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**ORIGINAL ARTICLE**

# Metabonomic profiling of diet-induced hyperlipidaemia in a rat model

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**Abstract**

This study describes the metabolic profiles of the development of hyperlipidaemia in a rat model, utilizing metabonomics by gas chromatography–mass spectrometry (GC-MS) determination coupled with multivariate statistical analysis. Rat plasma samples were collected before and during a high-lipid diet at days 0, 7, 14, 21 and 28, and were analysed for lipid levels using kit assays or metabonomics using GC-MS. Forty-one endogenous metabolites were separated, identified and quantified using GC-MS. The data matrix was processed by principal component analysis or partial least squares discriminant analysis. Dynamic modification of the rat metabonome can be clearly identified and tracked at different stages of hyperlipidaemia in the rat model. Potential biomarkers, including β-hydroxybutyrate, tyrosine and creatinine, were identified. The current work suggests that metabonomics is able to provide an overview of biochemical profiles of disease progress in animal models. Using a metabonomic approach to identify physiopathological states promises to establish a new methodology for the early diagnosis of human diseases.

**Keywords:** *Metabonomics; hyperlipidaemia; GC-MS; principal component analysis; partial least squares discriminant analysis; early diagnosis*



## Introduction

Hyperlipidaemia has been recognized as a major risk factor of coronary heart disease (CHD), the leading cause of death in the USA (LaRosa 2001). Hyperlipidaemia is defined as a disorder of lipid metabolism leading to abnormal elevation of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) or triglycerides (TG), or deficiency of high-density lipoprotein cholesterol (HDL-C) (Alwaili et al. 2009). As a progressive chronic and systemic disease (Lahoz & Mostaza 2007), atherosclerotic CHD begins in childhood and progresses to morbidity and mortality throughout the life span. Hyperlipidaemia plays an important role in development and progression of atherosclerosis and cardiovascular diseases. Not only severe hyperlipidaemia, but also moderate hyperlipidaemia is closely linked to cardiovascular disease (CVD) (Tannock 2008). Treatment of hyperlipidaemia by lipid-lowering therapy can decrease the risk of CHD, and may even prevent CVD (Goff et al. 2006). LDL-C is the primary target for the lipid-lowering therapy and CVD prevention (Raal 2009). The thresholds for initiating hyperlipidaemia therapy and target lipid levels have recently been revised to even lower lipid levels (Tannock 2008). However, the well-accepted surrogate biomarkers of atherosclerosis and cardiovascular events are not putatively predictable for these diseases as evidenced by outcomes from clinical trials (Raal 2009, Rosenson 2008). Hyperlipidaemia in the early phase is usually ignored as there is a lack of clear clinical symptoms and a diagnostic index. As hyperlipidaemia is a systemic progressive disorder, alterations of endogenous metabolites may

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provide a prognostic index at the initial stage of this disorder compared with those of the putative lipid levels at the symptomatic stage. Early diagnosis or prevention of, or intervention in hyperlipidaemia poses challenges to clinicians and scientists.

Metabonomic approaches provide insights in altered metabolite pathways and disease aetiology and pathophysiology (Holmes et al. 2008, Nicholson & Lindon 2008). As such, it plays an important role in the discovery of metabolite biomarkers for prognostic purposes (Nordstrom & Lewensohn 2009). Metabonomics, as a new approach of systems biology, offers a series of snapshots of metabolic profiles which are products of gene–environment–lifestyle interactions at the individual and the population levels (Lindon et al. 2004a, Nicholson et al. 2008). Using molecular spectroscopic and chemometric approaches, metabonomics can connect phenotype variation with disease risk factors to evaluate the influences of lifestyle, environment and genes in states of diseases and drug treatment (Lindon et al. 2004b, c, Trygg et al. 2007). In particular, metabonomic techniques have been applied to identify endogenous metabolites associated with a number of diseases, including cancer, diabetes, schizophrenia, inborn errors of metabolism and CVD (Giovane et al. 2008, Gowda et al. 2008, Khaitovich et al. 2008, Spratlin et al. 2009). Furthermore, it has opened new avenues for systemic biomarker discovery in early diseases (Denkert 2008, Nagaraj 2009).

There are two platforms commonly used in metabonomics approach – nuclear magnetic resonance (NMR) and mass spectrometry (MS). Although popular and effective (Griffin 2003, Lindon et al. 2004a), NMR has two significant disadvantages in metabonomic study: poor sensitivity and resolution, which tend to mask low-abundance analytes by high-concentration components (Gowda et al. 2008, Lewis et al. 2008). Gas chromatography (GC)-MS methods have shown benefits in both quantitative and high-throughput data analysis in metabonomics (A et al. 2005, Jonsson et al. 2005a, Nordstrom & Lewensohn 2009). The protocol of extraction and derivation has been developed for analysing human plasma using GC-MS. The established method has shown potential in identifying biological markers related to diseases (A et al. 2005, Jonsson et al. 2005a, b).

In this research, a GC-MS-based analytical strategy was applied with pattern-recognition techniques to investigate biochemical profiles and potential biomarkers of hyperlipidaemia in a rat model. Measurement of lipid levels using kit assays was performed simultaneously to detect the pathophysiological processes. The study indicates that this metabonomic approach could identify potential biomarkers and elucidate progressive stages of hyperlipidaemia in rats, which provides the basis for validation of biomarkers, mechanisms of pathophysiology, and early diagnosis of hyperlipidaemia in clinical studies.

## Methods

### *Chemicals and reagents*

The kits for measuring TG, TC, LDL-C and HDL-C were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

[$^2H_6$]-Salicylic acid (97%), used as the internal standard (I.S.), was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). *N*-methyl-*N*- trimethylsilyl-trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) were obtained from Fluka (Buchs, Switzerland), and methoxyamine from Supelco, Inc. (Bellefonte, PA, USA). Pure water was produced using a Milli-Q Reagent Water System (Millipore Corp., Billerica, MA, USA).

### *Animal study*

Male Sprague–Dawley rats, weighing 190–210 g, were purchased from Sino-British Sippr/BK Lab Animal Ltd (Shanghai, China). The animals were housed in stainless steel cages in a controlled environment (20°C, 50% relative humidity and 12 h light/12 h dark cycles) for at least 6 days prior to the experiment. Animal experiments were carried out in accordance with the Guidelines for Animal Experiments at Nanjing University of Technology (Nanjing, China), and the protocol was approved by the Animal Ethics Committee at Nanjing University of Technology.

