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Discrimination of Type 2 diabetic patients from healthy controls by using metabonomics method based on their serum fatty acid profiles

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Abstract

Metabonomics, the study of metabolites and their roles in various disease states, is a novel methodology arising from the post-genomics era. This methodology has been applied in many fields, including work in cardiovascular research and drug toxicology. In this study, metabonomics method was employed to the diagnosis of Type 2 diabetes mellitus (DM2) based on serum lipid metabolites. The results suggested that serum fatty acid profiles determined by capillary gas chromatography combined with pattern recognition analysis of the data might provide an effective approach to the discrimination of Type 2 diabetic patients from healthy controls. And the applications of pattern recognition methods have improved the sensitivity and specificity greatly.
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Keywords: Pattern recognition; Diabetes mellitus; Fatty acid profiles; Linear discriminant analysis; Artificial neural networks; Metabonomics

1. Introduction

Metabonomics is the method of studying, profiling, and fingerprinting metabolites in various physiologic states [1]. This method has recently demonstrated enormous potentials in many fields such as plant genotype discrimination [2–3], toxicological mechanisms, disease processes, and drug discovery [4–10]. One such recent application of this method included the rapid and noninvasive diagnosis of coronary heart disease [11–14]. In these methods, metabolite profiling is mainly used for the analysis of a class of metabolites. Metabolomics aims to include all classes of compounds and utilizes metabolic fingerprinting to maintain a rapid classification of samples according to their origin and biological relevance. In order to optimize and utilize metabonomics, a stable metabolite fingerprint must be achievable.

Fatty acids are known to be important biomedical indicators of the abnormal lipid metabolism in diabetes mellitus and extensive studies have been undertaken to investigate the changes of fatty acid profiles in diabetic patients [15–21]. In several studies it has been shown that the fatty acid composition of serum lipids [22] and of skeletal muscle phospholipids [22,23] influence insulin sensitivity. And a recent research noted that whatever the molecular mechanism, long-chain fatty acids may now be viewed as central nervous system signaling molecules, and fatty acids and carnitine palmitoyltransferase-1 as potential pharmaceutical targets for the treatment of obesity and diabetes.

Diabetes mellitus (DM) disease is a typical metabolism disorder disease. To test the power of metabonmics, in this study, the Type 2 diabetes mellitus (DM2) was investigated to know the possibility of distinguishing the DM2 patients from healthy persons based on metabolite profile. Based on serum lipid metabolites and metabonomics method developed, it was found that serum fatty acid profiles determined by

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Table 1
Data sets used in this study

Fraction	Patients	Controls	Total
Cholesterol esters	45	45	90
Free fatty acids	51	50	101
Phospholipids	40	49	89
Triglycerides	49	48	97
Combination	34	44	78

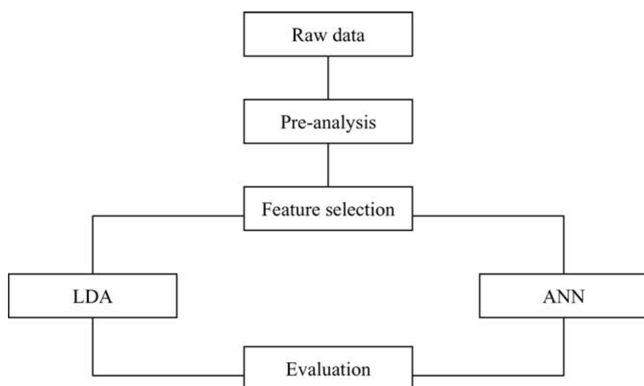

Fig. 1. Flow diagram of the general scheme followed for the data analysis.

capillary gas chromatography combined with pattern recognition analysis of the data might provide an effective approach to the discrimination of DM2 patients from healthy controls. And the application of bioinformatics methods has greatly improved the sensitivity and specificity.

2. Materials and methods

2.1. Data acquisition

In this study, 101 subjects (51 patients with Type 2 diabetes mellitus and 50 healthy controls) were collected before breakfast and determined in Zentrallab., Medizinishce Klinik, Germany. All people were adult. The control samples were confirmed without any underlined disease. Total lipids have been extracted from human serum with chloroform-methanol 2:1 (v/v) and separated into individual classes by TLC. After transesterification the fatty acid methyl esters were analyzed by capillary gas chromatography on an FFAP column. The quantitation of the fatty acids has been performed using internal and external standards [24].

