NMR-based metabonomic analyses of horse serum: detection of metabolic markers of disease

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Summary

Nuclear magnetic resonance (NMR)-based metabonomic studies use multivariate statistical analyses of NMR spectra from biological samples to detect metabolic profiles (metabonomes) associated with diseases, toxins or genetic variations. Osteochondrosis dissecans (OCD) is a developmental orthopedic disease that is heritable in Standardbreds, but the metabonome associated with it has not been identified. To test the hypothesis that NMR-based metabonomics would detect differences in the metabonomes of Standardbreds that did or did not develop OCD lesions, blood samples were collected from yearling Standardbreds in 2007 (Experiment 1, n = 40) and 2008 (Experiment 2, n = 71). Metabonomic analyses of serum NMR spectra revealed distinct separation ($R^2(Y)$ = 0.82) of the metabolic profiles of horses that had OCD (n = 20) vs half or full siblings that did not develop lesions (Control; n = 20), with excellent predictability (Q^2 = 0.83) in Experiment 1. In Experiment 2, there was strong separation of horses sampled in August vs September, which obscured the OCD vs Control comparisons and caused poor predictability of the models (Q^2 < 0.0) despite good separation in the model ($R^2(y)$ = 0.98). In Experiment 3, blood was drawn from 15 nursing foals in May, 2009. Hock radiographs were taken the following year to verify the presence or absence of OCD. Metabonomic analyses of serum NMR spectra from the foals revealed strong separation and high predictability ($R^2(Y)$ = 0.95, Q^2 = 0.90) of the metabonomic model. The metabolites associated with the clusters were identified by loadings plots for Experiment 3. It appears that an OCD metabonome exists but more analyses are necessary before it can be fully utilized.

Introduction

Metabonomics has been defined as "the quantitative measurement of the dynamic multi-parametric response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999). The spectra of biological fluids and tissues generated by nuclear magnetic resonance (NMR) spectroscopy reflects the full range of metabolites present in the sample as each molecule has a unique nuclear resonance pattern (Pelczer, 2005). A given substance is represented by one or more peaks that appear at specific locations along the chemical shift scale and the peaks' fine structures (singlet, doublet, triplet) reflect the unique interactions of protons in the chemical structure (Nicholson et al., 1995; Richards and Hollerton, 2011). Multivariate statistical analysis using principle component analysis (PCA), partial least squares with discriminant analyses (PLS-DA) and orthogonal PLS-DA (OPLS-DA) on the integrals (area under the curve) of the peaks is used to identify metabolite components which vary systematically across a sample set in a significant manner (Pelczer, 2005; Beckonert et al., 2007) and to provide metabolic profiles (metabonomes) associated with specific disease states, permitting identification of diagnostic metabolic markers and providing better understanding of the underlying disease processes (Trygg et al., 2007; Fonville et al., 2010). For example, metabonomic analyses of serum from both humans (Xang et al., 2009) and horses (Hodavance et al., 2007) with insulin resistance revealed distinct differences in their metabonomes compared with individuals with normal insulin sensitivity. In both species, the differences were based on alterations not only in glucose but also choline and a variety of amino acids. As specific amino acids are known to influence hepatic gluconeogenesis and glucose metabolism (Krebs et al., 2003), this information can result in better understanding of the metabolic pathway defects associated with the disorders. In combination with genomic and proteomic information, metabonomic information can also potentially result in the development of therapeutic interventions (Xang et al., 2003; Fonville et al., 2010).

