



## <sup>1</sup>H NMR metabolomics study for diabetic neuropathy and diabetes

Ja-Shil Hyun<sup>1,†</sup>, Jiwon Yang<sup>2,†</sup>, Hyun-Hwi Kim<sup>1</sup>, Yeong-Bae Lee<sup>2,\*</sup>, and Sung Jean Park<sup>1,\*</sup>

<sup>1</sup>College of Pharmacy and Gachon Institute of Pharmaceutical Sciences, Gachon University, 191 Hambakmoero, Yeonsu-gu, Incheon 21936, Republic of Korea

<sup>2</sup>Department of Neurology, Gil Medical Center, Gachon University College of Medicine, 21 Namdong-daero 774beon-gil, Namdong-gu, Incheon 21565, Republic of Korea

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**Abstract** Diabetes is known to be one of common causes for several types of peripheral nerve damage. Diabetic neuropathy (DN) is a significant complication lowering the quality of life that can be frequently found in diabetes patients.

In this study, the metabolomic characteristic of DN and Diabetes was investigated with NMR spectroscopy. The sera samples were collected from DN patients, Diabetes patients, and healthy volunteers. Based on the pair-wise comparison, three metabolites were found to be noticeable: glucose, obviously, was upregulated both in DN patients (DNP) and Diabetes. Citrate is also increased in both diseases. However, the dietary nutrient and biosynthesized metabolite from glucose, ascorbate, was elevated only in DNP, compared to healthy control. The multivariate model of OPLS-DA clearly showed the group separation between healthy control-DNP and healthy control-Diabetes. The most significant metabolites that contributed the group separation included glucose, citrate, ascorbate, and lactate. Lactate did not show the statistical significance of change in t-test while it tends to down-regulated both in DNP and Diabetes. We also conducted the ROC curve analysis to make a multivariate model for discrimination of healthy

control and diseases with the identified three metabolites. As a result, the discrimination model between healthy control and DNP (or Diabetes) was successful while the model between DNP and Diabetes was not satisfactory for discrimination. In addition, multiple combinations of lactate and citrate in the OPLS-DA model of healthy control and diabetes group (DNP + Diabetes patients) gave good ROC value of 0.952, which imply these two metabolites could be used for diagnosis of Diabetes without glucose information.

**Keywords** Diabetic neuropathy, diabetes, metabolomics, NMR

### Introduction

Diabetes mellitus (DM) is a metabolic disease with high blood sugar level and is well known to cause multiple complications. Diabetes is especially known to be one of common causes for several types of peripheral nerve damage. The peripheral neuropathy is characterized by muscle weakness with abnormal sensation such as tingling and numbness and sometimes can cause severe pains. The uncontrolled

† These authors contributed equally.

\* Address correspondence to: **Sung Jean Park** or **Yeong-Bae Lee**, College of Pharmacy, Gachon University, 191 Hambakmoero, Yeonsu-gu, Incheon, Republic of Korea, Tel: 82-32-820-4957; E-mail: psjnmr@gachon.ac.kr for **Sung Jean Park** or Department of Neurology, Gil Medical Center, Gachon University College of Medicine, Republic of Korea; E-mail: yeongbaelee@gachon.ac.kr for **Yeong-Bae Lee**.

neuropathic pains in diabetic patients significantly reduce the quality of life.

This disabling state frequently begins from the distal part of peripheral nerve including hands and feet and develops towards thigh, hips, and torso. This 'stocking-glove' neuropathy is the most representative among the various types of neuropathy caused by diabetes mellitus and called as diabetic neuropathy (DN). Interestingly, rigorous control of glucose level in type 1 diabetes reduce the DN occurrence while is less effective in type 2 diabetes, which imply the different physiology may play in the different type of diabetes.

Until now, the pathophysiological mechanism of DN has been studied extensively but only several potential causes for DN including the altered pathway of advanced glycation end-products (AGE), poly (ADP-ribose) polymerase (PARP), protein kinase C (PKC) and so on were suggested. All these variations result in inflammation and oxidative stress on the nervous system. However, the specific and preventive treatment for DN except for symptom-relief medication has not been found yet.