After a 6-day acclimation, rats were randomly assigned into a treatment (diet-induced hyperlipidaemia) group ($n = 8$) and a control group ($n = 8$); the control group was fed with standard chow, and the treatment group was fed with laboratory chow enriched with 1% (w/w) cholesterol and 15% (w/w) lard for 4 weeks (Xu et al. 2003). Rats were fasted for 10 h before blood was collected from the orbital plexus; they were allowed free access to food and water throughout the study except during the fasting period.

Blood samples were obtained predose and at days 7, 14, 21 and 28 postdose in fasting condition from rats fed the high-lipid diet. After addition of EDTA anticoagulant and centrifugation at 4000 rpm for 10 min, 1 ml of plasma of the supernatant was collected and divided into two parts for the kit assay for lipid levels and the GC-MS analysis, respectively. All samples were stored at –80°C until analysis.

### *Kit assays for lipid levels*

Kit assays for lipid levels were conducted using rat plasma samples according to the kit instructions. Plasma was

thawed and incubated at 37°C for 15 min. Lipid levels, including TC, TG, HDL-C and LDL-C, were determined using commercial kits with enzymatic methods.

### *Metabonomic study*

#### Pretreatment of plasma samples

One hundred microlitres of rat plasma was added to 400 µl methanol with 2 µg [$^2H_6$]-salicylic acid as I.S. The solution was vigorously vortexed for 10 min. After 1-h protein precipitation in an ice bath, the tube was centrifuged at 14000 rpm at 4°C for 10 min. Four hundred microlitres of the supernatant was transferred to a GC vial, and then evaporated to dryness under nitrogen at room temperature.

Thirty microlitres of methoxyamine in pyridine (15 µg µl$^{-1}$) was added to each GC vial, and then the solution was vigorously vortexed for 10 min. After oximation reaction for 16 h at room temperature, the samples were trimethylsilylated for another hour by adding 30 µl MSTFA with 1% TMCS as the catalyst. Finally, 40 µl heptane was added into the GC vial, and the solution was vortexed for 1 min before GC-MS analysis.

#### GC-MS analysis

One microlitre of the derivatized sample was injected splitless into a Finnigan TRACE DSQ gas chromatograph (Thermo Electron Corp., Austin, TX, USA) equipped with a 30 m × 0.25 mm ID, fused silica capillary column, which was chemically bonded with 0.25 µm DB5-MS stationary phase (J&W scientific, Folsom, CA, USA). The injector temperature was 270°C, the septum purge flow rate was 20 ml min$^{-1}$, and the purge was turned on after 60 s. The gas flow rate through the column was 1 ml min$^{-1}$. Then the temperature was increased from 70 to 240°C at a rate of 20°C per min, held on for 1 min, and then to the last 320°C at a rate of 20°C per min and held for 1 min. The transfer line temperature was 270°C and the ion source temperature was kept at 200°C. Ionization was achieved by a 70 eV electron beam at a current of 2.0 mA. Masses were acquired from *m/z* 50 to 650 at a rate of 30 spectra per s, and the acceleration voltage was turned on after a solvent delay of 170 s. Each run was 18 min in total.

#### Validation of assay method

*Linearity*. Plasma was diluted with water to relative concentrations of 0.063, 0.125, 0.250, 0.500 and 1.000 (v/v, plasma/plasma + water). One hundred microlitres of the diluted plasma, including 10 µl [$^2H_6$]-salicylic acid (1 mg ml$^{-1}$) used as I.S., was mixed with 400 µl of methanol. Extraction and derivatization were performed according to the procedures described in Pretreatment of plasma samples. After GC-MS analysis, the peak areas of endogenous metabolites were integrated. The peak-area

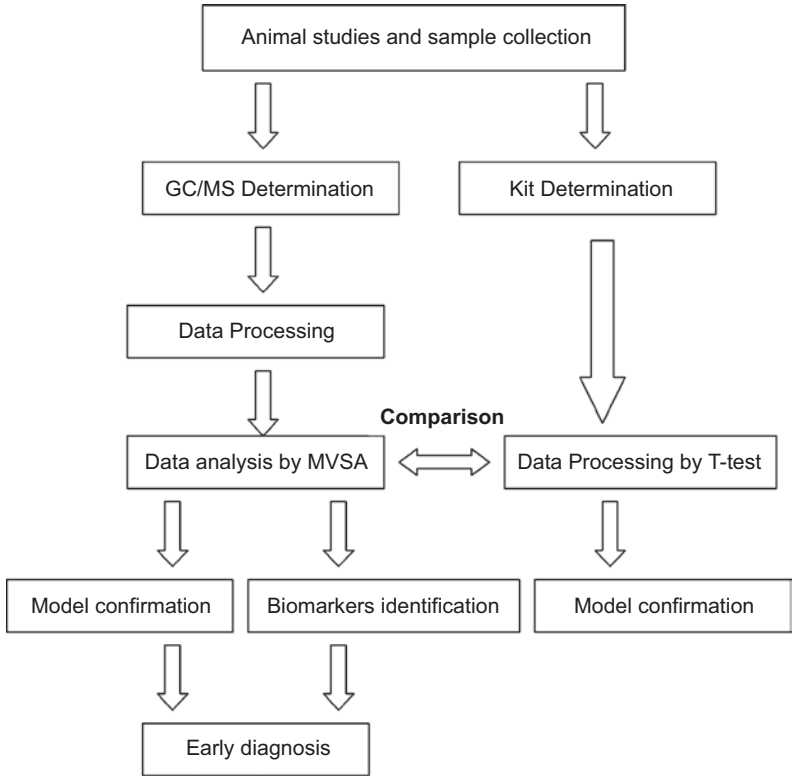


**Figure 1.** Study strategy for metabolic profiles and biomarker identification in diet-induced hyperlipidaemia in a rat model. MVSA, multivariate statistical analysis.

ratio of each metabolite to I.S. was calculated and linear correlation coefficients were calculated in the analysed concentration interval.

*Precision*. Precision was calculated using relative standard deviation (RSD) at three plasma dilutions: 0.063, 0.250 and 1.000 (v/v, plasma/plasma + water). The intraday precision values were determined in five replicates at each dilution, and the replicates were processed independently. The interday precision values were determined across each dilution on five different days. All the samples were treated according to the procedures described in Pretreatment of plasma samples.

*Sensitivity*. Limit of detection (LOD) and limit of quantification (LOQ) for plasma samples were investigated. Plasma dilutions of 0.02, 0.063, 0.125, 0.250, 0.5 and 1.000 (v/v, plasma/plasma + water) were treated according to procedures described in Pretreatment of plasma samples. LOD was defined as a level giving a signal-to-noise (S/N) ratio of 3, and LOQ was defined as a level giving a S/N ratio of 5.