For 78 of these subjects (34 DM2 patients and 44 healthy controls) complete sets of fatty acids data were available, and therefore they were used for pattern recognition. The number of data used for feature selection was summarized in Table 1.

2.2. Data processing

The general approach for the analysis of the data was shown in Fig. 1. Briefly, all fractions of fatty acids were pre-analyzed using principal component analysis. Feature selection was carried out using a mixed univariate/multivariate strategy to select fatty acids that were related to the classification. Based on the selected variables, linear discriminant analysis (LDA) and artificial neural networks (ANNs) were used for discrimination of DM2 patients from controls. The prediction ability of ANN and LDA was evaluated by applying the leave-n-out and the single splitting methods. To compare with the feature-selected data, the origin data were fed to ANN and LDA, respectively.

2.2.1. Feature selection

The general purpose of feature selection is to find the optimum combination of features, which provides the best classification result. The irrelevant variables that introduce noise should be eliminated. In this study, a mixed univariate/multivariate strategy was applied to reach this goal.

The importance of individual variables to discriminate between groups in the training sets was expressed by their *variance weights*. These weights were the ratio of between-class variance to within-class variance for the training groups. For two classes, K and L, the weight of the jth variable is obtained by using the equation

$$\text{Weight}_j = \frac{n_K n_L/n^2 \sum_{k=1}^{n_K}\sum_{l=1}^{n_L}(x_{kj}-x_{lj})^2}{n_K/n \sum_{k=1}^{n_K}\sum_{k'=1}^{n_K}(x_{kj}-x_{k'j})^2 + n_L/n \sum_{l=1}^{n_L}\sum_{l'=1}^{n_L}(x_{lj}-x_{l'j})^2} \quad (1)$$

where n_K and n_L are the number of individuals that are members of class K and L ($n_K + n_L = n$), x_{kj} is the value of this variable (k and k' are part of K, l and l' of L).

A backward stepwise strategy combined linear discriminant analysis and the variance weights of individual variables was carried out for feature selection. In each step one variable was deleted and LDA was carried out to the remaining variables. Correct recognition rate was calculated for each combination of features. And the subset of features with the highest correct recognition rate and with largest sum of variance weights was selected to next step. This process continued until a dropping of correct recognition rate occurred.

2.2.2. Classification

2.2.2.1. Linear discriminant analysis. The first approach to solving the classification problem was to use the more traditional method LDA. LDA is a statistical technique that can be used for the classification of individuals into mutually exclusive and exhaustive groups based on a set of independent variables [25]. The LDA involves finding a linear combination of the independent variables that minimizes the probability of misclassifying the individuals into their respective groups. The LDA was performed using the selected 10 fatty acids of the combination, which were listed in Table 3.

Table 2
Several statistical parameters and the weights of fatty acids for diabetic patients and healthy controls

