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Phospholipidomic identification of potential plasma biomarkers associated with type 2 diabetes mellitus and diabetic nephropathy

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ABSTRACT

Type 2 diabetes mellitus (T2DM) and its attendant complications, such as diabetic nephropathy (DN), impose a significant societal and economic burden. The investigation of discovering potential biomarkers for T2DM and DN will facilitate the prediction and prevention of diabetes. Phospholipids (PLs) and their metabolisms are closely allied to nosogenesis and aggravation of T2DM and DN. The aim of this study is to characterize the human plasma phospholipids in T2DM and DN to identify potential biomarkers of T2DM and DN. Normal phase liquid chromatography coupled with time of flight mass spectrometry (NPLC–TOF/MS) was applied to the plasma phospholipids metabolic profiling of T2DM and DN. The plasma samples from control ($n=30$), T2DM subjects ($n=30$), and DN subjects ($n=52$) were collected and analyzed. The significant difference in metabolic profiling was observed between healthy control group and DM group as well as between control group and DN group by the help of partial least squares discriminant analysis (PLS-DA). PLS-DA and one-way analysis of variance (ANOVA) were successfully used to screen out potential biomarkers from complex mass spectrometry data. The identification of molecular components of potential biomarkers was performed on Ion trap-MS/MS. An external standard method was applied to quantitative analysis of potential biomarkers. As a result, 18 compounds in 7 PL classes with significant regulation in patients compared with healthy controls were regarded as potential biomarkers for T2DM or DN. Among them, 3 DM-specific biomarkers, 8 DN-specific biomarkers and 7 common biomarkers to DM and DN were identified. Ultimately, 2 novel biomarkers, i.e., PI C18:0/22:6 and SM dC18:0/20:2, can be used to discriminate healthy individuals, T2DM cases and DN cases from each other group.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a typical metabolic disorder disease characterized by relative deficiency of insulin production and a decreased insulin action [1]. T2DM and its attendant complications threaten to overwhelm public health worldwide. It is predicted that T2DM could increase worldwide to more than 300 million individuals by 2025 [2]. Diabetic nephropathy (DN) as one of serious complications of DM is characterized by heavy proteinuria, kidney failure, and arterial hypertension. In recent years, DN continues to be a leading cause of end stage renal disease (ESRD) [3]. The number of ESRD cases caused by DN had increased dramatically over the last few decades [4–6]. Correspondingly, the treatment-related costs of T2DM and DN have increased heavily during recent decades. The investigation of discovering potential biomarkers for T2DM and DN will facilitate the prediction and prevention of diabetes. The purpose of this study is to discover some novel biomarkers which can be used as indicators for suggesting the progression and guiding the prevention and treatment of T2DM and DN.

Phospholipids (PLs) are the primary structural constituents of biological membranes, which contain a variety of fatty acyl and head group compositions, such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI),

Abbreviations: T2DM, type 2 diabetes mellitus; DN, diabetic nephropathy; ESRD, end stage renal disease; PLs, phospholipids; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine; NPLC–TOF/MS, normal phase liquid chromatography coupled with time of flight mass spectrometry; MVA, multivariate analysis; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; ANOVA, analysis of variance; ESI, electrospray ionization; QC, quality control; RSD, relative standard deviation; TCM, traditional Chinese medicine; LOD, limit of detection; LOQ, limit of quantitation; VIP, variable importance for projection; PKC, protein kinase C; PLA2, phospholipaseA2; SP, sorbitol pathway; MI, myo-inositol.

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phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SM) and lysophosphatidylcholine (LPC). In addition to this structural role, PLs take part in emulsifying neutral fat and cholesterol deposits in blood vessels [7,8], improving intelligence [9] and activating cell [10–12]. Moreover, PLs and their metabolism are closely associated with many diseases, like Alzheimer's, obesity and cancers [13–17]. Due to these important bio-functions, PLs have been given increased attention in many fields. A number of studies have demonstrated that metabolism disorder of lipid is directly associated with T2DM and DN. Some of PL molecules which have significant up-regulated or down-regulated expression in disease model have been already recognized as biomarkers of T2DM or DN [15–23]. However, there have been no reported PL biomarkers for distinguishing T2DM and DN.

Phospholipidomics can perhaps best be defined as the comprehensive and quantitative analysis of PLs in biological samples. The multivariate analysis (MVA) of complex PLs profiling are performed in phospholipidomics. The principal component analysis (PCA) method and partial least squares discriminant analysis (PLS-DA) method are mainly used in MVA, while PLS-DA is more appropriate than PCA for classification [24]. The phospholipidomic approaches have been increasingly used for many allied studies of PLs, such as biomarker discovery [21,22,25,26], efficacy evaluation of traditional Chinese medicine (TCM) [26].

In this paper, a HPLC-ESI/MS was employed to study the comprehensive PL metabolic profiling. Then, PCA was used to overview the distribution of all 112 human plasma samples and try to classify T2DM patients, DN patients and healthy controls. PLS-DA was not only used to discriminate the patients and controls, but also used to screen out biomarker candidates from complex mass spectrometry data. The key PL molecules which have the most contribution on the separation between sample classes were selected as potential biomarkers by PLS-DA. The one-way analysis of variance (ANOVA) was employed to determine the differences of metabolites between groups. Furthermore, the quantitative results obtained by the external standard method showed the different concentration levels of potential biomarkers in controls and patients.

2. Experimental

2.1. Chemicals and reagents

1-Stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (PE, C18:0/20:4, liver, bovine sodium salt), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phosphor-rac-(1-glycerol)] (PG, C16:0/18:1, sodium salt), l-α-phosphatidylinositol (PI, C18:0/20:4, liver, bovine-sodium salt), 1-stearoyl-2-oleoyl-*sn*-glycero-3-(phospho-L-serine) (PS, C18:0/18:1, sodium salt), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (PC, C18:0/18:0), sphingomyelin (SM, dC18:1/18:0, brain, porcine), and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC, C16:0) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 2,6-Di-tert-butyl-4-methylphenol was purchased from Sigma–Aldrich (St. Louis, USA). Hexane, 2-propanol, chloroform and methanol were of HPLC grade and obtained from J.T. Baker (Philipsburg, USA). Analytical grade formic acid, ammonia hydroxide and other reagents were purchased from Modern Dong Fang (Beijing, China). Ultrapure water was purified with a Milli-Q system (Millipore, Milford, MA).