Osteochondrosis dissecans (OCD) is a growth-related defect caused by abnormal calcification of cartilage matrices at the growth plates that result in articular cartilage defects (McIlwraith, 2004). Radiographically apparent lesions appear most commonly in young, rapidly growing horses between 3 to 12 months of age, although clinical signs of joint effusion and lameness may not surface until the animals are put into training (McIlwraith, 2004). Although defects can be induced by improper nutrition (Savage et al., 1993a; 1993b) and other environmental factors (NRC, 2007), it has been well documented that predisposition to tibiotarsal lesions is heritable in horses (Hoppe and Phillipson, 1985; Piermattei et al., 1993; Lykkjen et al, 2011). The incidence of lesions

in yearling and two year-old Standardbred horses was documented to be between 10 and 26% in European Standardbred trotters (Hoppe and Phillipson, 1985; Grondahl, 1992) and was 43 and 58% in two more recent studies conducted in Illinois, USA (Kapraun et al, 2008; Hilliken et al, 2011). A correlation between abnormal glucose/insulin metabolism and the presence of lesions has been reported in yearling Standardbreds (Ralston, 1996). However, the associated metabolic defects have not been well defined, although altered gene expression in the chondrocytes of affected cartilage has been implicated (Mirams et al., 2009). Even though the cartilage defects can be corrected surgically (McIlwraith, 2004) and Standardbred earnings as two and three year olds did not differ significantly between affected and non-affected controls (Hilliken et al, 2011), OCD represents a significant economic disadvantage (Jeffcott, 1996). Surgical correction costs more than US$2000. Of 92 Standardbred yearlings from a single farm sold at yearling auctions in 2008 in the United States, those in which hock OCD lesions had been surgically corrected (n = 30) sold for $9700 less on average than similarly bred yearlings that were assumed to be lesion free (n = 62). Therefore, it would be very beneficial if horses predisposed to the development of OCD could be identified before lesions develop so that dietary interventions could be developed to reduce or prevent the disease.

It was hypothesized that NMR-based metabonomic analyses would reveal distinct metabonomes associated with hock OCD lesions in young Standardbred horses and that the metabonomes of 2- to 3-month-old Standardbred foals would be predictive of future development of lesions. To test these hypotheses, three experiments were conducted with the following objectives: 1. determine if yearling Standardbred horses with and without OCD are metabolically distinct according to NMR-based metabonomic analyses of serum, 2. verify that differences observed in the first study are repeatable and measure plasma glucose and insulin concentrations associated with the metabonomes of each horse and 3. determine if NMR-based metabonomic analyses of serum from nursing Standardbred foals are predictive of the future appearance of OCD hock lesions.

Materials and methods

Animals and sampling

Experiment 1

Twenty matched pairs of Standardbred yearlings (18–20 months of age) from a Standardbred breeding farm in Hanover, PA, USA were used. To maintain confidentiality, each horse was assigned a random numerical identification number to be used in the statistical analyses. One member of each pair had undergone hock OCD surgery 2 to 9 months before the samples were taken; the other had no clinical evidence of OCD. Horses with the same sire (nine different sires were represented) and similarly bred dams were paired. All had been born on the farm and received the same basic health care. They had had access to the same pastures and given free access to alfalfa hay and fed approximately 2.75 kg of Purina Strategy® feed (Purina Mills, St. Lois, MO) per head per day since weaning. They were all group-fed in pastures until transferred to individual stalls for sale preparation 2 weeks prior to sampling. At the barn, they were all fed 1.5 to 2 kg of Strategy® feed and 0 to 0.5 kg of Ultium® feed (Purina Mills) (depending on body condition) twice daily and had free access to alfalfa hay, salt and water. They were exercised for 20 to 30 minutes in the mornings (08:00–12:00) using an automatic free-stall horse exerciser. They were fed at 07:30 on the day of sampling (September 21, 2007). The OCD status of the control horses was based only on the absence of clinical signs (swelling in the hock or lameness) and was not verified radiographically.

After swabbing the injection site with ethyl alcohol, blood samples were collected by venipuncture into sodium heparin Vacutainer tubes (Becton Dixon & Co., Franklin Lakes, NJ) for the collection of plasma and into one serum separator Vacutainer tube within a 3 hour period (12:45–14:30). Samples were immediately placed on ice and transported to the laboratory (a 6 hour drive), where the serum tubes were centrifuged at room temperature and the serum was aliquoted (1 mL per tube) into microcentrifuge tubes and stored at –80 °C pending NMR analysis.

