N = 15$ ) at week 4 were recorded from 2.3 to 7.7, significant increases (normalized peak intensities from 13.4 to 49.8) were found in the DM group ( $N = 15$ ). Means of biomarker level were significantly increased (589% at week 4, 826% at week 8 and 582% at week 12 respectively) in the DM group at  $m/z$  5,143 Da as compared with the control group at all time intervals ( $29.52 \pm 10.17$  vs  $4.28 \pm 1.32$  at week 4,  $P = 3.1E-6$ ;  $0.30 \pm 0.20$  vs  $0.03 \pm 0.03$  at week 8,  $P = 7.1E-5$ ;  $10.11 \pm 5.21$  vs  $1.48 \pm 0.38$  at week 12,  $P = 5.7E-7$ ). As shown in Fig. 4(3), biomarker at  $m/z$  5,143 Da was discovered to have high classification value (all of accuracy rate 100%) in the sera to differentiate the DM group and the control group at all time intervals.

### 3.3. Potential common biomarkers in the descending aorta and sera

Significantly, biomarkers of  $m/z$  5,143 ( $6.49 \pm 1.20$  vs  $4.64 \pm 0.73$ ;  $P = 9.8E-8$ ) and 5,161 ( $6.75 \pm 1.19$  vs  $4.45 \pm 0.86$ ;  $P = 2.1E-8$ ) Da were found to have high classification values (from 87% to 94%) in the descending aorta to differentiate the DM group and the control group at week 12. Means of biomarker level were also increased (from 40% to 52%) in the DM group as compared with the control group. These lysate biomarkers were found in anion exchange column fraction 5, exactly the same as the serum biomarkers. The identical molecular weights and chromatographic separation characteristics indicated that these peaks might be potential common biomarkers for both the lysate and serum samples.

### 3.4. Protein matching and validation

Potential serum biomarker at  $m/z$  10,018 Da found in this study matched with islet amyloid polypeptide (precursor) (IAPP) at MW 10,015 from *Rattus norvegicus* (rat) in the SWISS-PROT knowledgebase, with 0.03% higher than the molecular mass recorded by SWISS-PROT. The quantitative measurement of IAPP in serum was performed by EIA to validate the protein matching. Amylin concentrations of the DM rats' sera at week 12 were 51% higher than those of the normal controls ( $50.63 \pm 3.93$  ng/mL vs  $33.48 \pm 2.96$  ng/mL;  $P < 0.05$ ).

Simultaneously, another potential serum biomarker at  $m/z$  12,074 Da in this study was found to match



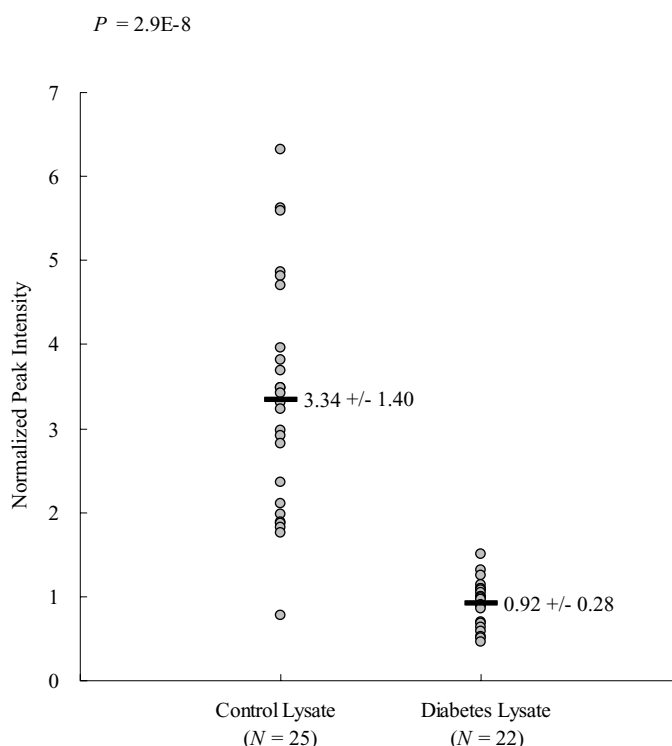


Fig. 3b. Levels of proteins in the descending aorta at  $m/z$  8,193 Da at week 12. The result were expressed as scatter plot. Statistical value:  $P = 2.9E-8$ . Diabetic group was compared with the control lysate.

