Waters

METABONOMICS AS A DIAGNOSTIC TOOL IN VETERINARY MEDICINE

Phillip D. Whitfield[1], Alexander J. German[2], Peter-John M. Noble[2], Robert J. Beynon[1], Rachel Burrow[2], Alistair I. Freeman[2], Hilary J. Major[3]
[1]Department of Veterinary Preclinical Sciences
[2]The Small Animal Teaching Hospital, Faculty of Veterinary Science, University of Liverpool, Liverpool L69 7ZJ, UK
[3]Waters Corporation, MS Technologies Centre, Atlas Park, Simonsway, Manchester M22 5PP, UK, Correspondence: hilary_major@waters.com

INTRODUCTION

Portovascular anomalies (portosystemic shunts) are the most common form of congenital liver disease in dogs and can result in the neurobehavioral syndrome, hepatic encephalopathy. Clinical signs include dementia, gait disturbance, seizures and coma. Whilst conventional clinical investigations are used to diagnose portovascular anomalies (PVAs) in dogs, additional techniques are required for accurate prognostic evaluation and to assess the response of patients to treatment. The reduced ability of the liver to detoxify and excrete waste products in dogs with portovascular anomalies will be reflected in dramatic changes in the levels of a wide range of low molecular weight metabolites in body fluids and tissues. The emerging post-genomic science of metabonomics is concerned with detecting global changes in the distribution and concentration of endogenous metabolites and may identify surrogate biomarkers of disease states. In this study, metabonomic-based strategies have been employed to assess and differentiate the perturbations of the metabolite profiles in the plasma of dogs with congenital portovascular anomalies and acquired hepatic disease.

EXPERIMENTAL

STUDY ANIMALS

- The study involved 25 dogs referred to the Department of Veterinary Clinical Sciences at the University of Liverpool.
- 15 were referred for the investigation of suspected hepatic disease.
- 9 of these were clinically diagnosed with congenital portovascular anomalies; 6 with an acquired hepatopathy.
- The remaining 10 dogs were referred for non-hepatic disorders and were assigned as controls.

LC/MS

- Plasma samples were protein precipitated prior to analysis by HPLC/MS.
- Data was acquired in both positive and negative ion electrospray modes using the following conditions:

HPLC CONDITIONS:

HPLC:	Waters® Alliance® HT 2795XC		
Column:	Waters Symmetry® C_{18} 2.1 x 100 mm, 3.5µm		
Mobile phase:	A: water + 0.1% formic acid		
	B: acetonitrile + 0.1% formic acid		
Flow rate:	600 µL/min split to 120 µL/min to MS		
Column temp:	40 °C		
Injection volume:	10 µL		
Gradient:	Time (min)	% A	% B
	0	100	0
	0.5	100	0
	4.0	80	20
	8.0	5	95
	9.0	5	95
	9.1	100	0

MS CONDITIONS:

MS:	Waters Micromass® Q-Tof micro™
Ionisation mode:	positive and negative ion electrospray
Capillary:	3200 V positive, 2600 V negative
Sample cone:	30 V
Source temperature:	120 °C
Desolvation Temperature	250 °C
Cone gas flow:	50 L/hr
Desolvation gas flow:	500 L/hr

MS Acquisition Parameters—LockSpray™ Enabled

Acquisition range:	m/z 50–850
Acquisition rate:	0.4 sec
Inter-scan time:	0.1 sec
Mode:	centroid
Lock reference:	leucine enkephalin
Lock mass:	556.2771 positive, 554.2615 negative

MS/MS Conditions

Collision gas:	argon
Collision energy:	25 eV

RESULTS

DATA PROCESSING USING MARKERLYNX

- Extraction of information from large data sets typical of Metabonomic analyses is facilitated by the use of the MarkerLynx™ Application Manager.
- MarkerLynx includes deconvolution and alignment of the peaks across samples.
- A table of m/z and retention times with associated intensities for all the compounds detected is generated.
- The reduced data set is analysed by principal component analysis (PCA) within MarkerLynx.
- The PCA results for the positive and negative ion data are shown in Figures 1 and 2 respectively.

Waters Metabonomics MS System.