### *Data analysis*

The data from kit assays are presented as the mean ± SD. Statistical significance between the groups was determined using the *t*-test.

All GC-MS data were processed using Xcalibur software (Thermo Electron Corp.). Peaks with a S/N ratio lower than 5 were rejected. Retention time correction was done by incorporating I.S. in order to minimize run-to-run errors. To obtain accurate peak areas for I.S. and specific peaks/compounds, two quantification masses for each component were specified and the data were reprocessed. The area of each peak was normalized to the area of the I.S. Mass spectra of all detected compounds were identified and compared with spectra in NIST 2.0 (2005), and the Wiley library, and in-house spectra library constructed in the Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University. The Human Metabolome database (http://www.hmdb.ca) (Wishart et al. 2007) and Lipid Maps Database (http://www.lipidmaps.org) were also used to search for potential metabolites in accordance with the measured mass.

Multivariate statistical analysis (MVSA) was carried out using SIMCA-P 11 software (Umetrics, Umeå, Sweden). The data matrix was constructed using the GC-MS response of each peak as variables with the observation/samples in columns and the peaks in rows. It can be represented in a K-dimensional space (where K is equal to the number of variables), projected and reduced to a few principal components (PC) that describe the maximum variation of different groups or samples. PC analysis (PCA) is a statistical method used in metabolomics to find the relationship among the samples/observations (Eriksson et al. 2001, 2004, Trygg et al. 2007). Partial least squares projection to latent structures and discriminant analysis (PLS-DA) was used to calculate models differentiating the groups. Statistically different peaks were calculated by the PLS-DA model with a confidence interval of 0.99 and significance level of 0.01. Data were visualized using the PC scores and loadings plot. Each dot on the scores plot represents an individual sample, and dots on the loadings plot represent peaks/compounds observed and identified in the chromatogram. The scores and loadings plot are corresponding and complementary (Jonsson et al. 2005b). Therefore, biochemical components responsible for the differences between samples detected in the scores plot can be extracted from the corresponding loadings.

## Results and Discussion

### *Measurement of lipid levels using kit assays*

The levels of TC, TG, HDL-C and LDL-C in the plasma of the controls and the treatment group determined by kits are shown in Table 1. The data of the control group

**Table 1.** Plasma lipid levels of rats in the control and treatment groups during the experiment.

| Group | Week | TC (mmol $L^{-1}$) | TG (mmol $L^{-1}$) | HDL-C (mmol $L^{-1}$) | LDL-C (mmol $L^{-1}$) |
|---|---|---|---|---|---|
| Control | 0 | 1.93 ± 0.34 | 1.48 ± 0.15 | 1.13 ± 0.15 | 1.39 ± 0.15 |
| | 1 | 1.94 ± 0.32 | 1.48 ± 0.17 | 1.15 ± 0.12 | 1.41 ± 0.16 |
| | 2 | 1.96 ± 0.37 | 1.49 ± 0.15 | 1.13 ± 0.16 | 1.37 ± 0.14 |
| | 3 | 1.95 ± 0.32 | 1.46 ± 0.16 | 1.11 ± 0.17 | 1.39 ± 0.13 |
| | 4 | 1.96 ± 0.40 | 1.48 ± 0.14 | 1.15 ± 0.14 | 1.40 ± 0.14 |
| Diet-induced hyperlipidaemia | 0 | 1.92 ± 0.33 | 1.50 ± 0.12 | 1.14 ± 0.15 | 1.40 ± 0.13 |
| | 1 | 1.89 ± 0.36 | 1.50 ± 0.10 | 1.14 ± 0.17 | 1.39 ± 0.14 |
| | 2 | 1.96 ± 0.31 | 1.48 ± 0.14 | 1.11 ± 0.18 | 1.41 ± 0.12 |
| | 3 | 3.95 ± 0.46*# | 1.48 ± 0.13 | 0.91 ± 0.12*# | 2.79 ± 0.23*# |
| | 4 | 6.17 ± 0.69*# | 1.49 ± 0.15 | 0.89 ± 0.15*# | 3.36 ± 0.31*# |

*Statistically different ($p$ <0.05) from diet-induced hyperlipidaemia group; #statistically different ($p$ <0.05) from within-group control.
TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

showed no significant differences among the five time points ($p$ >0.05). The lipid levels of the treatment group in the first 2 weeks showed no significant changes when compared with those predose ($p$ >0.05). At day 21, the levels of TC, TG and LDL-C increased significantly whereas the level of HDL-C decreased significantly compared with predose ($p$ <0.05).

The data obtained in the treatment group were compared with that of the control group at the same time point using the Student's $t$-test. In the first 2 weeks, there were no significant differences between the two groups ($p$ >0.05). At day 21, the lipid levels in the treatment group exhibited significant differences when compared with those in the control group ($p$ <0.05). The results of the kit assays indicated that hyperlipidaemia was established in the rat by day 21.

### *Metabonomic study*

The GC-MS chromatogram of the plasma samples obtained from the control and treatment groups at day 28 are shown in Figure 2A and B. More than 40 compounds were identified in the GC-MS chromatograms and confirmed as endogenous metabolites, including organic acids, fatty acids, amino acids, lipids, carbohydrates, etc.

#### Validation of assay method

*Linearity*. Twenty-three of the identified endogenous compounds were selected to investigate the linearity of the method. These compounds covered a wide span of GC retention times, and they belonged to different classes of compounds with various physicochemical properties, such as organic acids, amino acids and carbohydrates. The linearity of the response was determined by analysing plasma samples at five different concentrations, and was found to be generally high for most of the compounds investigated ($r$ >0.999), as shown in Table 2.

*Precision*. Twenty-three of the identified endogenous compounds were selected to investigate the precision of the method. The precision of the analysis was calculated as the RSD values of the peak area for each metabolite corrected by the peak area of the I.S. The RSD value of most of the 23 compounds was less than 15%, and less than 10% for many of them, as shown in Table 3. Generally, the lowest concentrations of plasma resulted in the highest precision value, and the precision value of interday was higher than that of intraday.

*Sensitivity*. Twenty-six of the identified endogenous compounds as described in the linearity and precision tests were selected to evaluate LOD and LOQ. These compounds could be detected at S/N greater than or equal to 3 when the plasma dilution (v/v, plasma/plasma + water) was 0.02, and defined as LOD. These compounds could also be detected at S/N greater than or equal to 5 when the plasma dilution (v/v, plasma/ plasma + water) was 0.063, and defined as LOQ.