2.2. Instrumentation

An Agilent 1100 series HPLC system (Agilent Technologies, PaloAlto, CA, USA) was used. 20 μL of the prepared sample was separated on a diol column (Nucleosil, 100-5 OH, Germany) (250 mm × 3.0 mm I.D., 5.0 μm) at 35 °C. The mobile phase consists of hexane (A) and 2-propanol/water/formic acid/ammonia (100/13/0.9/0.14 (v/v)) (B). In the isocratic elution program, 30% A and 70% B were used. The flow rate was set to 0.2 mL/min.

A TOF-MS (Agilent Corp., Waldbronn, Germany) equipped with electrospray ionization (ESI) source was online coupled to the Agilent 1100 HPLC system. The operation parameters were optimized as follows: negative-ion mode (ESI^-); capillary voltage, −4 kV; nitrogen drying gas, 9 L/min; nebulizer gas, 35 psi; gas temperature, 350 °C; mass range, m/z 400–1000. All data were acquired in profile mode.

An ion-trap mass spectrometer (Agilent Corp, Waldbronn, Germany) equipped with ESI source was online coupled to the Agilent 1100 HPLC system. The operation parameters are as follows: negative ionization mode (ESI^-); capillary voltage, −4 kV; nitrogen drying gas, 9 L/min; nebulizer, 35 psi; gas temperature, 350 °C compound stability, 80%; mass range, m/z 50–1000.

2.3. Sample collection and preparation

The samples were collected from 30 healthy controls including 11 females and 19 males (aged from 40 to 68 years), 30 patients with DM including 14 females and 16 males (aged from 41 to 75 years), and 52 patients with DN including 27 females and 25 males (aged from 24 to 63 years). The blood samples were put in EDTA-containing tubes to prevent platelet activation and phospholiphase activity. Two milliliters of blood were centrifuged at 2600 × *g* for 10 min at 4 °C. All of plasma samples were kept frozen at −80 °C before sample preparation. The related clinical characteristics of healthy individuals, DM patients and DN patients are shown in Table 1. There are no significant differences of shown parameters between DM and DN.

Before extraction, the samples should be thawed at room temperature. PLs were extracted by a modified Bligh and Dyer procedure [21,27]. Briefly, 350 μL of water was added to 150 μL plasma in a 20 mL glass bottle; then 2 mL of methanol with 0.01% (w/v) 6-di-tert-butyl-4-methylphenol and 4 mL of chloroform were successively added; the mixed solution were sonicated for 60 s both before and after adding chloroform. After sonication the solution was whirl mixed for 30 s and incubated for approximately 1 h at room temperature. Finally, 2 mL of water was added before the solution was mixed for 5 s and centrifuged at 2600 × *g* for 10 min at 4 °C. Three layers were observed in the glass bottle. The upper

Table 1
Clinical characteristics of healthy individuals, DM patients and DN patients.

Characteristic	Controls	DM	DN
n	30	30	52
Sex (M/F)	19/11	16/14	27/25
Age (years)	48.8 ± 5.6	59.5 ± 7.2	56.9 ± 8.5
Diabetes (type2/type1)	na	30/0	52/0
Diabetes duration (years)	na	10.6 ± 5.0	13.3 ± 6.9
Diabetic nephropathy duration (years)	na	na	2.9 ± 2.4
Fasting blood glucose (mmol/L)	5.6 ± 0.7	7.9 ± 1.0	7.1 ± 1.2
HbA1c (%)	5.8 ± 0.2	8.3 ± 0.4	8.6 ± 0.4
Total cholesterol (mmol/L)	5.3 ± 1.1	4.9 ± 0.8	5.2 ± 0.6
HDL-cholesterol (mmol/L)	1.2 ± 0.2	1.1 ± 0.3	1.2 ± 0.4
LDL-cholesterol (mmol/L)	3.3 ± 0.9	3.0 ± 0.6	3.1 ± 1.0
Triacylglycerol (mmol/L)	1.6 ± 0.6	1.7 ± 0.5	2.2 ± 0.7
Hypertension (absent/present)	na	14/16	35/17
BMI (kg/m^2)	16.5 ± 2.1	24.3 ± 2.6	25.0 ± 3.6
Smokers (current or former/never)	17/13	9/21	27/25
Family history of diabetes (absent/present)	29/1	19/11	29/23

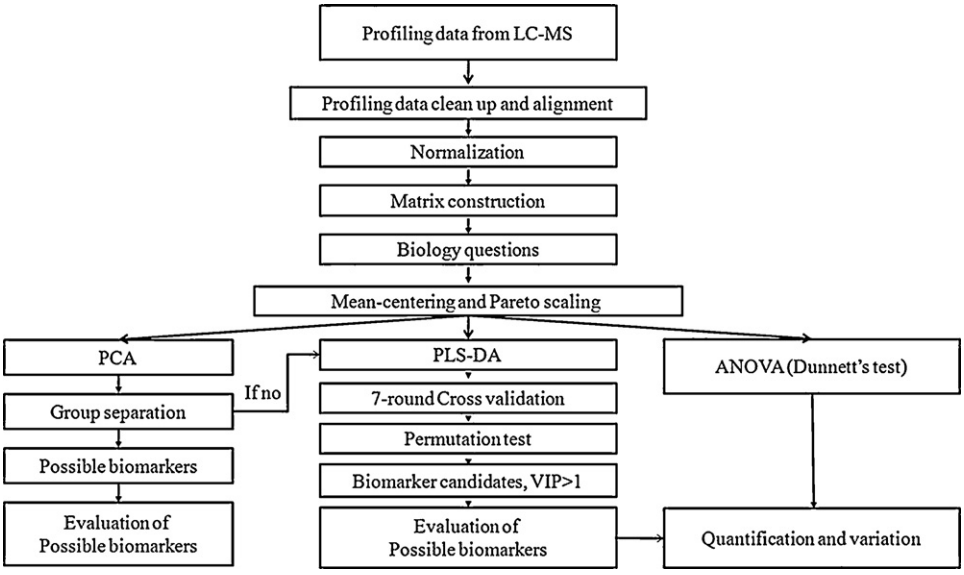

Fig. 1. Data analysis procedure of this phospholipidomic study.

layer is mainly methanol and water. The middle layer is mainly protein. And the low layer is chloroform phase containing PLs extracts. The upper layer and the middle layer were taken out carefully with pipette. 3 mL of chloroform phase was taken out and put in a 5 mL glass bottle. The solutions were dried by evaporation under nitrogen gas. The dried samples were stored at −20 °C until analysis. Prior to measurement, the extracted samples were re-dissolved in 40 μL chloroform/methanol (2:1 (v/v)) and 160 μL hexane/2-propanol (3:7 (v/v)).