Fatty acids	Cholesterol esters							Free fatty acids						
	Diabetic patients		Healthy controls		Weight	t	Significance ($\alpha = 0.05$)	Diabetic patients		Healthy controls		Weight	t	Significance ($\alpha = 0.05$)
	Mean	S.D.	Mean	S.D.				Mean	S.D.	Mean	S.D.			
C12:0	0.163	0.155	0.198	0.081	1.179	1.899	N	0.388	0.803	1.123	1.668	0.971	2.830	Y
C14:0	0.948	0.298	0.997	0.3	0.979	1.099	N	2.942	1.386	3.452	1.315	1.052	1.896	N
C15:0	0.238	0.093	0.225	0.055	1.118	1.141	N	0.298	0.163	0.395	0.173	1.117	2.901	Y
C16:0	13.43	1.372	11.75	0.804	*2.37*[a]	*10.022*	Y	26.777	4.188	27.988	3.958	1.025	1.493	N
C16:1	4.155	2.148	3.919	1.717	1.031	0.814	N	2.998	1.111	3.2	1.574	0.902	0.746	N
C18:0	1.037	0.336	0.944	0.162	1.22	2.365	Y	12.685	2.639	13.515	2.908	0.987	1.503	N
C18:1 N9	19.634	2.589	18.493	1.843	1.194	3.406	Y	33.16	9.818	29.804	5.882	1.195	2.079	Y
C18:1 N7	1.058	0.31	0.916	0.205	1.235	3.625	Y	2.025	0.567	1.459	0.803	1.195	4.098	Y
C18:2 N6	48.98	5.488	49.899	4.226	1.052	1.259	N	11.899	3.509	12.806	4.071	0.959	1.200	N
C18:3 N6	1.179	0.494	1.475	0.528	1.114	3.884	Y	0.147	0.123	0.175	0.118	1.003	1.167	N
C20:0	0.067	0.06	0.056	0.022	1.236	1.633	N	0.301	0.26	0.246	0.125	1.192	1.350	N
C18:3 N3	0.484	0.157	0.652	0.188	*1.371*	*6.507*	Y	0.814	0.305	1.208	0.812	0.985	3.240	Y
C20:2	0.219	0.054	0.236	0.066	0.958	1.891	N	0.42	0.236	0.56	0.244	1.125	2.931	Y
C20:3 N6	0.663	0.135	0.726	0.123	1.112	3.273	Y	0.321	0.104	0.335	0.547	0.784	0.180	N
C22:0	0.013	0.02	0.037	0.245	0.782	0.926	N	0.264	0.255	0.293	0.345	0.905	0.481	N
C20:4	5.597	1.914	6.793	1.237	*1.384*	4.979	Y	1.799	0.678	1.693	1.895	0.812	0.376	N
C20:5 N3	0.481	0.253	0.777	0.329	1.383	*6.766*	Y	0.807	0.694	0.366	0.216	*1.682*	4.294	Y
C24:0	0.075	0.051	0.091	0.079	0.901	1.614	N	0.476	0.341	0.447	0.376	0.947	0.406	N
C22:4	0.017	0.039	0.021	0.03	1.037	0.771	N	0.157	0.093	0.128	0.166	0.877	1.086	N
C22:5	0.015	0.019	0.029	0.025	1.063	4.230	Y	0.253	0.107	0.195	0.131	1.034	2.439	Y
C22:6	1.546	0.403	1.766	0.488	1.038	3.298	Y	1.068	0.504	0.611	0.523	*1.348*	4.472	Y

Fatty acids	Phospholipids							Triglycerides						
	Diabetic patients		Healthy controls		Weight	t	Significance($\alpha = 0.05$)	Diabetic patients		Healthy controls		Weight	t	Significance ($\alpha = 0.05$)
	Mean	S.D.	Mean	S.D.				Mean	S.D.	Mean	S.D.			
C12:0	0.079	0.396	0.053	0.081	1.266	0.449	N	0.749	0.824	1.418	1.405	1.136	2.898	Y
C14:0	0.522	0.497	0.373	0.077	*1.389*	2.071	Y	2.581	0.99	3.48	1.165	1.069	4.141	Y
C15:0	0.209	0.086	0.212	0.055	1.082	0.199	N	0.368	0.143	0.398	0.117	0.129	1.141	N
C16:0	25.73	5.999	26.512	1.55	1.262	0.878	N	29.515	5.065	28.188	4.805	4.887	1.337	N
C16:1	1.129	1.026	0.773	0.289	1.374	2.321	Y	4.031	1.376	4.29	1.472	1.410	0.905	N
C18:0	15.23	4.676	15.746	1.137	1.262	0.747	N	4.536	0.932	4.941	1.098	1.007	1.980	N
C18:1 N9	12.664	4.543	10.229	1.199	*1.582*	*3.605*	Y	37.62	3.603	36.97	4.481	4.019	0.796	N
C18:1 N7	1.359	0.311	1.195	0.183	1.336	3.095	Y	1.835	0.827	1.668	0.734	0.774	1.062	N
C18:2 N6	22.466	8.124	21.745	2.232	1.245	0.595	N	14.934	5.439	14.273	3.814	4.657	0.699	N
C18:3 N6	0.173	0.191	0.139	0.077	1.216	1.139	N	0.437	0.284	0.495	0.283	0.281	1.018	N
C20:0	0.282	0.09	0.318	0.053	1.236	2.347	Y	0.091	0.044	0.118	0.084	0.066	2.010	Y
C18:3 N3	0.217	0.22	0.201	0.071	1.223	0.480	N	0.79	0.37	1.018	0.504	0.437	2.570	Y
C20:2	0.079	0.118	0.097	0.106	1.007	0.757	N	0.431	0.091	0.454	0.106	0.098	1.159	N
C20:3 N6	2.781	0.95	3.179	0.546	1.259	2.476	Y	0.263	0.099	0.267	0.071	0.085	0.231	N
C22:0	1.196	0.434	1.304	0.236	1.176	1.493	N	0.018	0.019	0.028	0.036	0.028	1.734	N
C20:4	10.173	3.092	10.979	1.902	1.146	1.509	N	1.045	0.41	1.125	0.349	0.377	1.045	N
C20:5 N3	0.687	0.472	0.9	0.334	1.203	2.488	Y	0.173	0.18	0.175	0.085	0.140	0.070	N
C24:0	1.406	0.586	1.637	0.353	1.226	2.297	Y	0.02	0.025	0.023	0.037	0.031	0.474	N
C22:4	0.463	0.325	0.534	0.287	1.025	1.094	N	0.163	0.101	0.184	0.152	0.127	0.811	N
C22:5	0.441	0.15	0.591	0.128	*1.604*	*5.090*	Y	0.161	0.074	0.166	0.058	0.066	0.374	N
C22:6	2.715	0.871	3.283	0.845	1.193	3.111	Y	0.397	0.467	0.398	0.222	0.363	0.014	N