Differences were observed in overall compositions between plasma samples obtained from the control and diet-induced hyperlipidaemia groups by visual inspection of the GC/MS chromatograms. The levels of erythrose and some unidentified peaks (UI) were lower in the diet-induced hyperlipidaemia group in comparison with the control, while the levels of creatinine, oxalate and cholesterol were elevated, which indicated that the endogenous metabolite levels were altered due to high lipid interference. Visual inspection the GC-MS chromatograms of the plasma samples is a subjective process and interanimal variation can distort the data interpretation. Therefore, multivariate data analysis was performed to facilitate an overview of metabolite patterns and to identify potential biomarkers, which may lead to elucidation of the metabolic or pathophysiological processes of hyperlipidaemia.

#### Time-related metabonomics profiles

The data matrix of peak areas obtained in rats from both the treatment and the control groups was subject to PCA. To facilitate the visualization of the data structure, PCA can reduce a great number of variables into a smaller number of uncorrelated variables, which are called PCs. The first PC explains the greatest variability in the data, the second PC is independent of (orthogonal to) the first component, and best explains the remaining or residual variability of the data and so on. There are two kinds of plots in PCA, scores plot and loadings plot. The scores plot shows observations positioned in K-dimensions (equal to number of PCs) space. The loadings plot shows variables, or peaks which constitute the basis of the reduced dimension PC space. Each point on a PCA scores plot represents all the variables acquired in one spectrum, here 41 variables. Therefore, all of the sample points that cluster together have more similar spectra (and hence more similar biochemical profiles) than those that stand apart. However, plots should be scrutinized specifically to include or discard sample points which are far from the majority as outliers usually have negative influences on modelling. No serious outlier was found in the PCA scores plot for this dataset when overviewed.

The PCA scores plot of the whole dataset is presented in Figure 3. According to cross-validation, a two-component PCA model was calculated. This model explained 41.4% (R2) and predicted 30.5% ($Q^2$) response variables. All the samples (K0–K4) of the control group clustered at the top right corner of the plot, suggesting a stable and consistent metabonomic status for the control group. It was noted that the samples of the diet-induced hyperlipidaemia group at predose (M0) also clustered in the same area as those from the control group. This result was in accordance with the fact that all these samples were collected

from the rats which were obtained from the same source and fed with the same normal chow. The samples in the diet-induced hyperlipidaemia group at different time points tended to cluster in the various areas of the plot, showing a movement from right to left with time. In detail, the scores plot showed a time-dependent trajectory of metabonomic patterns at different time points during the time these rats were fed with a high-lipid diet. The samples collected at the first time point (M0, predose) were clustered within the region of right-middle, the samples at the second time point (M1, day 7 postdose) lay in the low-middle, and at the third time point (M2, day 14 postdose) they moved forward more toward the low-left. The two groups were quite different but did not

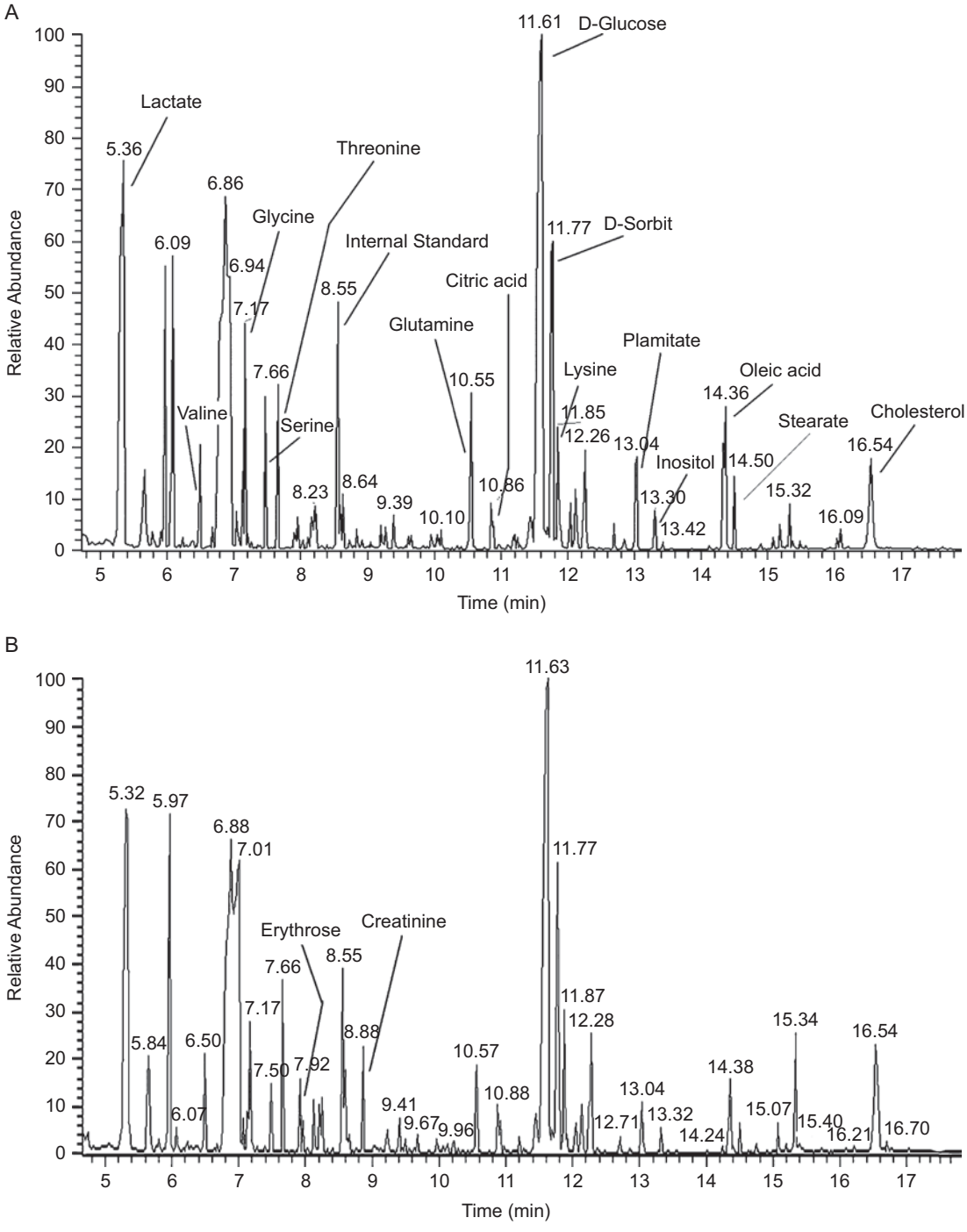


**Figure 2.** Comparison of gas chromatography–mass spectrometry (GC-MS) total ion current (TIC) chromatogram of plasma samples from the control group (A) and diet-induced hyperlipidaemia group (B) at day 28.