with resistin-like alpha (precursor) (RSNA) at MW 12,076 from *Rattus norvegicus* (rat) in the SWISS-PROT knowledgebase, with 0.02% lower than the molecular mass recorded by SWISS-PROT. The quantitative measurement of RSNA in serum was performed by ELISA to validate the protein matching. Resistin concentrations of the DM rats' sera at week 12 were 42% lower than those of the normal controls ( $18.93 \pm 1.17$  ng/mL vs  $32.49 \pm 1.36$  ng/mL;  $P < 0.001$ ).

#### 4. Discussion

Over the past decade, it has been obvious that the prevalence of diabetes is increasing rapidly. Nevertheless, it is easily detected and diagnosed [17]. There are a number of screening tests for DM, such as the oral glucose tolerance test, fasting plasma glucose test, random blood glucose test, glycosylated hemoglobin measurement, blood fructosamine measurement, urine glucose test, etc. However, it is still impossible to predict, eliminate or cure diabetes and its complications [18], this is partly attributed by a lack of understanding on its mechanism of pathogenesis. The complicated, chang-

ing pattern of protein expression contains important information about the pathologic process taking place in the cells of actual tissue [19], utilization of this information for the selection of suitable drug or targets in the field will be very useful [20].

The severity of diabetes and its complications progress with time in uncontrolled patients. An enormous challenge resides in the obvious fact that proteome is dynamic, nevertheless proteomic technologies provide a snap shot approach to cellular responses study [21].

Two dimensional polyacrylamide gel electrophoresis as a tool for the analysis of complex protein mixtures, though powerful, is a laborious, time-intensive process which can not be modified to capture the rapid dynamic changes in protein expression from hundreds or thousands of samples. Its inability to analyze very small proteins ( $< 10$  kDa), and unable to detect low or even moderately expressed proteins when analyzing lysate generated from few hundreds of cells become a great limitation for its development [22].

Hence, there is a need for introducing advance technologies capable of sensitive, high-throughput protein expression display which can validate the correlation