2.4. External standard quantitative method and its validation

2.4.1. Calibration procedure

Stock solutions of 1 mg/mL PE (C18:0/C20:4), PG (C16:0/C18:1), PS (C18:0/C18:1), PI (C18:0/20:4), PC (C18:0/18:0), SM (dC18:1/C18:0), and LPC (C:16:0), were prepared in volumetric flasks in chloroform/methanol (2:1 (v/v)) individually. A series of standard working solutions with the concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, and 5 μg/mL for PG and PS; 1, 2, 3, 4, 5, 10, 20, 30, 40, and 50 μg/mL for PE and PI; 5, 10, 20, 30, 40, 50, 60, 70, 80, and 100 μg/mL for PC; 1, 2, 5, 10, 20, 30, 40, 50, 60 and 70 μg/mL. SM and LPC were obtained by further dilution with hexane/2-propanol (3:7 (v/v)). Calibration curves were obtained by the plots of the peak-area versus the concentration of the calibration standards.

2.4.2. Precision studies

Quality control (QC) samples with high, middle, low PL concentrations were prepared in parallel. They were prepared in the following way. All standard solutions were evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue reconstituted in 150 μL pooled plasma was prepared by the method described as *sample preparation*. Intra-day accuracy and precision (each $n = 6$) were evaluated by analysis of QC samples at different times on the same day. Inter-day accuracy and precision (each $n = 6$) were determined by analysis of QC samples twice per day at three concentration levels over three consecutive days. The concentration of each sample is determined using calibration standards prepared on the same day. The method precision was determined by the relative standard deviation (RSD). The extraction recoveries were determined by comparing the peak areas obtained from QC samples subtracting blank plasma with the standard working solutions at the same concentration. Except for method validation, QC samples were analyzed randomly by LC-TOF/MS, a pair of blank/QC were placed every ten samples and at the beginning/end of the sequence.

2.5. Data processing

Under the negative ionization mode, LC-TOF/MS chromatograms were inspected for profiling the phospholipid species in plasma. After two-step format transformation (WIFF → CDF → RAW) by Agilent translate software and Waters's Dbridge software, the obtained LC-TOF/MS data (.wiff format) were converted into RAW data files (.raw format) which can be processed using the Micromass MarkerLynx Applications Manager (version 4.0, Waters Corp., Milford, USA). This applications manager incorporates a peak deconvolution package that allows detection of the mass, retention time and intensity of the peaks eluting in each chromatogram. The parameters were set as follows: minimum retention time 8.00 min, maximum retention time 40.00 min, mass range m/z 400–1000, mass tolerance 0.05 Da, intensity threshold 10 counts, retention time tolerance 0.10 min; noise elimination level was set at 6.00, and isotopic peaks were excluded for processing. The area of each peak, after being recognized and aligned, was normalized to the summed total ion intensity of each chromatogram. The resulting three-dimensional data, peak number (RT – m/z pair), sample name, and normalized ion intensity were introduced to SIMCA-P software (version 11.5 Demo, Umetrics, Umea, Sweden) for PCA and PLS-DA. Mean centered was used for data centering and Pareto scaling. ANOVA was performed with SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA) in succession to reveal the statistical differences for the variables among healthy controls, T2DM patients and DN patients. Fig. 1 shows the data analysis procedure of this phospholipidomic study.

The concentration values of potential biomarkers were obtained by external standard method. The calibration curves of PE m/z 766 (C18:0/C20:4), PG m/z 747 (C16:0/C18:1), PI m/z 885 (C18:0/C20:4), PS m/z 788 (C18:0/C18:1), PC m/z 834 (C18:0/C20:4) SM m/z 775 (d18:1/C18:0), LPC m/z 540 (C16:0), were obtained by the plots of the peak-area versus the concentration of the calibration standards. The concentrations of all compounds within the same class were calculated by the corresponding equations of linear regression. This method was validated for stability, repeatability, reproducibility, extraction recoveries. The detailed values shown in Table 2 indicated the good performance characteristics of this

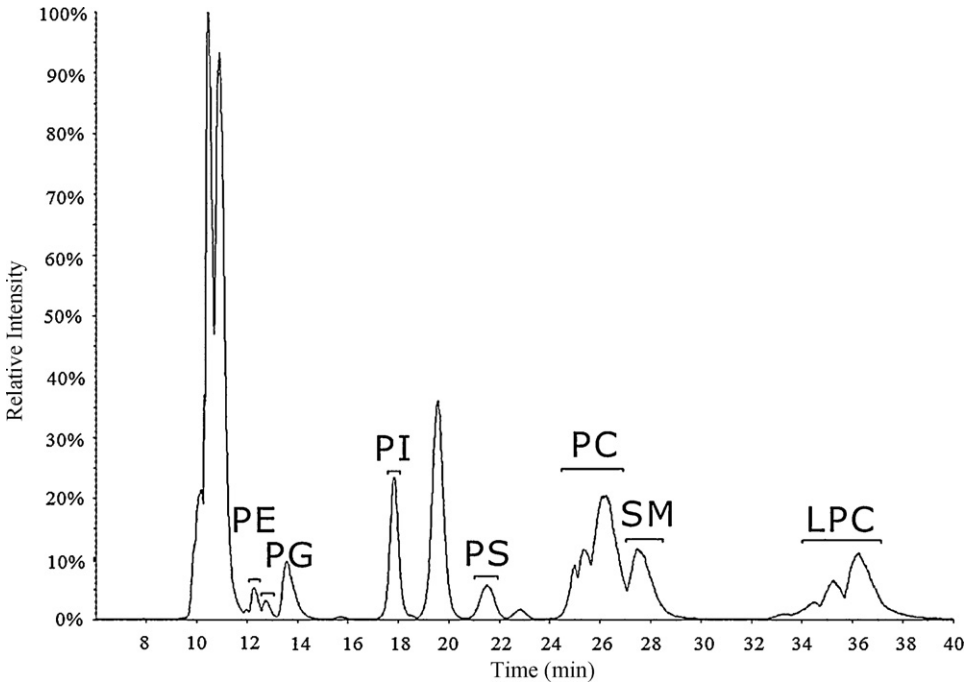

Fig. 2. Base peak intensity (BPI) chromatogram of phospholipids in human plasma extracts by normal phase-HPLC/TOF-MS. The elution order is as follows: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; LPC, lysophosphatidylcholine. The unknown peaks in the chromatogram do not affect the determination of retentions and peak areas of PL individuals.

Table 2
Performance characteristics of the external standard method for PLs quantification.