[a] The number written in italics font is the largest ones in these columns.

2.2.2.2. Artificial neural networks. The second approach involved training ANNs to classify the subjects into their respective groups. The ANNs used in this study consisted of a number of input nodes corresponding to the number of selected variables from each fraction of fatty acids and the combination of selected fatty acids of all fractions, five nodes in hidden layer and two output nodes representing the patient and control groups. According to the characteristics of the network's input–output transformation, the node's output value should lie in the range between 0 and 1. So all fatty acid variables acting as the network's input were normalized to interval of (0,1) using the Min-Max procedure [26]:

$$\text{Factor} = \frac{(x_{i,\max} - x_{i,\min})}{(l_{\text{high}} - l_{\text{low}})} \quad x_{i,\text{new}} = \frac{(x_{i,\text{old}} - x_{i,\min})}{\text{factor} + l_{\text{low}}} \qquad (2)$$

where $x_{i,\max}, x_{i,\min}$ are the maximum and minimum values for input variable $x_{i,\text{old}}$. l_{high} and l_{low} are two end of the interval, 0 and 1.

The normalized variables of each pattern were taken as inputs to train an ANN. Prior to the training of the ANN, the bias was set to 1 and the connection weights were set to small random values. The outputs for each pattern were set to [1,0] for diabetics and [0,1] for controls. A diabetic pattern was defined as being correctly classified if its outputs were [>0.75, <0.25] (compared with the target outputs [1,0]) and wrongly classified if its outputs were [<0.25, >0.75]. Other output values were interpreted as an uncertain classification. The classification of control patterns was interpreted similarly.

Optimization of ANNs was carried out by studies of the following parameters: the gain parameter of the sigmoid function, the value of learning rate and momentum term. It was found that, under the network architecture described above, combination of the gain of 1.0, learning rate of 0.9 and momentum of 0.7 could get better results.

2.2.3. Evaluation

The quality of pattern results should be carefully checked after the analysis. A relatively unbiased method called "leave-n-out" was carried out for the evaluation of prediction ability of LDA and ANNs in this study. The test set was selected randomly one third of the patterns from each group. The remaining patterns acted as the training set. This process was