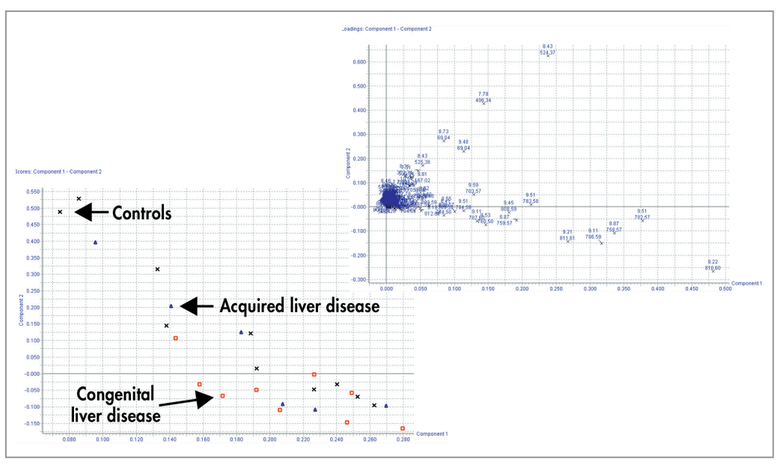

Figure 1. MarkerLynx Positive Ion PCA Plots.

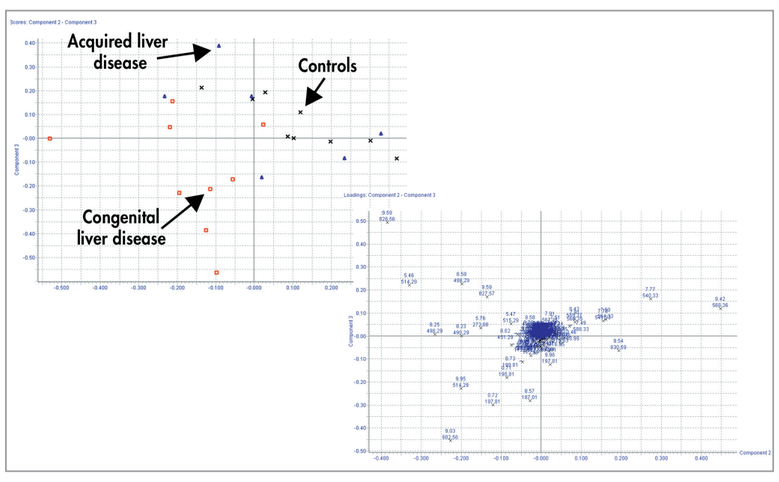

Figure 2. MarkerLynx Negative Ion PCA Plots.

- The MarkerLynx PCA scores plots show that the samples associated with the congenital liver disease (red squares) tend to cluster away from the other samples.
- Facile export of the marker table from MarkerLynx to SIMCA-P (Umetrics, Sweden) multivariate data analysis software package allowed the use of more advanced statistical analysis tools such as partial least squares discriminant analysis (PLS-DA).

PLS-DA RESULTS FOR CONTROLS AND CONGENITAL PORTOVASCULAR ANOMALIES

- PLS-DA is a supervised statistical analysis technique where the model is built based on class membership giving a maximum separation projection.
- Figures 3 and 4 show the PLS-DA plots for the controls (class 1) and the congenital PVA (class 2) samples in both positive and negative ion mode.

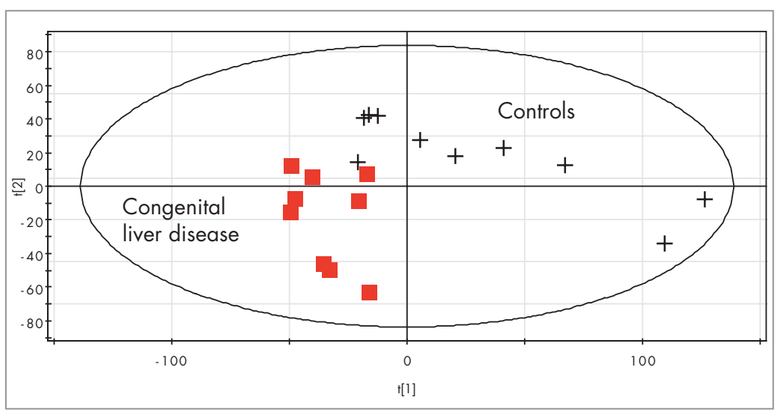

Figure 3. Positive ion PLS-DA scores plot of Controls (class 1) and Congenital PVA (class 2).

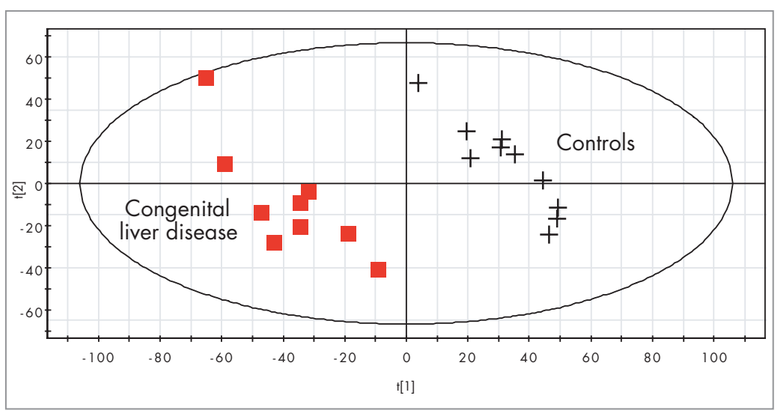

Figure 4. Negative ion PLS-DA scores plot of Controls (class 1) and Congenital PVA (class 2).