Compounds	Slope	Intercept	R^2	Linearity range (μg/mL)	RSD (%)		Recoveries (%)		
					Intra-day	Inter-day	High	Middle	Low
PE (C18:0/C20:4)	136,559	254,552	0.9989	1–50	<7	<9	91.2	93.5	109.2
PG (C16:0/C18:1)	1,000,000	2,000,000	0.9992	0.1–5	<5	<6	84.6	86.1	87.3
PI (C18:0/C20:4)	125,407	691,163	0.9983	1–50	<7	<8	81.6	88.8	80.6
PS (C18:0/C18:1)	344,649	114,993	0.9948	0.1–5	<4	<7	85.4	82.2	88.3
PC (C18:0/C18:0)	151,583	602,931	0.9977	5–100	<6	<6	89.3	97.7	106.4
SM (dC18:1/C18:0)	109,562	62,756	0.9999	1–70	<7	<10	91.4	93.1	112.5
LPC (C16:0)	472,308	−188,260	0.9992	1–70	<6	<8	89.3	94.8	106.3

quantitative method. The method meets the requirements of quantitative analysis. In other words, the established plasma PLs profiling were suitable for quantitative analysis and multivariate analysis. The significance of variation between each group in concentrations was determined using paired-sample *t*-test by Excel 2007 (Microsoft, USA). The *p*-values less than 0.05 were considered significant and values less than 0.01 were considered highly significant.

3. Results and discussion

3.1. Phospholipids profiling by NPLC–TOF/MS

Plasma phospholipids profiling were established by NPLC–TOF/MS under negative ionization mode. The negative ionization mode can give more information-rich data with lower noise and background than positive mode [28]. The isocratic

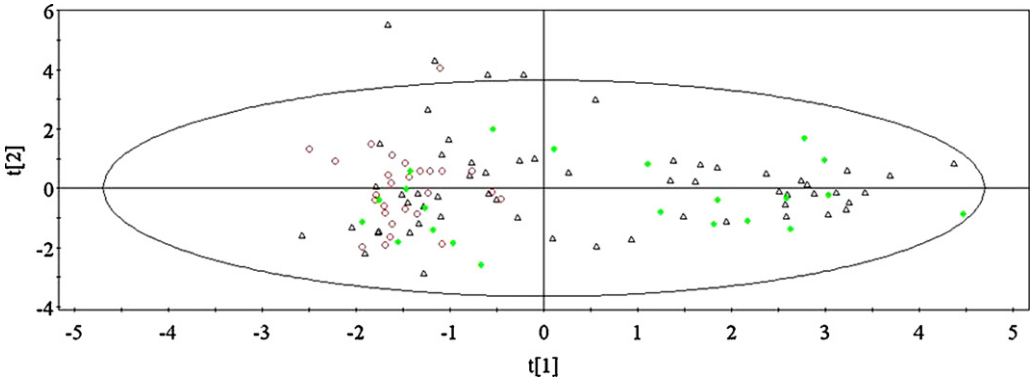

Fig. 3. Score plot from PCA of mean-centered data. Healthy controls (red circle, $n = 30$), T2DM (green diamond, $n = 30$), DN (black triangle, $n = 52$). It is shown that healthy controls can be separated clearly from most of patients. But there is no significant separation between each group.

elution at a relative low flow rate can not only avoid the problem of time shifting, but also save the column equilibration time between two injections [21]. The phospholipids profiling method used in this paper was a modification of the previous method we developed, which was successfully applied to study phospholipids metabolism disorder of diabetes nephropathy (Ref. [21]). The running time of the previous method is more than 50 min for one injection. And the relatively high amount of water mixed with n-hexane in mobile phase would result in retention time shift. In order to decrease the running time and avert retention time shift, we modified the solvents by increasing the buffer salt content and decreasing the proportion of water. Correspondingly, the proportion of the composition in solvent B (i.e., isopropanol/water/formic acid/ammonium) was changed from (86/13/0.8/0.12 (v/v/v/v)) to (100/13/0.9/0.14 (v/v/v/v)). Using the modified mobile phase, the single running time decreased to 40 min. Under this NPLC