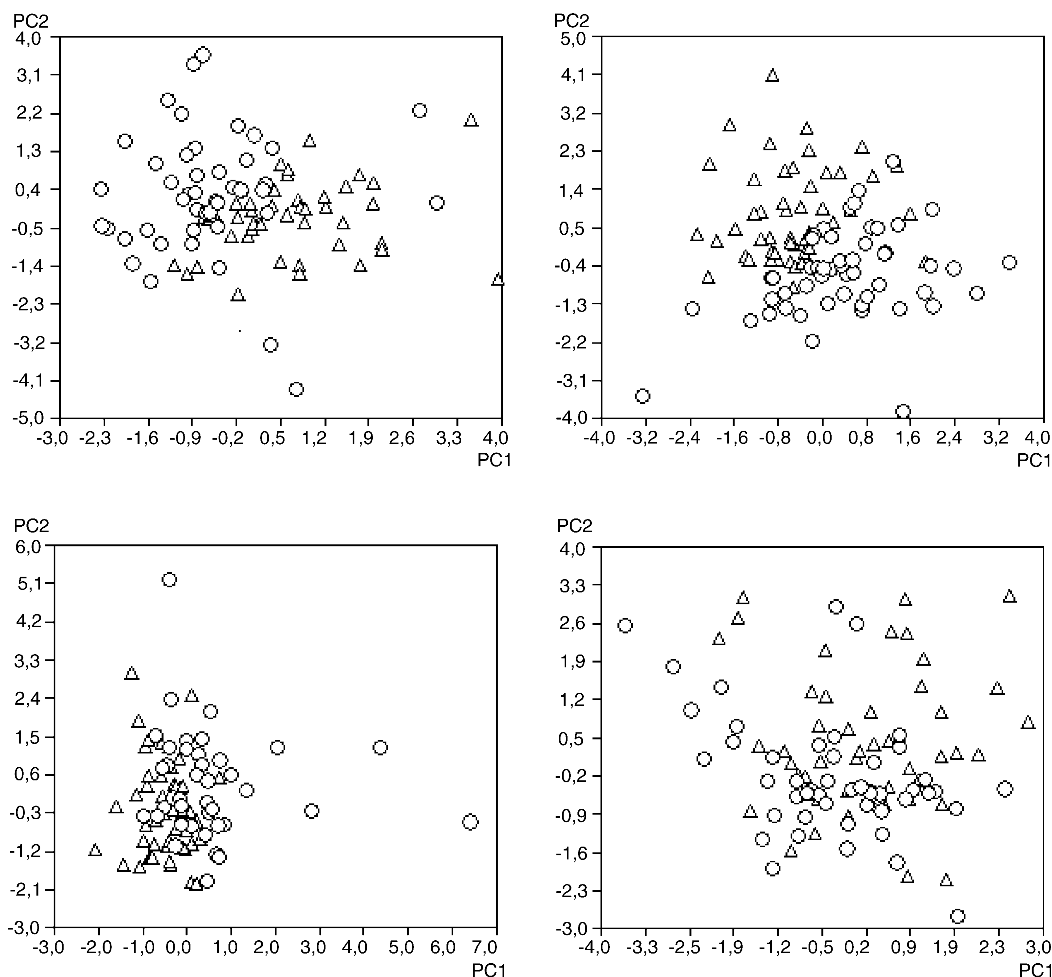

Fig. 2. PCA–NLMs of fractions (a) cholesterol esters, (b) free fatty acids, (c) phospholipids and (d) triglycerides. (◯) Type 2 diabetes mellitus patients; (△) Healthy controls. PC1, the first principal component; PC2, the second principal component.

Table 3
Feature selection results

Fraction	Selected variables
Cholesterol esters	C16:0, C18:1 N7, C18:3 N6, C18:3 N3, C20:4, C20:5 N3, C22:6
Free fatty acids	C12:0, C18:1 N9, C18:1 N7, C18:3 N3, C20:2, C20:5 N3, C22:5, C22:6
Phospholipids	C14:0, C16:1, C18:1 N9, C18:1 N7, C20:3 N6, C20:5 N3, C22:5, C22:6
Combination[a]	C16:0(CE), C18:3 N3(CE), C20:4(CE), C18:1 N9(FFA), C18:1 N7(FFA), C20:5 N3(FFA), C22:6(FFA), C16:1(PL), C18:1 N9(PL), C22:5(PL)

[a] CE, cholesterol esters; FFA, free fatty acids; PL, phospholipids.

performed three times, and each pattern was used once in the test set, but several times in the training set. An overall recognition rate and an overall prediction rate were then calculated from the training sets and test sets, respectively. Additionally, a single splitting method was employed for the evaluation of the prediction ability of ANN. Each group of patterns was randomly divided into one third, which was used as the test set and the two thirds was used as the training set. Each pattern was used only once in the test set or in the training set. To the origin data, only single splitting method was carried out for the evaluation of the prediction rates.

2.3. Software

The programs used in this study were from self-developed statistical software package written in DELPHI.

3. Results and discussion

3.1. Pre-analysis

First of all, each fraction of fatty acids was analyzed using principal component analysis followed by non-linear mapping (PCA–NLM). For the fractions of cholesterol esters, free fatty acids and phospholipids, PCA–NLM showed that the control patterns were quite well clustered while the DM patterns were more scattered (Fig. 2(a)–(c)). For the triglyceride fraction PCA–NLM showed that all patterns were scattered (Fig. 2(d)). The result indicated that triglyceride fraction of fatty acids might not reflect differences between DM patients and controls, while the other three fractions of fatty acids were suitable for classification.