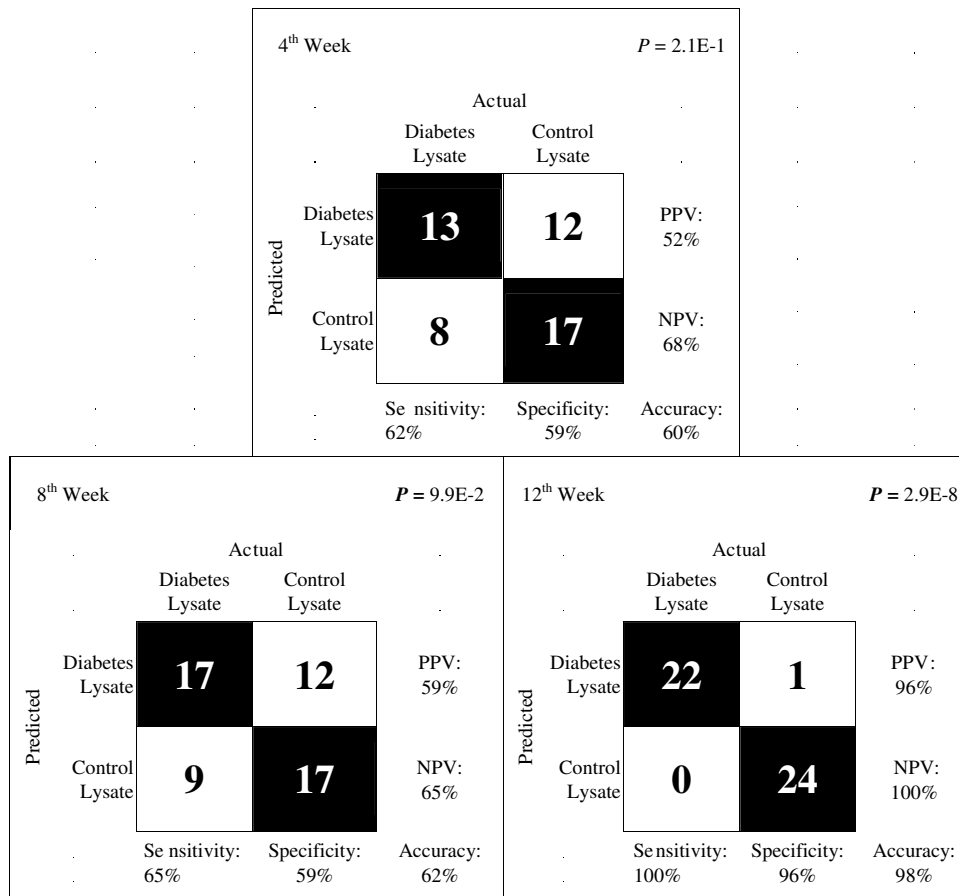


Fig. 3c. Classification values of  $m/z$  8,193 Da at all time intervals. The results were expressed as diagnostic tests. Statistical value:  $P = 2.1E-1$  at week 4,  $P = 9.9E-2$  at week 8,  $P = 2.9E-8$  at week 12. Diabetic group ( $N = 21, 26$  and  $22$  respectively) was compared with the control lysate ( $N = 29, 29$  and  $25$  respectively). PPV, positive predictive value; NPV, negative predictive value.

of phenotype with the presence or absence of a protein biomarker from the original microscopic samples. Once the validation of the protein expression profile (consisting of a multiplex of hundreds of proteins) correlates with a particular phenotype, a decision can then be made to pursue one or more of the protein candidates in greater detail [23].

Recent rapid development of proteomic technologies may provide a means to elucidate the mechanism of diabetes pathogenesis with high throughput and sensitivity [24,25]. Differential proteomic analysis has been extensively applied clinically for disease studies. However, most of the researches focus on oncological studies [26,27]. In view of this, we applied SELDI-TOF MS profiling of lysate and serum derived from DM rats in this study, as the superiority of SELDI was evident over multiple lysate/serum fractions and multiple array types [12].

The SELDI-TOF MS technology enables selective protein retention on proteinchip array surfaces by means of distinct chromatographic surfaces with detection sensitivity in the attomole ( $10^{-18}$ ) range. The analysis of the proteinchip, however, is quite different from traditional chromatographic techniques. Instead of optical detection, the bound proteins are combined with a charge and energy transfer molecule and assayed using laser desorption/ionization TOF mass spectroscopy. With TOF MS, it became possible to simultaneously identify hundreds or thousands of different proteins and peptides bound to a single site [28–30].

The technology has now been extended to construct a sensitive, reproducible protein biomarker display tool for the study of disease progression, and has been used as a model system for the rapid analysis and characterization of protein expression changes from microscopic-sized input material in discrete cell populations from human and animal tissues [31,32].

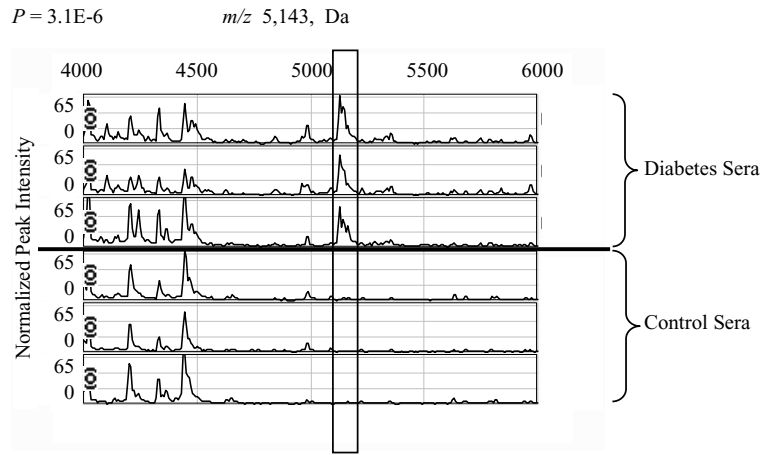


Fig. 4a. Peak view of selected spectra in the serum at  $m/z$  5,143 Da at week 4. Statistical value:  $P = 3.1E-6$ . Diabetic group was compared with the control serum.