**Table 2.** Linearity of endogenous compounds in rat plasma by gas chromatography–mass spectrometry.

| | Peak area ratios of plasma dilutions | | | | | |
|---|---|---|---|---|---|---|
| Compounds | 1 | 0.5 | 0.25 | 0.128 | 0.063 | Correlation coefficient |
| Propanoic acid | 5.828 | 3.998 | 2.987 | 2.341 | 1.942 | 0.9993 |
| Alanine | 0.313 | 0.206 | 0.162 | 0.133 | 0.115 | 0.9990 |
| Butanoic acid | 1.643 | 1.004 | 0.631 | 0.426 | 0.251 | 0.9958 |
| Valine | 0.216 | 0.113 | 0.0584 | 0.0314 | 0.0119 | 0.9993 |
| Urea | 3.896 | 2.347 | 1.553 | 1.108 | 0.823 | 0.9981 |
| Isoleucine | 0.478 | 0.312 | 0.228 | 0.183 | 0.141 | 0.9977 |
| Glycine | 0.863 | 0.528 | 0.328 | 0.223 | 0.186 | 0.9990 |
| Serine | 0.581 | 0.324 | 0.201 | 0.128 | 0.0874 | 0.9996 |
| Threonine | 0.682 | 0.412 | 0.236 | 0.143 | 0.112 | 0.9977 |
| Lysine | 0.154 | 0.0996 | 0.0711 | 0.0541 | 0.0498 | 0.9994 |
| Aspartic acid | 0.109 | 0.0623 | 0.0358 | 0.0196 | 0.0118 | 0.9981 |
| Proline | 0.880 | 0.493 | 0.291 | 0.174 | 0.112 | 0.9972 |
| Phenylalanine | 0.275 | 0.153 | 0.0867 | 0.0592 | 0.0378 | 0.9996 |
| Ribose | 0.204 | 0.114 | 0.0621 | 0.0421 | 0.0263 | 0.9994 |
| Glutamine | 0.458 | 0.297 | 0.216 | 0.167 | 0.148 | 0.9996 |
| Isocitric acid | 0.508 | 0.293 | 0.175 | 0.113 | 0.0721 | 0.9989 |
| Glucose | 7.581 | 3.987 | 2.115 | 1.006 | 0.556 | 0.9995 |
| Tyrosine | 0.350 | 0.244 | 0.187 | 0.154 | 0.135 | 0.9987 |
| Maltose | 0.349 | 0.193 | 0.116 | 0.0721 | 0.0486 | 0.9997 |
| Inositol | 0.771 | 0.447 | 0.256 | 0.193 | 0.134 | 0.9992 |
| Octadecadienoic acid | 0.949 | 0.561 | 0.368 | 0.254 | 0.211 | 0.9998 |
| Arachidonic acid | 0.172 | 0.113 | 0.0698 | 0.0511 | 0.0421 | 0.9966 |
| Cholesterol | 1.550 | 0.758 | 0.359 | 0.211 | 0.0874 | 0.9995 |

**Table 3.** Precision of the gas chromatography–mass spectrometry method for the determination of endogenous compounds in rat plasma.

| | Intraday precision | | | Interday precision | | |
|---|---|---|---|---|---|---|
| | RSD (%) at dilutions (v/v) of | | | RSD (%) at dilutions (v/v) of | | |
| Compounds | 0.063 | 0.25 | 1 | 0.063 | 0.25 | 1 |
| Propanoic acid | 7.50 | 4.74 | 3.72 | 8.35 | 7.60 | 3.50 |
| Alanine | 8.30 | 7.38 | 4.77 | 12.09 | 11.00 | 9.10 |
| Butanoic acid | 9.55 | 3.67 | 3.62 | 10.12 | 9.21 | 4.60 |
| Valine | 6.05 | 4.55 | 7.36 | 8.80 | 8.01 | 8.93 |
| Urea | 7.86 | 4.12 | 1.83 | 9.08 | 8.26 | 8.25 |
| Isoleucine | 9.70 | 4.64 | 8.90 | 10.58 | 9.63 | 9.12 |
| Glycine | 5.60 | 4.57 | 6.06 | 5.64 | 5.13 | 5.92 |
| Serine | 5.61 | 4.20 | 2.89 | 7.66 | 6.97 | 7.54 |
| Threonine | 10.59 | 3.36 | 4.77 | 11.70 | 10.65 | 3.28 |
| Lysine | 10.35 | 5.77 | 6.24 | 9.85 | 8.96 | 2.16 |
| Aspartic acid | 7.17 | 5.00 | 2.80 | 8.10 | 7.37 | 4.54 |
| Proline | 6.79 | 6.24 | 1.18 | 6.51 | 5.92 | 7.70 |
| Phenylalanine | 9.06 | 5.20 | 3.34 | 9.38 | 8.54 | 5.45 |
| Ribose | 13.54 | 7.24 | 4.61 | 14.97 | 13.63 | 3.29 |
| Glutamine | 9.35 | 7.51 | 6.25 | 13.21 | 12.02 | 5.04 |
| Isocitric acid | 8.86 | 6.11 | 2.13 | 9.39 | 8.55 | 4.47 |
| Glucose | 11.85 | 8.50 | 2.10 | 13.86 | 12.61 | 3.62 |
| Tyrosine | 9.38 | 4.97 | 2.60 | 10.88 | 9.90 | 5.77 |
| Maltose | 11.05 | 8.78 | 4.65 | 11.89 | 10.82 | 3.01 |
| Inositol | 6.94 | 4.44 | 4.61 | 8.05 | 7.33 | 2.86 |
| Octadecadienoic acid | 11.56 | 8.37 | 6.76 | 12.47 | 11.35 | 3.16 |
| Arachidonic acid | 7.99 | 3.78 | 3.62 | 8.85 | 8.06 | 4.16 |
| Cholesterol | 10.96 | 7.69 | 3.20 | 12.04 | 10.96 | 2.55 |

RSD, relative standard deviation.