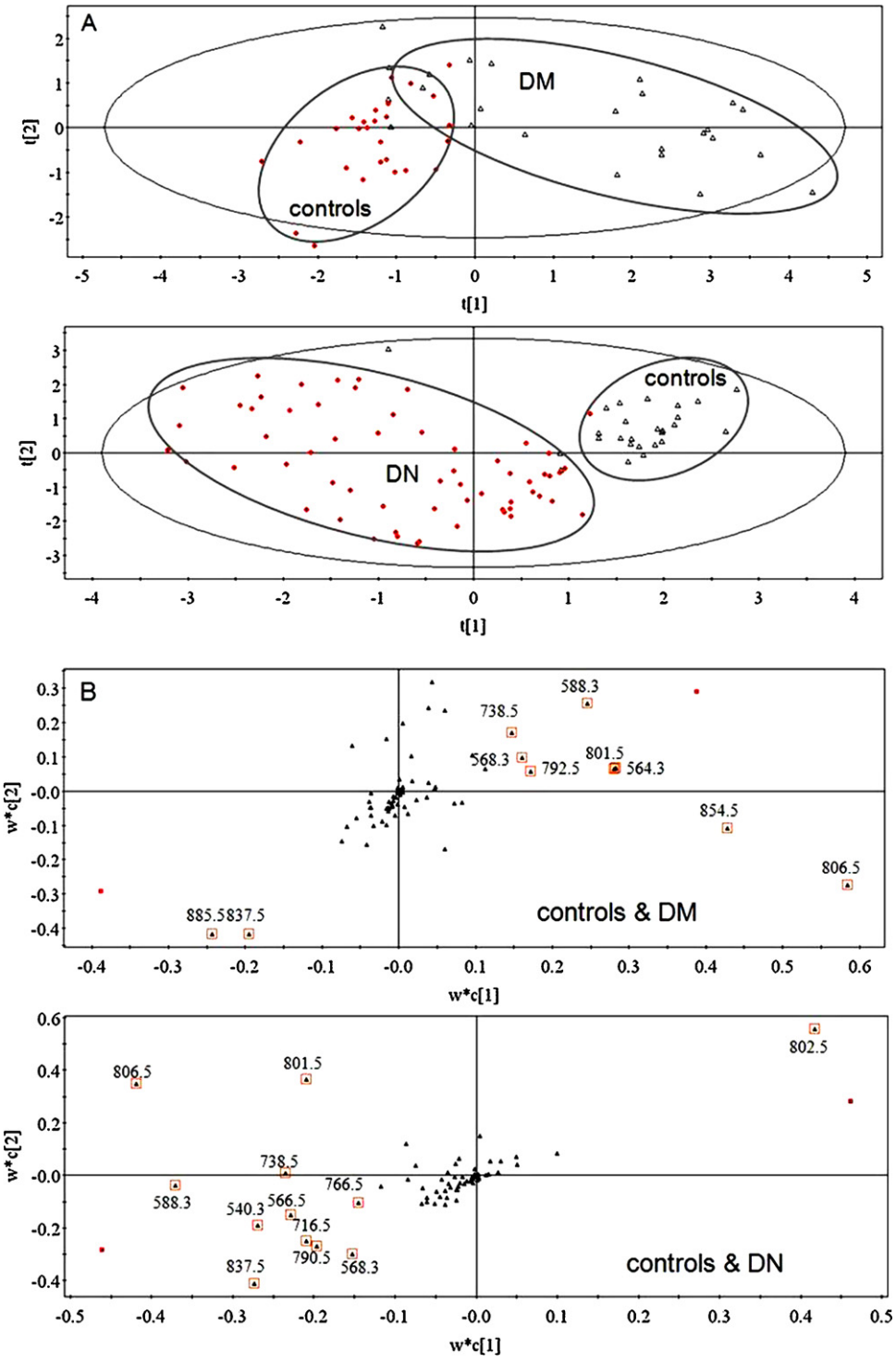

Fig. 4. (A) Score, (B) loading and (C) VIP plots from PLS-DA using mean-centered data. The top half figures in both A, B and C were obtained by analysis of healthy controls and T2DM subjects, while the bottom half ones were obtained by analysis of healthy controls and DN subjects. Assignment: (B) PL species marked with red square frame contributed to the separation of controls and cases. (C) The variables with dark color correspond with the marked PL species in loading plot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

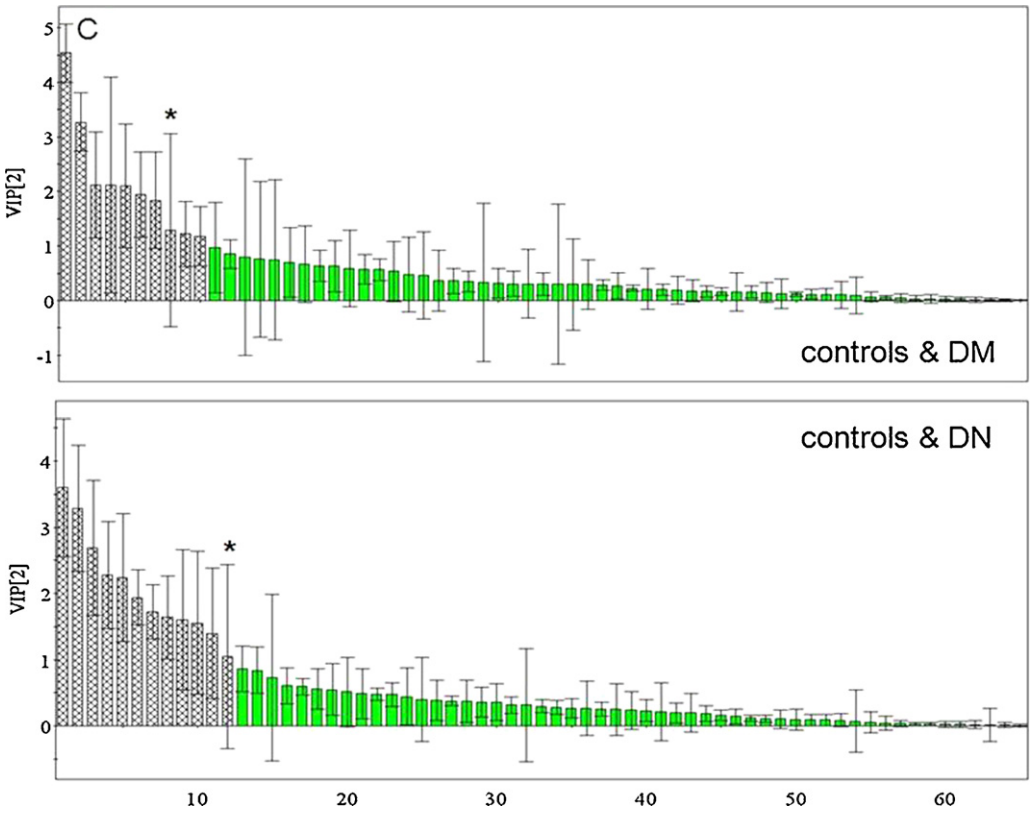

Fig. 4. (*Continued*).

condition, an efficient between-class separation of 7 PL classes was achieved. The elution order of PLs was generated as PEs, PGs, PIs, PSs, PCs, SMs and LPCs (Fig. 2). The peak widths of each class at peak base were respectively recorded as 0.5 min for PEs (12.0–12.5 min), 0.7 min for PGs (12.5–13.2 min), 1.6 min for PIs (17.1–18.7 min), 1.6 min for PSs (20.7–22.3 min), 3.6 min for PCs (24.1–27.7 min), 3.4 min for SMs (26.4–29.8 min), 6.9 min for LPCs (32.6–39.5). The retention time of each species within a class depend on their own polarity decided by the length and unsaturation degree of fatty acid chains. Although only one peak can be observed for one class, the retention times of PL individuals have minor differences. However, the retention time difference of compounds within the same class is less than that in two different classes, which can be used to align the retention times of PL species in the extracted ion chromatogram. Due to between-class separation, there are no significant visible changes between the base peak intensity chromatograms of controls, T2DM and DN. We must therefore employ multivariate analysis to characterize the different profiles from controls and cases.

3.2. Multivariate analysis and potential biomarkers

3.2.1. PCA

In order to overview the distribution of all 112 human plasma samples, a PCA model was used to classify T2DM patients, DN patients and healthy controls. More than one hundred variables (PL molecules) from 30 healthy individuals, 30 T2DM patients and 52 DN patients were set to be the row of X matrix, 112 plasma samples were set to be the column of X matrix. Mean-centered and par-scaled (scaled to square root of standard deviation) mathematical methods were performed to pretreat the chromatographic data sets. After that, they were applied to PCA. It can be seen that from the score plot (Fig. 3), healthy controls were separated clearly from most of patients, while T2DM and DN patients were jumped together. The PCA results suggest that phospholipids metabolic abnormality happened in patients group.