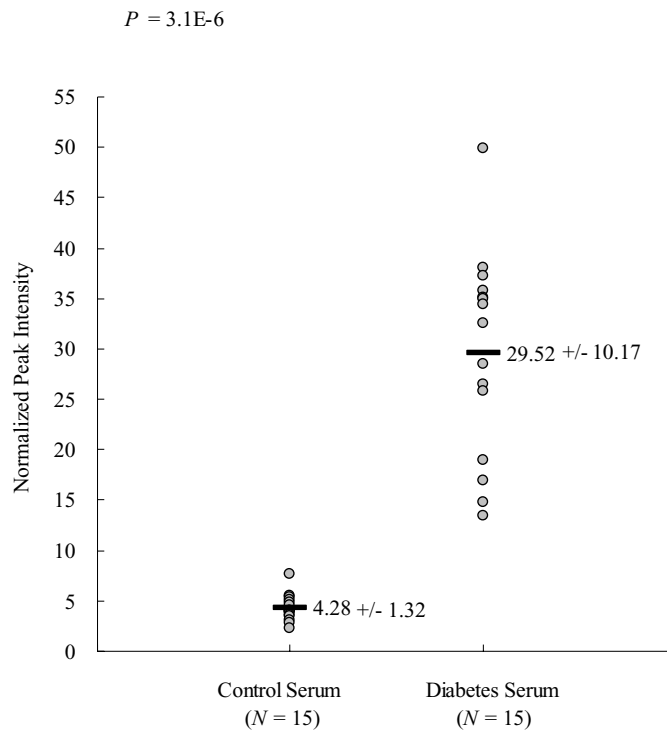


Fig. 4b. Levels of proteins in the serum at  $m/z$  5,143 Da at week 4. The results were expressed as scatter plot. Statistical value:  $P = 3.1E-6$ . Diabetic group was compared with the control serum.

It is now known that most of the diabetic complications are related to underlying vascular diseases of the microvasculature and macrovasculature. Aorta represents tissue of the macrovasculature, so the proteome of descending aorta may provide an index for the development of diabetic macrovasculopathy [6,33,34]. In the present study, aortas were obtained from rats 4 weeks,

8 weeks and 12 weeks after STZ injection. These time points were chosen based on our previous findings that ROS generation and glutathione (GSH, the major endogenous antioxidant) depletion occurred at very early stage following by the occurrence of oxidative stress [6].