In this study, we aimed to identify metabolic characteristic of DN patient and diabetes patients to figure out the metabolic relationship between diseases by the NMR-based study. We monitored the quantitative alteration of serum metabolites in both diseases and compared the several metabolites with the healthy controls. The statistical analysis of NMR metabolites showed some fluctuation of metabolites and the ROC curve analysis showed the probable model of discrimination between diabetes and controls.

## Experimental Methods

**Patients-** Twenty-one type 2 diabetic patients with or without symptoms of neuropathy (11 diabetic neuropathic patients and 10 diabetic patients) and ten healthy control subjects were enrolled in the study. Diabetic neuropathy was diagnosed based on the clinical symptoms, physical examination, and nerve conduction study. Severity of pain and neuropathy

was assessed by a self-administered questionnaire of the Neuropathic Pain Scale (NPS, 10-item) and the Michigan Neuropathy Screening Instrument (MNSI, 15-item).<sup>1,2</sup> Physical assessment was also performed according to the MNSI examination that includes inspection and assessment of vibratory sensation and ankle reflexes.<sup>2</sup> Nerve conductions were studied in four motor nerves (median, ulnar, tibial, and peroneal nerve) and three sensory nerves (median, ulnar, and sural nerve) in the ipsilateral upper and lower extremity. Serum lipid profile (total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol), glycated hemoglobin, serum blood urea nitrogen, and serum creatinine were measure in all patients and healthy control subjects. This study was approved by the local committee of Institutional Review Board (IRB number: GAIRB2015-33) and all the subjects gave written informed consent.

**NMR sample preparation** - Blood samples (10 ml) were collected and all blood was drawn by the same operator. Whole blood was centrifuged at 12,000 × g for 10 minutes and supernatant was removed. Serum was collected in serum-separator tubes and stored at -80°C until NMR test. The 400 ul serum aliquots of healthy controls and patients were mixed with the 100 ul stock solution of NMR buffer containing 550 mM sodium phosphate buffer. The final NMR samples contained 100 mM sodium phosphate buffer (pH 7.0), 2 mM of trimethylsilyl-propanoic acid (TSP) and 10% D<sub>2</sub>O. To analyze the metabolites, 1D Carr-Purcell-Meiboom-Gill (CPMG) NMR spectra (cpmgpr1d) were obtained at 298 K on a Bruker ASCENDIII 600 spectrometer equipped with a cryoprobe.<sup>3</sup> The CPMG pulse sequence generated spectra edited by T<sub>2</sub> relaxation times, reducing broad resonances from high molecular weight compounds, improving the observation of low molecular weight metabolites. The water signal was removed by a presaturation method using low-power irradiation on the water resonance. <sup>1</sup>H-NMR spectrum for each sample consisted of 128 scans with following parameters: spectral width = 12019.2 Hz, spectral size = 65,536 points, pulse width (90) = 13.2 μs and

relaxation delay (RD) = 2.0 s. Each free induction decay (FID) was zero-filled to 64,000 points and transformed with line broadening (LB) = 0.3 Hz.

*Spectral processing and metabolite identification* - Initially,  $^1\text{H}$  NMR spectra were manually phased and baseline corrected using Bruker Topspin 3.2 software (Bruker GmbH, Karlsruhe, Germany) and referenced to TSP at 0.0 ppm. The post-processing baseline correction was conducted with Mnova (Mestrelab Research, Santiago, Spain). In each spectrum, the algorithm of multipoint baseline correction was used for building the baseline model. Because the NMR spectral bins of each spectrum can be easily influenced by small change of pH and/or ionic strength and the broad bin size consisting of more than one resonance often makes the result difficult to interpret, the peak alignment using segment and pair-wise peak alignment by Mnova was applied before binning. In addition, a variable bin size ranging from 0.005 ppm to 0.09 ppm was used so that each single bin contains single metabolic information as much as possible. The  $^1\text{H}$  NMR spectra were segmented into variable-sized spectral regions (bins) between 0.94 and 8.48 ppm. The chemical shift region of 4.69-5.20 ppm containing residual water was excluded. The lipid or protein contaminated region (1.10-1.33, 1.52-1.68, 1.78-1.90 and 5.28-5.70 ppm) was also removed from the spectra to clarify the contribution of metabolites.<sup>4</sup> The integrated bins were used as the variables for statistics. The assignment of bins was achieved using Chenomx NMR suite 7.7 (Chenomx Inc., Edmonton, Canada) and evaluated in  $^1\text{H}$ - $^{13}\text{C}$  HSQC and 2D  $^1\text{H}$ - $^1\text{H}$  TOCSY spectra.