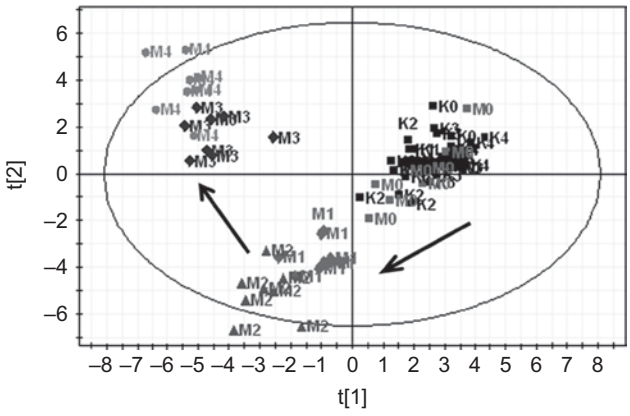


**Figure 3.** Principle component analysis (PCA) scores plot of rat plasma samples from the diet-induced hyperlipidaemia and control groups. K, control group ($n$ = 8); M, diet-induced hyperlipidaemia group ($n$ = 8); 0 ~ 4, sampling time points: 0, predose; 1, 2, 3, 4, denotes days 7, 14, 21, 28 postdose, respectively. Arrows indicate the trajectory of metabonomics patterns with time.

lie far from their original region, which indicated a visible difference but not a significantly different metabonome from that of their start point. Remarkable differences appeared at the fourth time point (M3, day 21 postdose) and the fifth time point (M4, day 28 postdose) at which the samples were a long way from time point one (M0), two (M1) and three (M2). Separation of the samples at the different time points is very clear, and shows a distinct displacement in the plot. Thus, in this PCA map, each spot represents a sample, and each assembly of samples indicates a particular metabolic pattern at different time points. It suggests that a stable rat model of hyperlipidaemia was established after being induced with a high-lipid diet for 3–4 weeks.

Although the lipid levels of the diet-induced hyperlipidaemia group determined by kit assays presented no significant changes in rats fed with a high-lipid diet at days 7 and 14, these samples displayed significantly different metabolic characteristics compared with those of rats at predose. The metabonomic profiles of hyperlipidaemia showed an observable movement at days 7 and 14 from the day 0 position, whereas the kit assays for determination of lipid levels showed no significant change for these time points. In other words, in comparison with the conventional kit assays, the metabonomics method can detect and describe the alternation of the endogenous metabolites in plasma more sensitively and accurately during the formation process of hyperlipidaemia. It suggests that an early-stage prognosis or diagnosis of hyperlipidaemia is possible using the metabonomics approach. In addition, the samples collected at different time points in the diet-induced hyperlipidaemia group clustered in different regions in the scores plot, which may help us examine the model objectively and determine disease progress and severity.

**Potential biomarker identification**

Because individul samples collected from rats may exhibit great variation on metabolome, it would be difficult to calculate a good PCA model for separating various groups and consequently identifying the intrinsic differences among the groups. A PLS-DA model was applied to the dataset to discern the difference between the control and high-lipid diet treatments. The objective with PLS-DA was to develop a model that was able to differentiate two classes of observations on the basis of their variables (X). The Y matrix encoded class membership by a set of 'dummy' variables, herein of one column with one or zero for each class. Then a PLS model was fitted between X variables and artificial Y. In this way, a discriminant plane was obtained where the observations were well separated according to their class membership. A very good two-component model was computed (Figure 4), which could explain 99.1% and predicted 97.6% of the data at the first PC. From the scores plot, it could be seen that the diet-induced hyperlipidaemia group was separated clearly from the control group. Although it was a valid model to show the inner difference between the control and diet-induced hyperlipidaemia groups, the scores plot provided little mechanistic insight on a molecular basis, and none of the biochemical significance of the clustering. However, the data could be investigated in more detail by examining the loadings to identify which variables were responsible for the categorization in the scores plot. In the corresponding loadings plot, the metabolites responsible for the alternation in the scores plot were position-correlated with each other. The substances related to the diet-induced hyperlipidaemia group were found on the left side, while the substances related to the control group appeared on the right. M2.DA1 and M2.DA2 in the plot were positioning signs representing the control and diet-induced hyperlipidaemia groups, respectively. For the metabolites, the closer to the M2.DA1/M2.DA2 in the figure, the higher concentration in the control/ diet-induced hyperlipidaemia group. Because names of many metabolites overlapped in the loadings plot, the loading column plot was also constructed for easy visualization. In the loading column plot, each column represented a metabolite detected in the GC-MS chromatogram with an error bar to show the statistical difference. A positive value indicated a relatively greater concentration of metabolite present in the samples from the control group, whereas a negative value implied a relatively higher concentration in the samples from the diet-induced hyperlipidaemia group. By using this PLS-DA model, statistically significant variables were identified between the groups by the first PC, which is the most reliable and covers the most significant variables. In the loadings plot and the loading column plot elevated levels of cholesterol, β-hydroxybutyrate, creatinine, sorbitol, oxalate, etc., and a decrease of erythrose, D-mannopyranoside,

lysine, tyrosine, two UIs, etc., were observed in the diet-induced hyperlipidaemia group, which was in agreement with the visual inspection of the GC-MS chromatogram. Finally, the potential biomarkers identified in the scores and loadings plots of PLS-DA from the samples in the diet-induced hyperlipidaemia group predose and in the fourth week (day 28) postdose, are given in Figure 5. The results are similar to the above findings.

Hyperlipidaemia is characterized by abnormally high levels of cholesterol, which was a target determined in the diet-induced hyperlipidaemia model in rats. However, at the early stage of hyperlipidaemia, the cholesterol levels did not increase statistically significantly according to measurements using the kit assays, while metabolic profiles displayed a distinct modification of the metabolome from week 0 to weeks 1, 2, 3 and 4. It indicates that determination of major metabolites using GC-MS was more sensitive to this early drift than the conventional method (i.e. determination of cholesterol levels using kit assays). The possible reason may lie in a boost of or rapid inhibition of certain metabolic pathways in which a number of the metabolites are involved. These metabolites contributed significantly to the movement of the sample location in the score plots, displaying a dynamic track at the diverse time points of the diet-induced hyperlipidaemia model in rats.