In this study, 7 single potential biomarkers in the de-

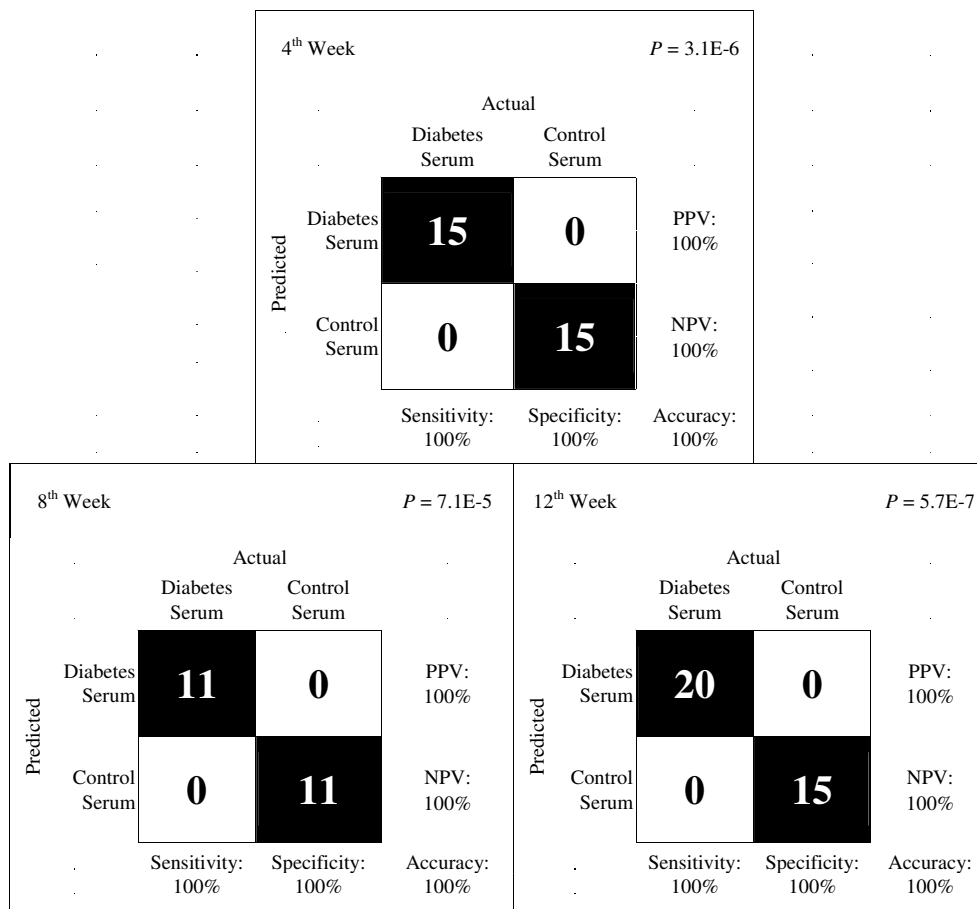


Fig. 4c. Classification values of  $m/z$  5,143 Da at all time intervals. The results were expressed as diagnostic tests. Statistical value:  $P = 3.1E-6$  at week 4,  $P = 7.1E-5$  at week 8,  $P = 5.7E-7$  at week 12. Diabetic group ( $N = 15, 11$  and  $15$  respectively) was compared with the control serum ( $N = 15, 11$  and  $20$  respectively). PPV, positive predictive value; NPV, negative predictive value.

scending aorta, as well as 7 single potential biomarkers in the sera were found. All of these biomarkers were found to have high classification values to differentiate the DM group and the control group at different time intervals. Those biomarkers that have high classification capacities and statistical values may play a role in the development of diabetes and its complications associating to macrovasculature.

A multiple biomarkers panel was found in the descending aorta at as early as week 4 of disease onset. Upon close examination, no significant individual biomarker was found in the descending aorta between the DM rats and control rats at week 4. Alternatively, a multimarker panel was discovered through multivariate analysis. The biomarker levels in the descending aorta of diabetic rats at  $m/z$  10,003 Da were significantly decreased at week 8 and week 12. All the biomarker levels in the descending aorta of DM

rats were lower than the biomarker levels in control rats at week 8. These results therefore suggested that before week 8, diabetic complications associating with macrovasculature were not obvious. However, as time proceeded, there were significant proteomic changes correlated with macrovascular complications. These results agreed with the other finding [35] and implied that there were obvious changes in the descending aorta in different durations of DM. Further investigation of this finding may provide potential biomarker(s) and prophylactic drug target for the early development of macrovascular complications.

The other potential biomarker at  $m/z$  8,193 Da was found to have a gradual trend at the 3 time points. The classification values to differentiate the DM group and the control group were gradually increasing from 60% to 98% from week 4 through week 8 to week 12. The statistical values also changed from  $2.1E-1$  to  $2.9E-$

8. This may indicate that the potential biomarker is corresponding to the progression of the disease.

Significantly, a triplet of potential biomarkers (at  $m/z$  5,125, 5,143 and 5,161 Da) was discovered simultaneously in the sera of DM rats at as early as week 4 of the disease onset. These results therefore indicate that the discovered peaks may be potential biomarkers showing the association with early DM. Besides, the same peaks were also found at week 8 and week 12 in the sera, as well as at week 12 in the aorta lysate of DM rats. Identification of the associated protein(s) may provide insight for the pathogenesis of DM.

The potential biomarker at  $m/z$  10,018 Da in this study was found to match with IAPP at MW 10,015 from *Rattus norvegicus* (rat) in the SWISS-PROT knowledgebase. This diabetes-associated peptide selectively inhibits insulin-stimulated glucose utilization and glycogen deposition in muscle, while not affecting adipocyte glucose metabolism [36]. Previous researches have indicated that IAPP has positive correlation with DM [37,38]. Our result was further validated by the Amylin (Rat) EIA Kit, where the amylin concentrations of the DM rats' sera at week 12 were 51% higher than those of the normal controls.