*Statistical analysis* - The statistical analysis was carried out using the SIMCA 15 (Umetrics, Umea, Sweden).<sup>5</sup> and SPSS 23 (SPSS, Inc., an IBM Company, Chicago, Illinois, USA). The spectra were classified into controls and clinically diagnosed case subjects (diabetic neuropathy and diabetes). The integrated bins were normalized using probabilistic quotient normalization (PQN) algorithm to facilitate comparison of samples. In order to provide a

reasonable balance of contributions from high and low amplitude signals, the spectral integrals (bins) were scaled by the procedure called pareto-scaling: each variable is mean-centered and divided by the square root of the standard deviation.<sup>6,7</sup>

The multivariate analysis was performed as follows. To clarify the separation between groups, bin data were processed using a supervised pattern recognition method, orthogonal partial least squares discriminant analysis (OPLS-DA).<sup>8,9</sup> The S-plot is an easy method to visualize significant features (variables) of an OPLS-DA model of two classes. The metabolites affected with the group separations were identified by the corresponding S-plot, in which each point represented a single bin data. The axes plotted in the S-plot from the predictive component are the covariance  $p[1]$  against the correlation  $p(\text{corr})[1]$ , representing the magnitude (modeled covariation) and reliability (modeled correlation), respectively.<sup>10</sup> The efficiency and reliability of OPLS-DA models was validated using 500-random permutation test.<sup>11</sup> The quality of the models is described by  $R^2$  and  $Q^2$  values.  $R^2$  is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and  $Q^2$  is defined as the proportion of variance in the data predictable by the model and indicates predictability.<sup>12</sup> Corresponding Mahalanobis p-values for PCA and OPLS-DA score plots were calculated with PCA/PLS-DA utilities to determine the statistical significance of group separation in the OPLS-DA score plots.<sup>13</sup> An observed p-value of 0.05 was used to identify statistically significant group separation. The OPLS-DA models were further characterized by their p-values obtained from CV-ANOVA (Analysis Of Variance testing of Cross-Validated predictive residuals) implemented in SIMCA 15.<sup>14</sup>

Univariate analysis was also performed to identify how significantly each bin affects the difference between groups. The Kruskal-wallis test (non-parametric analysis) was used for the comparative analysis.<sup>15</sup> For the multiple testing