Experiment 2

Eighty yearling Standardbred horses were used from the same farm in Hanover, PA, USA and under the same conditions as Experiment 1. Each horse was assigned a random numerical identification number to be used in the statistical analyses. Fifty of the horses were paired based on sire, dam and incidence of OCD lesions; half of the horses had undergone surgery to treat hock OCD lesions 1 to 9 months before the samples were taken (OCD group) and their matched pairs (the Control group) had never exhibited clinical signs of hock OCD. The other horses were chosen because they were either half or full siblings to the horses used in Experiment 1. The absence of hock OCD lesions was verified radiographically in 22 of the Control horses. Management practices on the farm and at the training facility were the same as for Experiment 1.

Samples were taken by venipuncture on August 21, 2008 (n = 41) and September 10, 2008 (n = 39). Although the horses sampled in August were being prepared for local yearling auctions and the horses sampled in September were destined for the elite Harrisburg sale, the management and feeding of both groups were the same. Alcohol swabs were not used to clean the injection site, as had been done in Experiment 1. The plasma tubes were centrifuged immediately and placed on ice. The serum tubes were not processed until return to the laboratory, i.e., within 6–7 hours of collection. Multiple aliquots of serum and plasma were taken from each tube and frozen at –80 °C pending the

NMR, glucose and insulin analyses.

Experiment 3

Eighteen nursing foals (1 to 3 months of age) from the same farm as in the previous experiments were brought into individual box stalls with their dams 3 to 4 hours before samples were drawn on May 15, 2009. Each horse was assigned a random numerical identification number to be used in the statistical analyses. The horses had free access to alfalfa hay and no attempt was made to control nursing activity by the foals. The foals were restrained manually and blood samples drawn by venipuncture as in Experiment 2. All sample handling was as in the previous experiments. The presence or absence of OCD lesions was documented radiographically or by surgery in 15 of the horses at 12 to 20 months of age. The remaining three foals were sold before radiographs could be taken and their data were not included in the analyses.

NMR analyses

The frozen samples were first kept at –10 °C overnight and then at room temperature for several hours before they were transferred to the NMR spectrometer. The vials were gently inverted several times during the room temperature incubation to insure homogeneity. The stability of the samples was not compromised by this process (data not shown). No buffers or other additives were added to the samples before analysis. The sample volume used was 0.5 mL.

NMR data acquisition

NMR experiments were conducted using a Bruker Avance-II spectrometer (Bruker-Biospin, Billerica, MA) set at 500 MHz and equipped with an inverse TCI CryoProbe (Bruker-Biospin, Billerica, MA) at a controlled temperature of 25 °C. A 1 mm outside-diameter capillary insert was filled with D_2O and kept in place and centred by a Teflon plug in the 5 mm outside-diameter sample tube to provide the deuterium lock. The chemical shift of the residual water was used for calibration at the first approximation. Homogeneity adjustment (shimming) was done using a pulsed-field gradient method (TopShim) and the ^1H signal of the sample itself (Weiger et al., 2006). The first spectra obtained had only the water peaks suppressed using excitation sculpting (ES) (Hwang et al., 1995). The second spectra were obtained by applying an added relaxation filter that used a long 50 ms spin-lock pulse that suppressed resonances of large molecules while retaining small molecules based on differential relaxation properties. This enables augmentation of sharp peaks from small molecules that are otherwise obscured (Tang et al., 2004).

The water suppressed, relaxation filtered spectra were processed using MNova v.6.1.1 software (Mestrelab Research S.L., Santiago de Compostela, Spain). The ^1H-NMR spectra were referenced to the alpha-glucose anomeric proton resonance at δ 5.233 ppm. Phasing, baseline corrections and peak alignment were performed manually by the authors on all the spectra in MNova. Spectral binning was performed using a bin size of 0.02 ppm. Metabonomic multivariate cluster analyses (SIMCA-P+, v.12.0.1, Umetrics, Umeå, Sweden) were performed on spectra using unsupervised principle component analysis (PCA), as well as supervised projection to latent structure discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA). Validation was conducted when using supervised methods to minimize spurious correlations (Trygg et al., 2007). The residual water peak and contaminant alcohol peaks, if any, apparently from the use of the alcohol swabs, were excluded from the statistical analysis (Fonville et al., 2010). Metabolite NMR peaks were assigned based on their chemical shifts and multiplicities using previously published data (Nicholson et al., 1995) and results of spiking experiments using alanine and valine to verify congruence with data from other species (Pelczer and Ralston, unpublished data). Validity of the models generated by the analyses was evaluated according to the R^2 (goodness of fit, percentage variation explained by the model) and the Q^2 (fraction of the total variation of the Xs that can be predicted by the model, predictability), compared with cross-validation wherein randomly selected data elements are left out of the model then entered as data to be predicted and compared to the original R^2 and Q^2 values of the original model. This process is iterated until all data elements are evaluated. The cross-validation R^2 and Q^2 values should all be lower than the original values in a valid model (Fonville et al., 2010).