3.2. Feature selection

Some statistical parameters of fatty acids of all four fractions are listed in Table 2. From Table 2, it can be expected that palmitic acid, eicosatetraenoic acid, eicocapentaenoic acid in the cholesterol esters fraction; eicosapentaenoic acid, docosahexaenoic acid in the free fatty acids fraction and oleic acid, docosapentaenoic acid in the phospholipids fraction may be more relevant for classification, while other fatty acids may be irrelevant in this study. Additionally, *t*-test was also employed to analyze the data, the same important fraction set could be found (Table 2).

Compared with the fatty acids of the other three fractions, almost all of the fatty acids of the triglyceride fraction gave low levels of variance weight, which meant they had poor classification ability in this study. This result was also supported by the pre-analysis described above. So the triglyceride fraction was deleted in further evaluation.

From Table 2, it was found that variables in the phospholipids fraction showed higher variance weights than those in other fractions, it seemed that they have higher classification power. However, the higher difference level of the standard deviations between patient and control groups (S.D. of patient group > S.D. of control group) would result in higher different dispersion of the two groups (see Fig. 2c). In such a case, when a linear statistical discriminant method (e.g. LDA) was applied, some patterns of the more disperse group (diabetic group) would be catalogued as members of the more condensed group (control group).

After the mixed univariate/multivariate method described in Section 2.2 was applied to each fraction of fatty acids, the subsets of variables were selected (Table 3). The selected subsets of fatty acids of all fractions were combined to generate a new data set. From the combined data, 10 fatty acids were selected as the variables, which were more relevant for differentiation of diabetic patients from controls (Table 3).

3.3. Results of classification

LDA is a statistical technique that can be used for the classification of individuals into mutually exclusive and exhaustive groups based on a set of independent variables [25]. The classification results yielded were shown in Table 4. LDA correctly identified 96.2% of the cases; the sensitivity and

Table 4
Evaluation results for LDA and ANN (after the features were selected)

	Method	Recognition rate	Prediction rate	Sensitivity	Specificity
	LDA	96.2% (75/78)	88.5% (69/78)	85.3% (29/34)	90.9% (40/44)
ANN	Leave-n-out	97.4% (76/78)	89.7% (70/78)	88.2% (30/34)	90.9% (40/44)
	Single splitting	96.2% (50/52)	96.2% (25/26)	90.9% (10/11)	93.75% (14/15)

Table 5
Evaluation results for LDA and ANN (based on the origin data)

Method	Prediction rate (%)	Sensitivity (%)	Specificity (%)
LDA, single splitting	65.4	36.4	65.0
ANN, single splitting	80.8	90.9	91.67

specificity were 85.3% and 90.9%, respectively. The same data sets analyzed by LDA were fed to ANNs, and the classification results were also given in Table 4. A little higher recognition rate, sensitivity and specificity could be found in the ANN results especially, when single splitting method was performed.

In spite of the little difference between the LDA and ANNs methods, both of them performed strong classification ability for the specific task in this study. It is partly because the features used for classification were selected based on a linear statistical strategy. From this point, it might be deduced that there was a linear relationship between serum fatty acid levels and DM2.

3.4. Evaluation

The evaluation results using the leave-n-out method and single splitting method (employed in ANNs) were given in Table 4. Both LDA and ANNs yielded good prediction rates (88.5%, 89.7% and 96.2%, respectively). It was found that the differences between recognition rates and prediction rates were at relatively low level, it indicated that over-training that results in a greatly decreased ability to identify new patterns in test sets did not occur.

3.5. Comparison with the original data

To validate the features selected, the original data were also fed to the ANN and LDA. And the results are given in Table 5. Comparing with Table 4, it could be found that much better results were obtained after features were selected, especially in LDA method. So it could be concluded that the feature-selection is essential especially to LDA, while the ANN method is not so sensitive for its powerful classification ability.

4. Conclusions

In this study, it is suggested that metabonomics method of serum fatty acid determined by capillary gas chromatography may provide an effective approach to the discrimination of DM2 patients from healthy controls. It was shown that, using univariate and multivariate strategy, it is possible to find a selected set of serum fatty acids to provide an optimal discrimination. And the metabonomics method was helpful to find the biomarkers of DM2 and to discover the mechanism of the disease.

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