**Table 1.** Demographic and clinical characteristics of the patients

	Healthy control (HC)	Diabetic neuropathic patient (DNP)	Diabetic patient (D)
Number of patients	10	11	10
Number of male (n, %)	3, 30%	5, 45.5%	7, 70%
Age (years)	61.9 / 17.2 (51.5-83)	64.4 / 9.6 (46-78)	65.5 / 9.2 (55-84)
Disease duration (years)	NA	13.0 / 10.0 (3-30)	7.1 / 5.6 (1-20)
BMI (kg/m <sup>2</sup> )	25.0 / 3.8 (19.1-31.2)	22.9 / 3.1 (18.2-27.3)	25.8 / 3.8 (21.1-32.6)
HbA1c (%)	5.5 / 0.2 (5.2-5.8)	7.1 / 1.6 (5.7-11.2)	7.6 / 2.0 (5.4-12.5)
TC (mg/dL)	183.6 / 24.1 (134.0-214.0)	175.0 / 38.9 (115.0-228.0)	139.3 / 34.6 (84.0-192.0)
TG (mg/dL)	207.0 / 170.4 (58.0-657.0)	230.0 / 134.6 (83.0-443.0)	128.8 / 45.2 (69.0-185.0)
HDL (mg/dL)	50.3 / 9.9 (37.0-68.0)	42.3 / 13.8 (32.0-81.0)	41.5 / 10.8 (33.0-65.0)
LDL (mg/dL)	114.5 / 27.1 (68.0-167.0)	88.1 / 36.3 (34.0-140.0)	80.9 / 28.9 (32.0-124.0)
BUN (mg/dL)	19.8 / 4.7 (12.8-27.1)	18.6 / 9.1 (9.0-40.5)	15.2 / 7.4 (7.3-31.3)
Cr (mg/dL)	0.7 / 0.2 (0.5-1.1)	0.8 / 0.3 (0.4-1.7)	0.9 / 0.4 (0.5-2.0)
NPS	NA	32.2 / 17.4 (9-65)	0
MNSIq	NA	4.5 / 2.4 (0-8)	0.7 / 1.2 (0-3)
MNSIp	NA	2.8 / 1.7 (0-5)	1.7 / 1.5 (0-4.5)

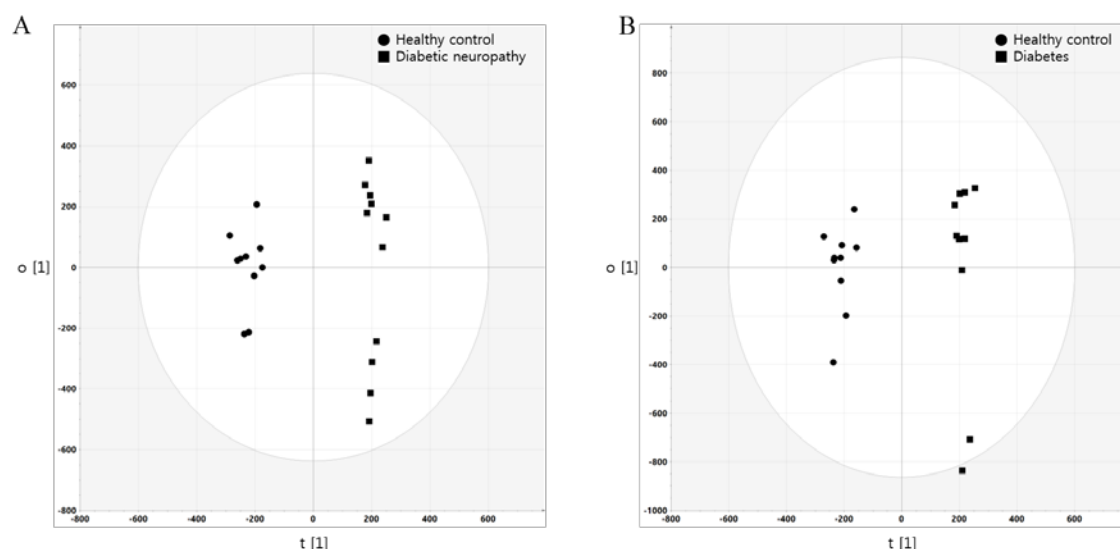
Continuous variables were presented as mean / standard deviation (range).

BMI, Body mass index; HbA1c, Glycosylated hemoglobin; TC, Total cholesterol; TG, Triglycerides; HDL, High-density lipoprotein cholesterol; LDL, Low-density lipoprotein cholesterol; BUN, Blood urea nitrogen; Cr, Creatinine; NPS, Neuropathic Pain Scale; MNSIq, Michigan Neuropathy Screening Instrument questionnaire; MNSIp, Michigan Neuropathy Screening Instrument physical examination; NA, Not applicable.

correction, acquired p-values were adjusted using Benjamini and Hochberg False Discovery Rate (FDR) method.<sup>16</sup>