3.2.2. PLS-DA

PLS-DA is a sophisticated supervised clustering method, which can establish the optimal position to place a discriminant surface that separates classes best and generate the matrices of scores and loadings [29]. PLS-DA is more appropriate than PCA for classification and PLS-DA is most effective in separating two groups [24]. In our study, PLS-DA analysis of the pretreated chromatographic data sets was therefore carried out to sharpen separation of healthy controls and patients and to screen out potential biomarker candidates. A two-component PLS-DA model was constructed to identify and reveal the differential metabolites in response to controls and patients. To estimate the predictive ability of our model, we used 7-fold cross-validation, resulting in a goodness of fit of (R^2) 0.765 and a goodness of prediction of (Q^2) 0.656 for this model.

The score plot, loadings plot and VIP plot from PLS-DA are shown in Fig. 4. From score plot in Fig. 4A, we can see the fact that the better separation between healthy control and DN patients was achieved than between healthy controls and T2DM patients, which indicates that DN has a higher degree of abnormal PL metabolism than T2DM. The loadings plot indicates that the most influential ions are responsible for separation between sample classes; the ions having the greatest influence in the PLS-DA scores plot are those farthest away from the main cluster of ions. In turns, these compounds (marked with red square frame in Fig. 4B) might be the candidates for biomarkers. In Fig. 4B, the ions with m/z 806.5, 854.5, 801.5, 885.5, 837.5, 588.3, 564.3, 792.5, 568.3, 738.5, were considered as biomarker candidates of T2DM and the ions with m/z 806.5, 802.5, 837.5, 801.5, 738.5, 588.3, 540.3, 790.5, 566.5, 716.5, 568.3, 766.5, were considered as biomarker candidates of DN. Furthermore, the variable importance for projection (VIP) plot summarizing the contribution of a metabolite ion to the

generation of the model has been applied [30]. The most important metabolites for the discrimination were assigned by sorting the ions according to their VIP value to select among the 65 variables. Metabolite ions with VIP value >1.0 were considered as biomarker candidates. As shown in Fig. 4C, two metabolites (marked with an asterisk) showed great confidence intervals, suggesting that their contribution to the PLS-DA model might be caused by analytical variation. These two biomarker candidates (*m*/*z* 792.5, 766.5) should be excluded from biomarker candidates. Finally, the potential biomarkers (totally 14) from PLS-DA analysis were listed as follows: *m*/*z* 806.5, 802.5, 854.5, 801.5, 885.5, 837.5, 540.3, 568.3, 564.3, 716.5, 790.5, 566.5, 588.3, 738.5. Among them, the ions at *m*/*z* 854.5, 885.5, 564.5 are T2DM-specific biomarkers; the ions at *m*/*z* 802.5, 540.3, 716.5, 790.5, 566.5, are DN-specific biomarkers; the ions at *m*/*z* 806.5, 801.5, 588.3, 568.3, 738.5, 837.5, are common biomarkers to T2DM and DN.

3.2.3. ANOVA

The analysis of variance (ANOVA) is the most widely used method of statistical analysis of quantitative data [31]. Based on the relative intensities of the metabolites from the normalized profiling data, One-Way ANOVA Post Hoc Tests was used to reveal the significant differences of the 65 metabolites between each group. After that, we can find out some metabolites as potential biomarkers. From Dunnett's T3 results, the ions at *m*/*z* **854.5**, **885.5**, **564.5**, have significant differences between controls and T2DM; the ions at *m*/*z* **802.5**, **540.3**, **716.5**, **790.5**, **566.5**, 750.5, 747.5, have differences between controls and DN; the ions at *m*/*z* **806.5**, **801.5**, **588.3**, **568.3**, **738.5**, **837.5**, 909.5, 773.5, are common biomarkers to T2DM and DN. The ions at *m*/*z* **801.5**, 909.5 were significant different between groups. The above ions written in bold ***m*/*z*** were all of the potential biomarkers found out by PLS-DA.

Totally, 18 PLs species were discovered as potential biomarkers by PLS-DA and ANOVA, namely *m*/*z* 854.5, 885.5, 564.5, 802.5, 540.3, 716.5, 790.5, 566.5, 750.5, 747.5, 773.5, 806.5, 801.5, 588.3, 568.3, 738.5, 837.5, 909.5. Among them, 3 DM-specific biomarkers, 8 DN-specific biomarkers and 7 common biomarkers to DM and DN were identified. 2 novel biomarkers (PI $[M-H]^-$ *m*/*z* 909 and SM $[M-HCOO]^-$ *m*/*z* 801) can be used to distinguish T2DM, DN and healthy individuals. The significances of these biomarkers are shown in Table 3.

3.3. Structural identification of potential biomarkers

The structural identification of potential PLs biomarkers were carried out by MS^n experiment under the anion module on Ion trap-MS/MS. In the negative ionization mode, the species of PG, PE (pPE), PI, and PS were all detected as the $[M-H]^-$ ions. The other three classes containing choline polar heads, namely PC, LPC and SM, were detected as the formate adducts $[M+HCOO]^-$ when having formic acid in mobile phase. The MS^2 and MS^3 were mainly used to detect the fragments for determining the fatty acid chains. The main fragments of PLs were lyso-phospholipids and carboxylate anions. All types of fragments might be used for species determination.

Take the PC specie at *m*/*z* 802.5 as an example to illustrate the identification process. PC species were recorded as $[M+HCOO]^-$ ions in negative ionization mode. The molecular ion of PC was detected at *m*/*z* 802.5. In the MS^2 spectrum (Fig. 5A), *m*/*z* 742.3 $[M-CH_3]^-$ was detected. In the MS^3 spectrum (Fig. 5B), lyso-phospholipids and carboxylate anions were detected. The fatty acid fragment ions at *m*/*z* 279.0, 254.9 correspond to $[M-H]^-$ of linoleic acid (C18:2) and $[M-H]^-$ of palmitic acid (C16:0) respectively. The deacylated ion at *m*/*z* 480.1 corresponds to LPC (C16:0) $[M-15]^-$ resulting from the loss of one fatty acid. The identification of species was based on the molecular ion and *sn*-1 and *sn*-2 carboxylate anions observed. For diacyl species, both carboxylate anions corresponding to *sn*-1 and *sn*-2 substituents were present in the negative ionization mode. The phospholipids isolated from animals often contain a saturated fatty acid at the *sn*-1 position and an unsaturated fatty acid at the *sn*-2 position [32]. However, phospholipids with two unsaturated fatty acids are present in blood, although at a very low concentration with exception of some PC species [33,34]. Accordingly, it is supposed that the $[M+HCOO]^-$ ion at *m*/*z* 802.5 was identified as C16:0/C18:2. Similarly, the structural identification of other potential biomarkers was also carried out by MS^n experiment. The identified molecular compositions of these biomarkers are present in Table 3.