Simultaneously, another potential biomarker at  $m/z$  12,074 Da in this study was found to match with RSNA at MW 12,076 from *Rattus norvegicus* (rat) in the SWISS-PROT knowledgebase. Resistin is a hormone produced by adipocytes, resistin-like alpha is a secreted protein that has a restricted tissue distribution with highest levels in adipose tissue [39]. This finding was in line with the previous report that resistin had reverse correlation with DM [40]. Subsequently, serum resistin was measured using the Rat Resistin ELISA assay to validate the protein matching. Resistin concentrations of the DM rats' sera at week 12 were 42% lower than those of the normal controls.

It is still premature to say whether the biomarkers discovered in this study would finally prove to have real association with the pathogenesis of DM and its macrovasculopathy. Our findings are a discovery-phase study that lays a cornerstone for further researches in this field. To get an insight into the pathogenesis, issues such as the defining of genetic risks, and the understanding of the origins of complications should be considered as utmost important. Meanwhile, further research on the protein profiling in kidney and retina lysate of DM rats is being carried out with the aim to elucidate through these additional studies. Once the pathogenesis of diabetic complications is established, some pilot studies on human sera and tissues will also

be attempted. First, this would strengthen and validate the usability of the animal model, and secondly, provide evidence for the presence of detectable and predictive biomarkers in human diabetics suffering from macrovascular complications. It is envisaged that the findings of all these studies can finally contribute to direct bedside applications. It is not hard to foresee a future in which physicians can use disease markers found by proteomic analysis at different stages of disease management [41].

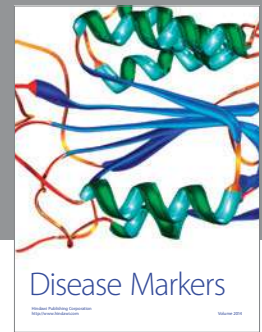
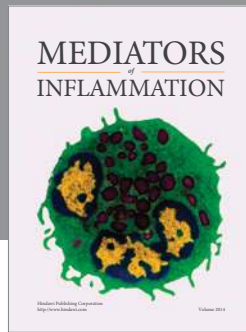
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### References

- [1] Z. Dagher, Y.S. Park, V. Asnaghi, T. Hoehn, C. Gerhardinger and M. Lorenzi, Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy, *Diabetes* **53** (2004), 2404–2411.
- [2] L. Quagliaro, L. Piconi, R. Assaloni, L. Martinelli, E. Motz and A. Ceriello, Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NAD(P)H oxidase activation, *Diabetes* **52** (2003), 2795–2804.
- [3] P.C. Sharpe, W.H. Liu, K.K. Yue, D. McMaster, M.A. Catherwood, A.M. McGinty and E.R. Trimble, Glucose-induced oxidative stress in vascular contractile cells: comparison of aortic smooth muscle cells and retinal pericytes, *Diabetes* **47**(5) (1998), 801–809.
- [4] P.C. Moore, M.A. Ugas, D.K. Hagman, S.D. Parazzoli and V. Poitout, Evidence against the involvement of oxidative stress in fatty acid inhibition of insulin secretion, *Diabetes* **53** (2004), 2610–2616.
- [5] S.M. Setter, R.K. Campbell and C.J. Cahoon, Biochemical pathways for microvascular complications of diabetes mellitus, *The Annals of Pharmacotherapy* **37**(12) (2003), 1858–1866.
- [6] K.K. Yue, W.S. Chung, A.W. Leung and C.H. Cheng, Redox changes precede the occurrence of oxidative stress in eyes and aorta, but not in kidneys of diabetic rats, *Life Sciences* **73** (2003), 2557–2570.
- [7] K.K. Yue, S.N. Leung, P.M. Man, W.F. Yeung, W.S. Chung, K.W. Lee, A.W. Leung and C.H. Cheng, Alterations in antioxidant enzyme activities in the eyes, aorta and kidneys of diabetic rats relevant to the onset of oxidative stress, *Life Sciences* **77** (2005), 721–734.
- [8] D.K. Arulmozhi, A. Veeranjanyulu and S.L. Bodhankar, Neonatal streptozotocin-induced rat model of type 2 diabetes mellitus: A glance, *Indian Journal de Pharmacologie* **36**(4) (2004), 217–221.
- [9] S. Vorderwulbecke, S. Cleverley, S.R. Weinberger and A. Wiesner, Protein quantification by the SELDI-TOF-MS-based proteinchip system, *Nature Methods* **2** (2005), 393–395.