- The PLS-DA scores plots show that separation of the controls from the congenital PVA samples can now be achieved.
- The negative ion model was relatively strong (R^2Y=0.962, Q^2=0.603) with the 2 classes well resolved in component 1 with no outliers.
- The models were validated by excluding an observation (sample) from each class in turn, a new model generated and the excluded observations predicted.
- Validation of the negative ion model showed that 15 out of the 19 samples were correctly classified (p>0.65) and the other 4 were borderline (close to 0.65).
- Coefficients plots indicate the RT and mass of the species up-regulated and down-regulated.
- Figure 5 shows the positive ion coefficients plot for the species down-regulated in the congenital PVA samples.

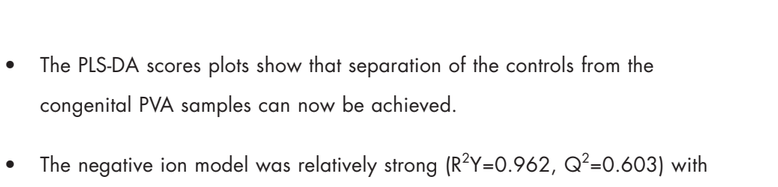

Figure 5. Positive ion coefficients plot of species down-regulated in congenital PVA samples.

- The species at m/z 496.33, RT 7.82 minutes and m/z 524.36, RT 8.48 minutes were postulated to be the phospholipids palmitoyl and stearoyl lysophosphatidylcholine respectively.
- Their structures were confirmed by exact mass LC/MS/MS comparison against standards (see figures 7 and 8).

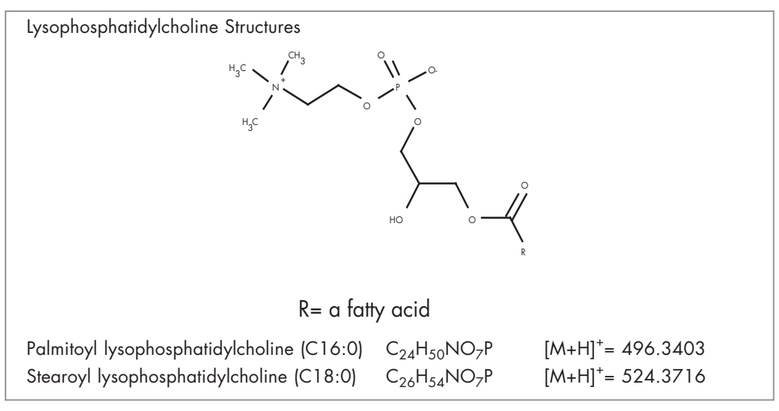

Figure 6. Lysophophatidylcholine Structures.

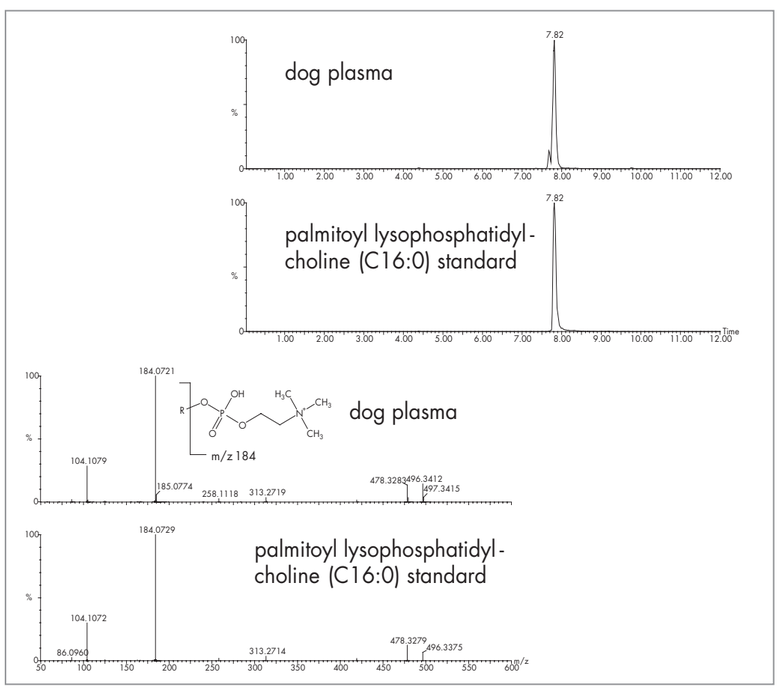

Figure 7. Products of m/z 496 at 7.82 minutes.