3.4. Quantification of potential biomarkers

An external standard method was carried out to determine plasma concentration of targeted compounds. One representative phospholipids molecule with proper concentration in the plasma in each class, such as PE (C18:0/20:4), PG (C16:0/18:1), PI (C18:0/20:4), PS (C18:0/18:1), PC (C18:0/18:0), SM (dC18:1/18:0), and LPC (C16:0), were selected as standards for quantification. The concentrations of all species within the same class were estimated according to the calibration curves of representative PL since the phospholipids species within the same PL class have the similar structure and similar signal response. And these data are just used for comparing the differences between cases and controls, so the relative quantification data satisfied the estimation. The slopes, intercepts and R^2 values of equations of linear regression are present in Table 2. The limit of detection (LOD) for different classes range from 0.03 μg/mL to 0.08 μg/mL. The lower value of linearity range of each class is higher than limit of quantitation (LOQ).

Predictably, the PL species within the same class have similar variation trends with the progress of T2DM and DN. But some single specie showed the different change trend. The concentrations of 18 proposed biomarkers are shown in Table 3. Compared with controls, LPCs, PEs, PG, SMs, one PC specie and one PI specie were up-regulated in patients, whereas pPE, PS and two PC species were down regulated. It is important to note the concentration variations of two novel biomarkers, *m*/*z* 801.5 (SM, dC18:0/20:2), 909.5 (PI, C18:0/22:6). An increasing trend of SM biomarker from controls through T2DM to DN is observed in Fig. 6, and a decreasing trend of PI biomarker was observed. It is supposed marker species variation reflected the development of diabetes. The possible biochemical mechanisms of abnormal PLs metabolism were then investigated.

3.5. Abnormal phospholipids metabolism in diabetes

According to PLS-DA score plot and the concentration variation of potential biomarkers, this phospholipidomic study indicates the abnormal phospholipids metabolism happened in T2DM and DN. At present, the relationship between the up- or down-regulation of PLs and diabetes is still not very clear. Herein, the possible mechanisms of abnormal PLs metabolism are reviewed. Three pathways, activation of the sorbitol pathway, increase in oxidative stress, activation of protein kinase C (PKC), which are activated upon high ambient glucose concentration in diabetes [35–37], have been identified. And the activation of phospholipaseA2 (PLA2) is related with the activation of PKC. Phospholipases are important enzyme in the body which can catalyze the decomposition of phospholipids to produce free fatty acids. So the activated PLA2 will accelerate the decomposition of phospholipids. The results of PCs and LPCs obtained in our experiment are consistent with this mechanism. With the development of T2DM the PLA2 was activated so the concentrations of PCs decreased. For LPC which was produced by PC

Table 3
List of potential biomarkers, molecular composition and their plasma concentrations in healthy controls, DM and DN.

Classes	m/z ESI$^-$	Fragments (m/z)		Molecular composition	Significances			Concentration (μg/mL)		
		MS^2	MS^3		Controls and T2DM	Controls and DN	T2DM and DN	Controls ($n=30$) mean ± st/$n^{1/2}$	DM ($n=30$) mean ± st/$n^{1/2}$	DN ($n=52$) mean ± st/$n^{1/2}$
LPC	540.3	480.3	255.3	C16:0	(↑)	<0.01 (↑)	(↑)	420.40 ± 17.88	536.64 ± 39.03	635.25 ± 45.67
LPC	564.3	504.3	279.3	C18:2	<0.01 (↑)	(↑)	<0.01[a] (↓)	50.99 ± 10.05	84.48 ± 12.53	61.53 ± 5.62
LPC	566.3	506.3	281.3	C18:1	(↑)	<0.01 (↑)	(↑)	71.23 ± 12.13	93.90 ± 12.62	109.55 ± 24.26
LPC	568.3	508.3	283.3	C18:0	<0.01 (↑)	<0.01 (↑)	(↑)	137.63 ± 12.39	186.44 ± 23.79	210.99 ± 37.45
LPC	588.3	528.3	303.3	C20:4	<0.01 (↑)	<0.01 (↑)	(↓)	44.94 ± 9.20	75.79 ± 10.90	73.93 ± 13.99
PC	802.5	742.5	255.3, 279.3	C16:0/18:2	<0.01 (↑)	<0.01 (↑)	(↑)	727.21 ± 39.91	908.83 ± 66.96	997.43 ± 78.95
PC	806.5	746.5	255.3, 283.3	C16:0/18:0	<0.01 (↓)	<0.01 (↓)	(↑)	24.55 ± 2.48	17.70 ± 1.65	18.00 ± 0.55
PC	854.5	794.5	283.3, 303.5	C18:0/20:4	<0.01 (↓)	<0.01 (↓)	(↑)	194.75 ± 11.96	135.28 ± 17.71	164.10 ± 2.57
PE	716.5	434.5, 460.5 255.3, 281.3		C16:0/18:1	(↑)	<0.01 (↑)	<0.01[a] (↑)	0.44 ± 0.03	0.47 ± 0.11	0.64 ± 0.07
PE	738.5	434.5, 482.5 255.3, 303.3		C16:0/20:4	<0.01 (↑)	<0.01 (↑)	(↑)	70.87 ± 8.83	96.62 ± 11.63	98.25 ± 15.02
PE	750.5	446.5, 303.3		pC18:0/20:4	(↓)	<0.01	<0.01[a] (↓)	606.11 ± 85.16	593.76 ± 77.24	264.94 ± 29.19
PG	773.5	283.3, 279.3 494.5, 490.5		C18:0/18:2	<0.01 (↑)	<0.01 (↑)	(↑)	0.98 ± 0.25	1.76 ± 0.70	2.04 ± 0.54
PI	837.5	255.3, 283.3 419.5, 581.5		C16:0/18:0	<0.01 (↓)	<0.01 (↓)	(↑)	4.80 ± 0.81	3.51 ± 0.35	4.76 ± 0.43
PI	885.5	283.3, 303.3 419.5, 581.5		C18:0/20:4	<0.01 (↓)	(↓)	(↓)	564.89 ± 44.12	427.64 ± 44.10	488.02 ± 46.07
PI	909.5	283.3, 327.5 419.5, 581.5		C18:0/22:6	<0.01 (↓)	<0.01 (↓)	<0.01 (↓)	7.06 ± 0.40	5.10 ± 0.36	3.97 ± 0.48
PS	790.5	283.3, 505.5	283.3	C18:0/18:0	(↓)	<0.01 (↓)	<0.01[a] (↓)	11.56 ± 1.65	11.12 ± 2.50	5.75 ± 1.10
SM	747.5	687.5	449.5	dC18:1/16:0	(↑)	<0.01 (↑)	(↑)	585.76 ± 19.89	664.94 ± 62.96	730.95 ± 88.25
SM	801.5	741.5	451.5	dC18:0/20:2	<0.01 (↑)	<0.01 (↑)	<0.01 (↑)	0.45 ± 0.12	0.80 ± 0.15	1.28 ± 0.24

"↑" means up-regulation of metabolites in the latter group as compared to the former one, whereas "↓" represents down-regulation of metabolites.
[a] These species are significant between T2DM and DN, but they cannot distinguish healthy individuals from T2DM or DN.