- [10] E.T. Fung and C. Enderwick, Proteinchip clinical proteomics: computational challenges and solutions, *BioTechniques Suppl* **3** (2002), 34–38 and 40–41.
- [11] T.T. Yip, J.W. Chan, W.C. Cho, T.T. Yip, Z. Wang, T.L. Kwan, S.C. Law, D.N. Tsang, J.K. Chan, K.C. Lee, W.W. Cheng, V.W. Ma, C. Yip, C.K. Lim, R.K. Ngan, J.S. Au, A. Chan and W.W. Lim, Protein chip array profiling analysis in patients with severe acute respiratory syndrome identified serum amyloid A protein as a biomarker potentially useful in monitoring the extent of pneumonia, *Clinical Chemistry* **51**(1) (2005), 47–55.
- [12] T.T. Yip and L. Lomas, SELDI proteinchip array in oncoproteomic research, *Technology in Cancer Research and Treatment* **1**(4) (2002), 273–280.
- [13] J. Li, Z. Zhang, J. Rosenzweig, Y.Y. Wang and D.W. Chan, Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer, *Clinical Chemistry* **48** (2002), 1296–1304.
- [14] W.Y. Loh and Y.S. Shih, Split selection methods for classification trees, *Statistica Sinica* **7** (1997), 815–840.
- [15] L.A. Boardman and J.F. Peipert, Screening and diagnostic testing, *Clinical Obstetrics and Gynecology* **41**(2) (1998), 267–274.
- [16] J. Krauth, The interpretation of significance tests for independent and dependent samples, *Journal of Neuroscience Methods* **9** (1983), 269–281.
- [17] J.B. Meigs, K. Williams, L.M. Sullivan, K.J. Hunt, S.M. Haffner, M.P. Stern, C. Gonzalez Villalpando, J.S. Perhantidis, D.M. Nathan, R.B. D'Agostino Jr., R.B. D'Agostino Sr. and P.W. Wilson, Using metabolic syndrome traits for efficient detection of impaired glucose tolerance, *Diabetes Care* **27** (2004), 1417–1426.
- [18] J.B. Brown, G.A. Nichols and A. Perry, The burden of treatment failure in type 2 diabetes, *Diabetes Care* **27** (2004), 1535–1540.
- [19] G. Chambers, L. Lawrie, P. Cash and G.I. Murray, Proteomics: a new approach to the study of disease, *The Journal of Pathology* **192**(3) (2000), 280–288.
- [20] J.E. Hale, V. Gelfanova, J.R. Ludwig and M.D. Knierman, Application of proteomics for discovery of protein biomarkers, *Briefings in Functional Genomics and Proteomics* **2**(3) (2003), 185–193.
- [21] M. Tyers and M. Mann, From genomics to proteomics, *Nature* **422** (2003), 193–197.
- [22] J.R. Sydor and S. Nock, Protein expression profiling arrays: tools for the multiplexed high-throughput analysis of proteins, *Proteome Science* **1**(1) (2003), 3.
- [23] R.E. Jenkins and S.R. Pennington, Arrays of protein expression profiling: towards a viable alternative to two-dimensional gel electrophoresis, *Proteomics* **1** (2001), 13–29.
- [24] W.C. Cho, K.K. Yue and A.W. Leung, Proteomics – leading the postgenome era, *China Biotechnology* **25** (2005), 33–38.
- [25] H.J. Issaq, T.D. Veenstra, T.P. Conrads and D. Felschow, The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification, *Biochemical and Biophysical Research Communications* **292** (2002), 587–592.
- [26] W.C. Cho, T.T. Yip, C. Yip, V. Yip, V. Thulasiraman, R.K. Ngan, T.T. Yip, W.H. Lau, J.S. Au, S.C. Law, W.W. Cheng, V.W. Ma and C.K. Lim, Identification of serum amyloid A protein as a potentially useful biomarker to monitor relapse of nasopharyngeal cancer by serum proteomic profiling, *Clinical Cancer Research* **10** (2004), 43–52.
- [27] E.F. Petricoin, A.M. Ardekani, B.A. Hitt, P.J. Levine, V.A. Fusaro, S.M. Steinberg, G.B. Mills, C. Simone, D.A. Fishman, E.C. Kohn and L.A. Liotta, Use of proteomic patterns in serum to identify ovarian cancer, *Lancet* **359** (2002), 572–577.