The significant metabolites used for biomarker analyses for a single or multiple biomarkers based on

the ROC curve. ROC analysis was performed to evaluate the values of different metabolites for disease discrimination by assessing the area under the ROC curve (AUC), the true positive rate (sensitivity), and the false positive rate (1-specificity).<sup>17</sup> The



**Fig 1. OPLS-DA model for two group comparison.** The OPLS-DA models of two group comparison are shown in A (healthy control- Diabetic neuropathy) and B (healthy control-diabetes). The 95 % confidence ellipse of the group is depicted. The circle in the score plot represents the healthy control sample and the square represents the diabetic neuropathy (A) and diabetes (B), respectively. The values of  $R^2Y$ ,  $Q^2$  and p-value of CV-ANOVA were 0.983, 0.745 and 0.025 (A) and 0.984, 0.843 and 0.002 (B). The Mahalanobis p-values for two group comparison were  $3.0583e^{-10}$  and  $1.3211e^{-14}$  respectively (A and B).

algorithm for ROC curve calculation of multivariate biomarker was based on OPLS-DA algorithm. ROC curves were generated using 7-fold internal cross validated predicted y-values from OPLS-DA model in the SIMCA program (Ver. 15). It is important to find the most appropriate combination of metabolites which can produce an effective prediction power. The potential biomarker candidates were selected based on the contribution to classification between groups based on values of variable importance in project (VIP) of all variables and the results of univariate analysis. The VIP value of each variable in the model was calculated to indicate its contribution the separation. A higher VIP value represents a stronger contribution to classification between groups.<sup>18</sup>

## Results and Discussion

*Metabolic features between healthy people and DN (or Diabetes) patients: multivariate- and univariate-analysis* - We obtained serum samples and

clinical data from 10 healthy controls (HC) 11 diabetic neuropathic patients (DNP) and 10 diabetic patients (D). Demographic and clinical characteristics are summarized in Table 1. There were no significant differences in age, sex ratio, body mass index, and blood test results between groups, except score of NPS and MNSI questionnaire between diabetic patients with or without symptoms of neuropathy. OPLS-DA was used as a supervised statistical method to clarify the separation between groups. The 2D score plot of OPLS-DA of healthy control-diabetic neuropathy and healthy control-diabetes are shown, respectively (Fig. 1). Each model showed a clear separation of two groups along the components with predictive abilities ( $R^2 = 0.983$ ,  $Q^2 = 0.745$  and  $R^2 = 0.984$ ,  $Q^2 = 0.843$ ) and p-value of CV-ANOVA (0.025 and 0.002). All observed  $R^2$  and  $Q^2$  values of OPLS-DA model were higher than those of the permuted test, revealing predictability and goodness of fit. The Mahalanobis p-value between two groups in each OPLS-DA score plot were  $3.0583e^{-10}$  and  $1.3211e^{-14}$  respectively.

**Table 2.** Statistical analysis of the non-parametric Kruskal-Wallis test

Assigned metabolite	Chemical Shift (ppm)	Multiple comparison (adjusted <i>p</i> -value <sup>*</sup> )	FDR <sup>***</sup>	Metabolic change <sup>****</sup>	
				DNP	D
Glucose	3.53 (m) 3.57 (dd) 3.87 (m) 4.68 (d)	HC-DNP <sup>**</sup> (<0.001) HC-D (0.002)	<0.05 0.024	Δ	Δ
Ascorbate	4.53 (d)	HC-DNP (<0.001)	<0.05	Δ	—
Citrate	2.70 (d) 2.56 (d)	HC-DNP (0.007), HC-D (0.007) HC-DNP (0.006), HC-D (0.006)	0.038, 0.029 0.036, 0.026	Δ	Δ

\* Adjusted *p*-value was calculated by Bonferroni's correction.