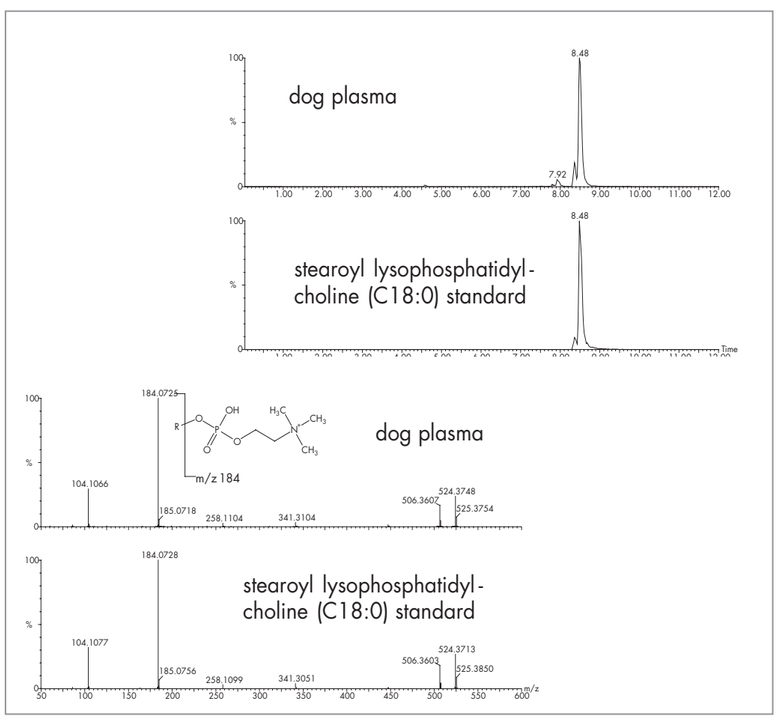

Figure 8. Products of m/z 524 at 8.48 minutes.

PLS-DA RESULTS FOR CONGENITAL PORTOVASCULAR ANOMALIES AND ACQUIRED LIVER DISEASE

- PLS-DA models were generated and validated for the congenital (class 2) and acquired liver disease (class 3) samples in both positive and negative ion (see figures 9 and 10).
- It was possible to distinguish between classes with 11 of the 15 samples correctly predicted. 3 were border-line to being correctly predicted and one was predicted as the wrong class in both ionisation modes.
- There was a significant increase in specific bile acids in the dogs with portovascular anomalies however no single biomarker was identified rather a combination of metabolites.

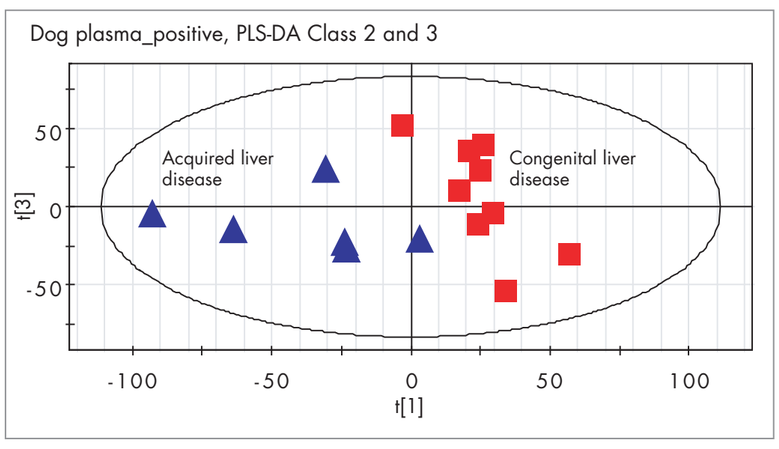

Figure 9. PLS-DA scores plot for positive ion Congenital and acquired liver diseases.

Figure 10. PLS-DA scores plot for negative ion Congenital and acquired liver diseases.

CONCLUSIONS

- Disturbances in the profiles of low molecular weight endogenous metabolites in the plasma of dogs with both congenital and acquired hepatic disease were characterised.
- Pattern recognition tools were able to distinguish groups of dogs with congenital portovascular anomalies and acquired hepatic disease.
- Several potential biomarkers were identified.
- A much larger study is required to confirm the findings of this initial study.
- This study shows that Metabonomics has the potential to be a powerful, non invasive, diagnostic tool for veterinary medicine.