Several potential biomarkers were identified in the metabonomic study on diet-induced hyperlipidaemia. It was estimated that rapid metabolism of cholesterol was established due to a possible *in vivo* 'emergency mechanism' and that cholesterol synthesis *in vivo* was inhibited by high levels of exogenous cholesterol. For the metabolism of cholesterol a large amount of adrenalin is needed. As adrenalin is synthesized from tyrosine, and tyrosine cannot be synthesized *in vivo*, the lowered level of tyrosine explained its rapid depletion. Furthermore, due to the inhibited synthesis of cholesterol, excessive acetyl-CoA was converted into

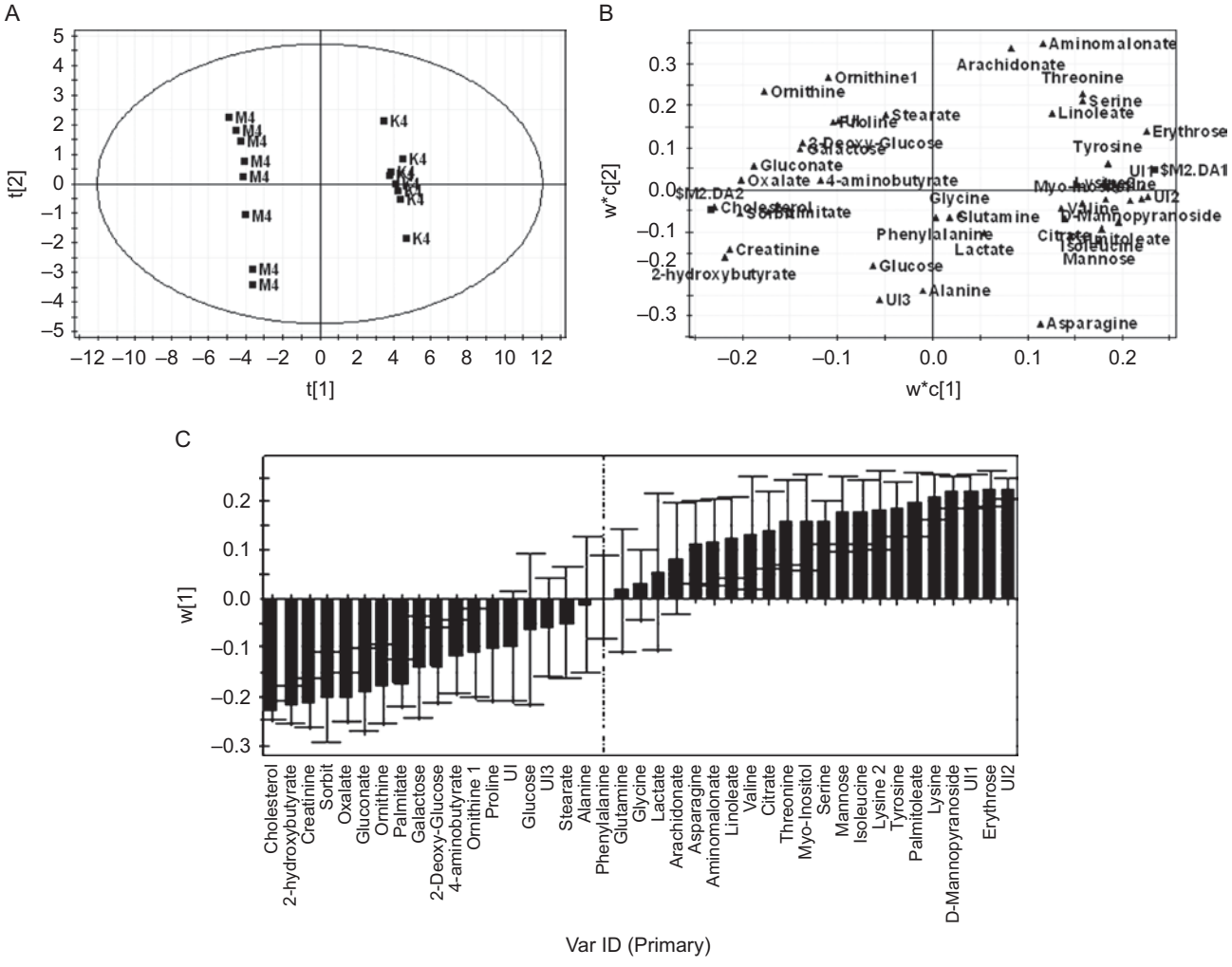


**Figure 4.** Scores plot (A), loadings plot (B) and loading column plot (C) of Partial least squares projection to latent structures and discriminant analysis for diet-induced hyperlipidaemia and control groups at day 28 postdose. K, control group ($n = 8$); M, diet-induced hyperlipidaemia group ($n = 8$).