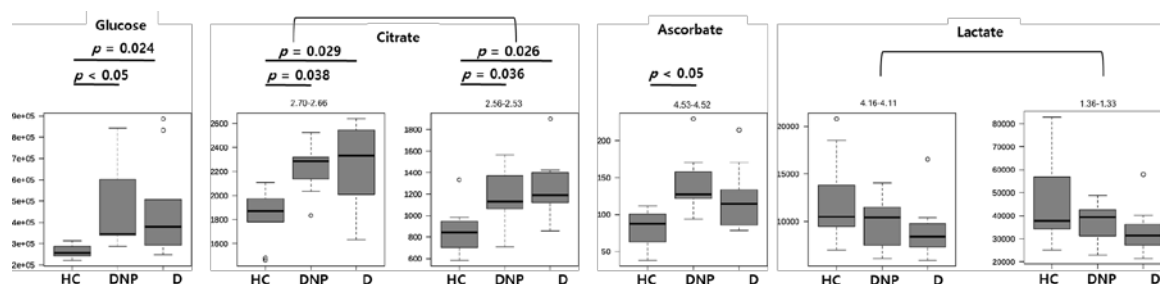
\*\* HC, healthy control; DNP, Diabetic neuropathic patient; D, Diabetic patient

\*\*\* FDR was calculated by Benjamini-Hochberg method.

\*\*\*\* Compared to the values of healthy controls, Δ indicates increase and — indicates no significant change.

To further evaluate the statistical capability of the metabolites to differentiate between groups, means of metabolite bins were compared as explained in the material and methods. Table 2 shows the result of univariate analysis, that is, the non-parametric Kruskal-Wallis test among healthy control, diabetic neuropathy and diabetes. The bin table created for the multivariate analysis was used as input variable for calculating *p*-value and adjusted *p*-value was calculated by Bonferroni's correction.<sup>19</sup> Null hypotheses of no difference were rejected if the adjusted *p*-values by Benjamini and Hochberg FDR

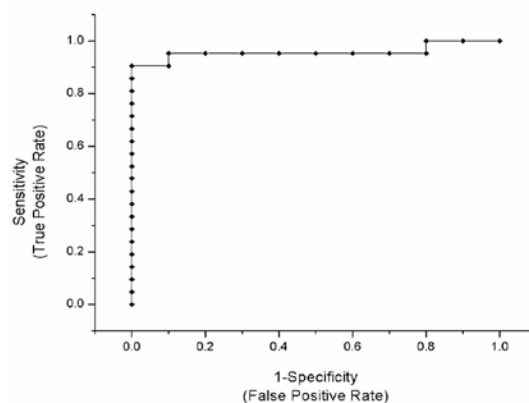
were less than 0.05. As a result, three metabolites listed in the Table 2 showed significant difference between groups. Two metabolites of glucose and citrate were higher in the DNP and D group compared to the HC group. The ascorbate was significant only for HC-DNP comparison. Moreover, lactate affected group separation in the OPLS-DA model seemingly tends to be down-regulated in HC, while it did not meet the FDR cutoff value. We also performed the univariate analysis between DNP and D group, but we could not find any significant metabolites in the comparison.