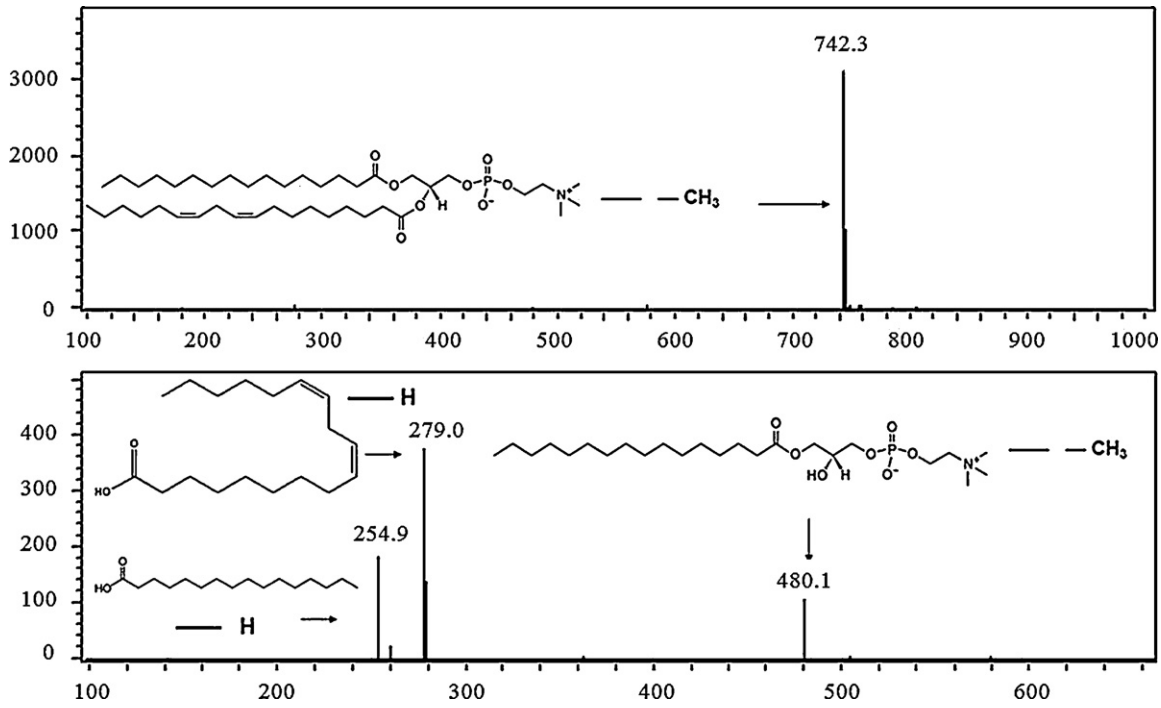

Fig. 5. MS^2 and MS^3 spectrum of PC C16:0/18:2.

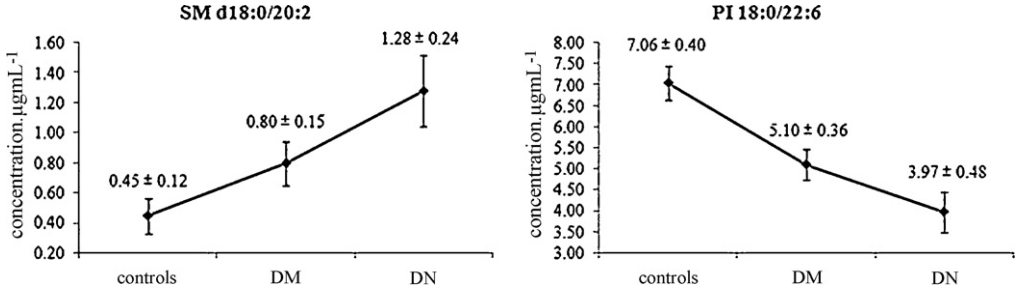

Fig. 6. The plasma concentration levels of two biomarkers for discriminating controls, T2DM and DN.

losing one fatty acid chain under the catalysis of phospholipase, so when PC decomposed, the concentrations of LPCs increased. But the reason for increased PC (C16:0/18:2) specie is largely unknown.

Indeed, abnormal inositol phospholipids metabolism happens in diabetes [38,39]. In this study, the abnormal phosphatidylinositol (PI) metabolism was characterized by a decreased trend of PI biomarkers in T2DM and DN. This phenomenon could be related to the activation of the sorbitol pathway (SP). The possible mechanism is described as follow: SP activation → degradation of intracellular inositol → reduction of myo-inositol (MI) → reduction of PI synthesis.

Sphingomyelin (SM) marker was up-regulated in T2DM and DN. It is reported that glucocorticoids have a large and specific effect on sphingolipids metabolism in diabetes, which can increase membrane sphingomyelin [40]. Furthermore, the fact that glucocorticoids levels are increased in diabetes patients has been proven by a clinical study [41]. On the basis of these specific evidences, we reach a conclusion that SM is increased with the development of T2DM and DN.

The variations of biomarkers may suggest a possible role of PI and SM in prevention and treatment of T2DM and DN. Hopefully, our quantitative results will provide reference ranges for the prediction and diagnosis of T2DM and DN and assessment of therapeutic effect.

4. Conclusion

In this paper, based on multivariate data analysis of plasma phospholipids profiling and targeted quantification, a phospholipidomic approach was successfully used to find out and identify potential biomarkers in type 2 diabetes mellitus and diabetic nephropathy. The combination of partial least squares discriminant analysis and one-way analysis of variance was successfully applied to screen out biomarker candidates from complex mass spectrometry data. The structural identification of potential phospholipids biomarkers was realized by Ion trap-MS^n experiment. Moreover, the quantitative analysis determined the plasma concentration levels of potential biomarkers in healthy controls and patients. The two novel biomarkers suggest that phospholipids can be used as indicators for predicting the progress of type 2 diabetes mellitus and diabetic nephropathy.

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