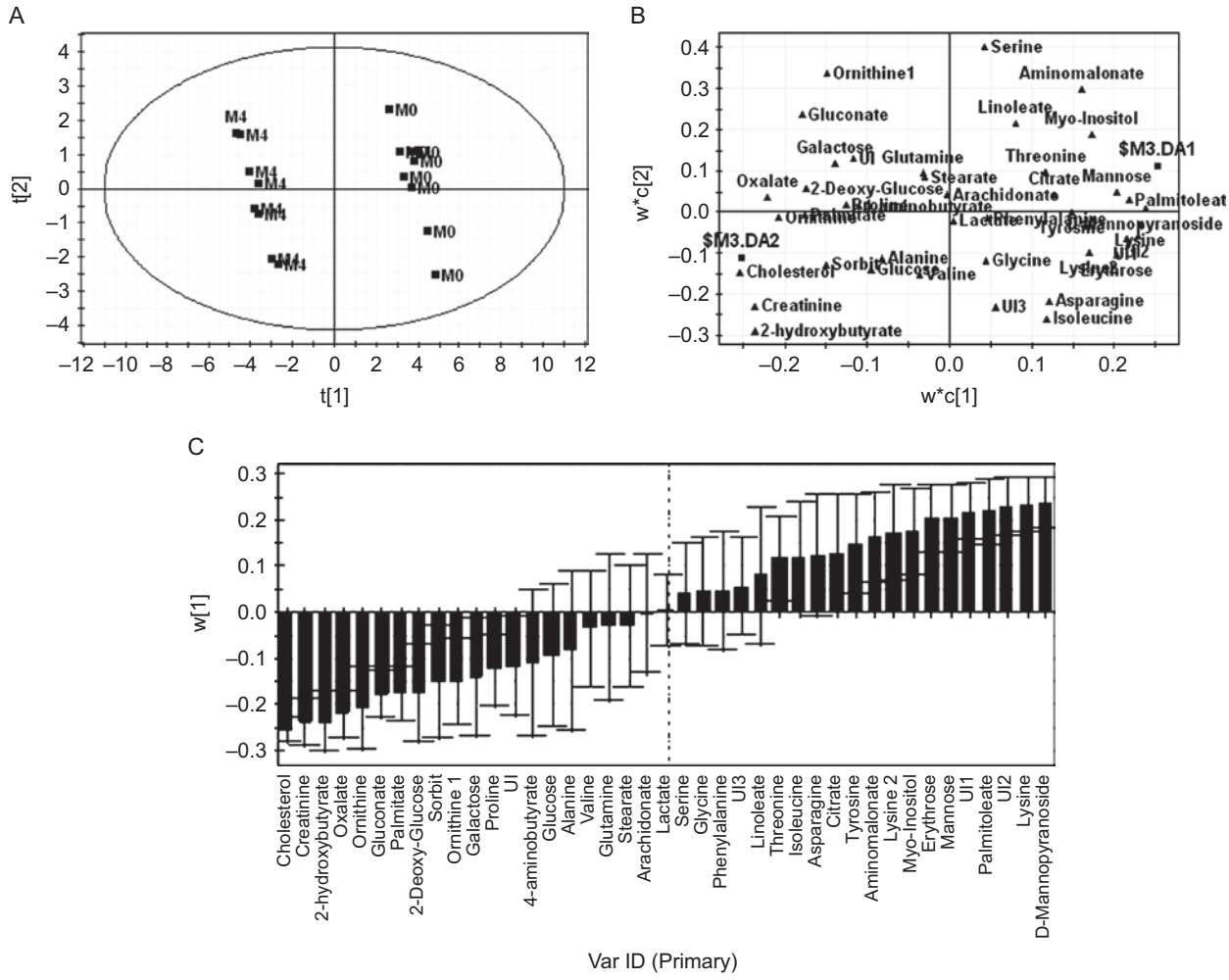


**Figure 5.** Scores plot (A), loadings plot (B) and loading column plot (C) of partial least squares projection to latent structures and discriminant analysis for diet-induced hyperlipidaemia group predose and at day 28 postdose. M, diet-induced hyperlipidaemia group; 0 and 4, the sampling time points: 0, predose; 4, day 28 postdose.

ketone body, such as acetoacetic acid, β-hydroxybutyric acid and acetone. Therefore, an increased level of β-hydroxybutyric acid was observed in the diet-induced hyperlipidaemia group, while acetoacetic acid and acetone were not detected which may be due to the volatile properties of these compounds leading to their loss from the samples during sample preparation. Creatinine is usually produced at a relatively constant rate by the human body. Although serum creatinine is a commonly used indicator of renal function, a rise in serum creatinine levels is observed only when marked damage occurs in functioning nephrons. Therefore, an increased level of plasma creatinine observed in the diet-induced hyperlipidaemia group might indicate renal damage caused by hyperlipidaemia. Thus, β-hydroxybutyrate, tyrosine and creatinine may serve as potential biomarkers in hyperlipidaemia. Validation of these biomarkers in animals and humans is warranted in future studies.

### *Metabonomic profiling: elucidating high-calorie and high-lipid diet as a cardiovascular risk factor*

Compared with the commercial kit method only determining the levels of some routine biomarkers, the status of samples from the different groups at different time points on the PCA scores plot was easily observed and the hyperlipidaemia effect on the rat metabolic system was discerned directly as well. Although the cholesterol levels of rats in the diet-induced hyperlipidaemia group determined by the kit assays presented no significant changes at weeks 1 and 2 after they had been fed with a high-lipid diet, these samples displayed significantly different metabolic characteristics compared with those of the predose state. In other words, in contrast to the commercial kit assays, the metabonomics method could detect and describe sensitive, complete and accurate changes of the endogenous metabolites in a quantitative manner during the build-up process of hyperlipidaemia. Metabonomics provided integrated information on a

wide range of metabolites without preselecting analytes to detect. It monitored the global outcome of all the influential factors in a 'snapshot' of metabolites using the whole picture view of the organism without predefining the effect of any single factor on the global outcome. Furthermore, individual factors could be identified when using MVSA.

Metabolic phenotypes are the products of the effects of lifestyle (e.g. diet), genes and environment (Lewis et al. 2008), while diseases are caused by the complex interaction of all these three factors (Nicholson et al. 2008). Metabonomic profiling links metabolic phenotypes to disease states at the individual and population levels. A high-lipid diet is one risk factor for hyperlipidaemia and cardiovascular diseases. A high-calorie and high-lipid diet contains processed carbohydrates and saturated fat, which can result in transient abnormal surge of TGs, blood glucose and free fatty acids (Raal 2009). These substances, caused by an excessive intake of such food, would be elevated to high amounts overwhelming the metabolic capacities of the mitochondria in muscles and tissues and inducing oxidant stress due to overproduction of free radicals (O'Keefe & Bell 2007, Raal 2009). The ensuing postprandial dysmetabolism triggers a cascade of atherogenic changes, including oxidant stress, inflammation, endothelial dysfunction and other biochemical alterations (O'Keefe & Bell 2007, Raal 2009). This transient abnormal elevation can even cause non-reversible glycosylation of proteins and thereby atherogenic changes. Given the risks of postprandial hyperlipidaemia caused by a high-lipid diet, close examination on metabolic phenotypes (e.g. novel biomarkers) using metabonomic profiling is warranted in animal and human studies. In this research project, diet-induced hyperlipidaemia was studied in the fasting state using kit assays and metabonomic profiling. This metabolic phenotype study revealed novel biomarkers requiring validation in animal and human studies.

In conclusion, a hyperlipidaemia model in rats was established by induction with a high-lipid diet. Its metabolic profile was investigated using commercially available kit assays and a systematic biological method, metabonomics, by means of GC-MS and MVSA. The results suggest that kit assays do not provide a sufficiently early prognosis and diagnosis of the presence of hyperlipidaemia in the rat model. Meanwhile, the GC-MS analysis coupled with MVSA demonstrated a distinct modification of metabonomics in the first 2 weeks compared with predose. The identified biomarkers indicated the perturbed metabolism and rapid response to high levels of cholesterol at the early phase of hyperlipidaemia. In addition, the present work indicates that the metabonomic approach could be used as an efficient tool to investigate the biochemical changes in hyperlipidaemia and to identify potential biomarkers for this disease (e.g. β-hydroxybutyrate, creatinine). Moreover, as a complementary method to the conventional measurement using kit assays for the study of a large set of metabolites, the development of the metabonomic approach into an early prognostic or diagnostic technique used in clinics is promising. Using a metabonomic approach to identify physiopathological states should establish a new methodology for early diagnosis in human diseases.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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