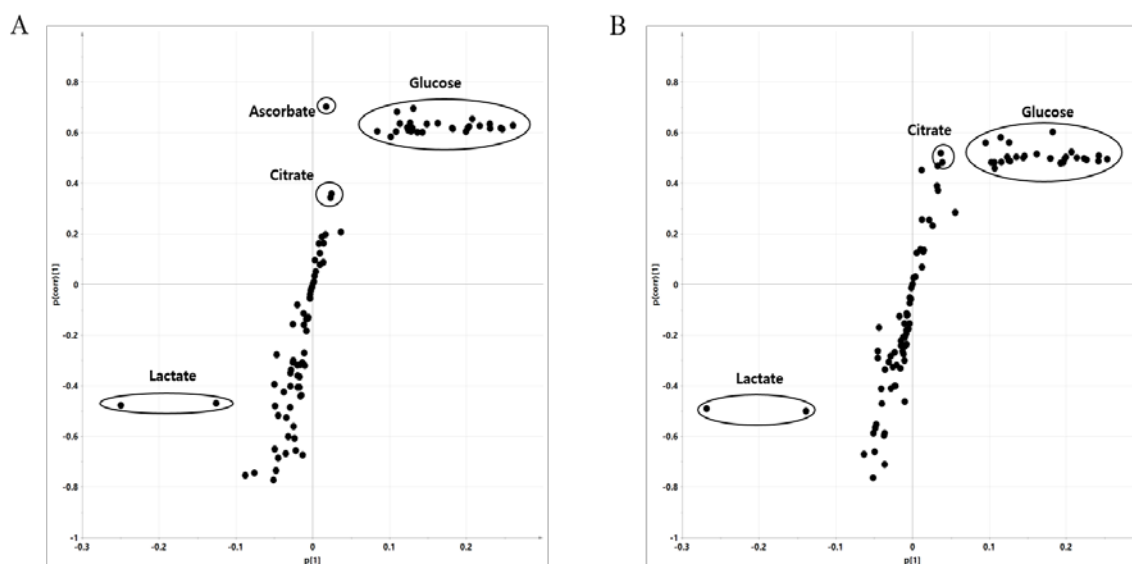
**Fig 2. Box and Whisker plots of metabolites with significant difference.** Box and whisker plots of four metabolites are illustrated (HC, healthy control; DNP, Diabetic neuropathic patient; D, Diabetic patient). Lactate is not statistically significant but affecting group separation. The groups of which the comparison was identified as significant are linked with lines. The horizontal line in the middle portion of the box is median value. The bottom and top boundaries of boxes represent lower and upper quartile. The open circles represent outliers.

Box and whisker plots of three statistical significant metabolites (glucose, citrate and ascorbate) and lactate, contributing to group separation, were illustrated (Fig. 2). The groups of which the comparison was identified as significant are linked with lines. The most significant contribution of metabolites for group separation were glucose, lactate and citrate as shown in the S-plot of the OPLS-DA model (Fig. 3).

*A potential single or multiple biomarkers based on ROC curve* - We explored the discriminant candidates of metabolites that separate each disease group (DNP + D) against control group using ROC curve analysis. Glucose was excluded in ROC analysis because the DNP and D group had also diabetes. The optimal number and composite of biomarkers was determined by monitoring AUC values obtained from the OPLS-DA and results from univariate analysis.



**Fig 4. The ROC curve analysis for two composite metabolites (lactate and citrate).** The AUC values were obtained from OPLS-DA models of healthy control – diabetic group (diabetic neuropathy + diabetes) with combination of metabolites. All NMR signals of glucose were not included in the metabolite list to remove the effect of glucose signal on the discrimination. The combination of two metabolites in the group comparison of healthy control – diabetic group (diabetic neuropathy + diabetes) provided the AUC value, 0.952.