- [28] T.W. Hutches and T.T. Yip, New desorption strategies for the mass spectrometric analysis of macromolecules, *Rapid Communications in Mass Spectrometry* **7** (1993), 576–580.
- [29] H. Kuwata, T.T. Yip, M. Tomita and T.W. Hutches, Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin, *Biochemical and Biophysical Research Communications* **1**(1429) (1998), 129–141.
- [30] H. Kuwata, T.T. Yip, C.L. Yip, M. Tomita and T.W. Hutches, Bactericidal domain of lactoferrin: detection, quantitation and characterization of lactoferrin in serum by SELDI affinity mass spectrometry, *Biochemical and Biophysical Research Communications* **245** (1998), 764–773.
- [31] V. Espina, K.A. Dettloff, S. Cowherd, E.F. Petricoin and L.A. Liotta, Use of proteomic analysis to monitor responses to biological therapies, *Expert Opinion on Biological Therapy* **4** (2004), 83–93.
- [32] N. Tang, P. Tornatore and S.R. Weinberger, Current developments in SELDI affinity technology, *Mass Spectrometry Reviews* **23** (2004), 34–44.
- [33] Y.L. Lu, S.J. Hu, Z.J. Shen and Y.C. Shao, Changes of macrovascular endothelial ultrastructure and gene expression of endothelial nitric oxide synthase in diabetic rats, *Chinese Medical Journal* **117**(8) (2004), 1165–1169.
- [34] G. Ozansoy and F.B. Akin, Effects of gemfibrozil treatment on vascular reactivity of streptozotocin-diabetic rat aorta, *The Journal of Pharmacy and Pharmacology* **56**(2) (2004), 241–246.
- [35] M. Zhou, H.J. Zhong and X.Y. Wu, Changes of ultrastructure and function of the aortic endothelium in streptozotocin-diabetic rats and effect of perindopril, *Bulletin of Hunan Medical University* **26**(1) (2001), 41–44.
- [36] M. Nishi, S.J. Cha, S. Nagamatsu, G.I. Bell and D.F. Steiner, Conservation of the sequence of islet amyloid polypeptide in five mammals is consistent with its putative role as an islet hormone, *Proceedings of the National Academy of Sciences of the United States of America* **86**(15) (1989), 5738–5742.
- [37] B. Ahren and M. Gutniak, No correlation between insulin and islet amyloid polypeptide after stimulation with glucagon-like peptide-1 in type 2 diabetes, *European Journal of Endocrinology* **137**(6) (1997), 643–649.
- [38] M. Stridsberg, C. Berne, S. Sandler, E. Wilander and K. Oberg, Inhibition of insulin secretion, but normal peripheral insulin sensitivity, in a patient with a malignant endocrine pancreatic tumour producing high amounts of an islet amyloid polypeptide-like molecule, *Diabetologia* **36**(9) (1993), 843–849.
- [39] C.M. Steppan, E.J. Brown, C.M. Wright, S. Bhat, R.R. Banerjee, C.Y. Dai, G.H. Enders, D.G. Silberg, X. Wen, G.D. Wu and M.A. Lazar, A family of tissue-specific resistin-like molecules, *Proceedings of the National Academy of Sciences of the United States of America* **98**(2) (2001), 502–506.
- [40] A. Schaffler, C. Buchler, U. Muller-Ladner, H. Herfarth, A. Ehling, G. Paul, J. Scholmerich and B. Zietz, Identification of variables influencing resistin serum levels in patients with type 1 and type 2 diabetes mellitus, *Hormone and Metabolic Research* **36**(10) (2004), 702–707.
- [41] E.F. Petricoin, K.C. Zoon, E.C. Kohn, J.C. Barrett and L.A. Liotta, Clinical proteomics: translating bedside promise into bedside reality, *Nature Reviews* **1** (2002), 683–695.



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