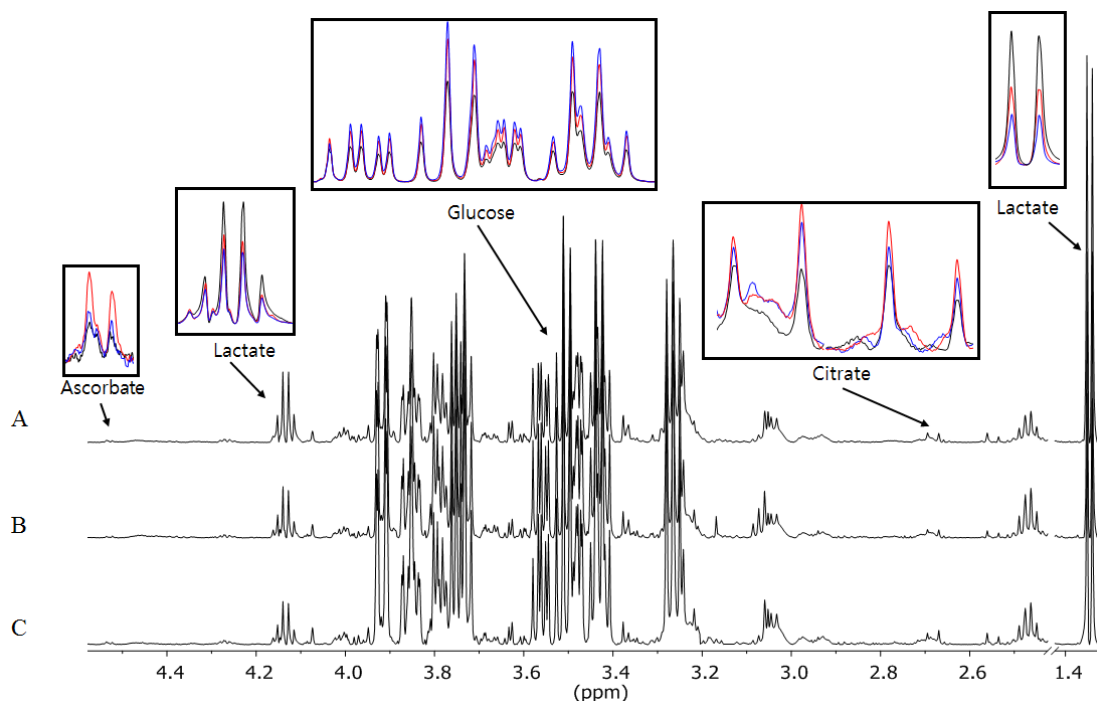


**Fig 3. The S-plot from OPLS-DA model between two groups of healthy control-diabetic neuropathy group (A) and healthy control-diabetes group (B).** The S-plot between two groups of healthy control-diabetic neuropathy group (A) and healthy control-diabetes group (B) from OPLS-DA are shown and metabolites that were highly contributed to the group separation are depicted on the plots. The important metabolites ( $p < 0.05$ , FDR  $< 0.05$ ) with the strongest association to disease are depicted on the S-plot. Lactate was also depicted because it contributed group separation, although it was not significant in univariate analysis.

The potential biomarker candidates for diabetes discrimination were lactate and citrate that showed the highest VIP value (2.7 and 1.5) in the model. The AUC values of these two metabolites (lactate and citrate) were 0.738 and 0.904, respectively. Based on this result, the potential model was constructed with two metabolites including lactate and citrate. The AUC of 0.952 can be obtained with the combination of these two metabolites (Fig. 4). The results of ROC curve analysis using OPLS-DA algorithm may suggest that the combination use of metabolites provide a useful tool to distinguish patients group (DNP+D) from healthy control without the information of serum glucose level. The discrimination model between healthy control and DNP (or Diabetes) was successful while discrimination between DNP and Diabetes was not successful. The failure in making a good model for discrimination of DNP and Diabetes may result from

the small number of metabolite features. In other words, more biomarkers should be added to the model for clear separation.

**The metabolite identification in NMR spectra-** The metabolites were basically identified in the 1D spectrum (Fig.5). The metabolite, glucose usually shows multiple proton peaks around 3.5 ppm and resultantly has multiple bins in the metabolite panel. The lactate shows unique peaks at 4.1ppm and 1.3 ppm, which are generally strong in intensity. The proton signals from L-ascorbate is highly overlapped in 3.5~4 ppm with other molecules while the double peaks at 4.5 ppm clearly show the 4H in the furan ring. Citrate was easily identified with doublet peaks positioned at 2.5 ppm and 2.6 ppm. As shown in Fig.5 the original nmr spectra which are not normalized yet showed the increase level of glucose, citrate, and ascorbate in DNP and Diabetes compared



**Fig 5. Three representative <sup>1</sup>H NMR spectra from the serum samples of healthy control (A), diabetic neuropathy (B) and diabetic group (C).** 1H CPMG spectra were processed using Mnova 10.0.<sup>20</sup> Statistically significant metabolites (ascorbate, glucose, and citrate) were labeled on the spectrum. Lactate was also labeled because it contributed group separation. The enlarged spectra for ascorbate, citrate, glucose and lactate are depicted in the figure. The black line in the enlarged figure represent the spectra of healthy control group, the red line represents the spectra of diabetic neuropathy group and the blue line represent the spectra of diabetic group.



to the healthy group while lactate was *vice versa*. The interference of intrinsic lipid signals from blood was not significant for identification and quantification of the described metabolites.

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