Plasma glucose and insulin analyses

Plasma glucose was measured using a colorimetric assay (Glucose-SL reagent; Diagnostic Chemicals Limited, Oxford, CN) at an absorbance of 340 nm. Each sample was analysed in duplicate, as were the standards. The concentrations of the glucose standards were 0, 1, 3, 10, 30 and 100 mg glucose per dL.

Plasma samples were analysed for insulin using Mercordian Insulin ELISA kit 10–1113–10 (American Laboratory Products Company, Salem, NH). Each sample was analysed in duplicate according to the manufacturer's instructions. The concentrations included in the insulin standard curve were 0, 1, 3 and 10 μIU insulin per dL. Optical densities were read at 450 nm within 30 minutes of the cessation of incubation. Mean α and β glucose and insulin concentrations were compared between horses with OCD and controls using a two-tailed Student's *t*-test. Significance was set at $P < 0.05$

Results

The forages and feeds used in the three experiments were not analysed chemically, but because all horses were fed the same diets in each experiment, nutrition was not considered to have contributed significantly to the observed differences.

Experiment 1

A representative ^1H-NMR spectrum of horse serum with

excitation sculpting (ES) water suppression is shown in Figure 1 wherein selected peaks are identified based on comparison with literature data (Nicholson et al., 1995; Martin et al., 2009). Some components were verified in horse serum by spiking experiments using valine and arginine (data not shown). Alcohol (a mixture of ethanol and methanol) was identified as a contaminant in the majority of the samples and these peaks were suppressed in the statistical analyses. This type of contamination has been observed in other metabonomic studies (Lykkejen et al., 2010).

Two aliquots from each sample were subjected to NMR. Data from seven samples were discarded as outliers because of hemolysis of the original sample. The PLS-DA scores plot for the data revealed good class (Control vs OCD) separation and convincing model validation (Figure 2). With only two components in the analysis, the R^2 was 0.662 and the Q^2 was 0.32. The duplicate aliquots were located close to each other and there were no significant outliers.

There was even more distinct separation in the OPLS-DA scores plots of the OCD horses vs the Controls (Figure 3). The duplicate aliquots clustered together and there were no significant outliers. With only one component in the analysis, the R^2 was 0.82 and the Q^2 was 0.83.

Experiment 2

Due to an error in handling, the samples obtained in August for glucose analysis were inadvertently lost. However, the mean blood insulin concentration of horses sampled in August was 14.0 ± 0.9 μIU/dL for the OCD group (n = 21) and 13.0 ± 1.2 μIU/dL for the Control group (n = 20) (P > 0.5). Plasma glucose concentrations of horses sampled in September were not different between the OCD group (85.4 ± 2.6 mg/dL, n = 9) and the Control group (82.4 ± 3.9 mg/dL, n = 30, P > 0.5). In horses sampled in September, insulin concentrations did not differ between OCD and control groups (OCD group: 11.1 ± 0.9 μIU/dL, n = 9; Control group: 17.0 ± 1.6 μIU/dL, n = 30; P > 0.05). Insulin concentrations did not differ between horses when grouped by date (August: 13.7 ± 0.7 μIU/dL; September: 15.6 ± 1.4 μIU/dL; P > 0.2).

For the NMR analyses, data from nine horses were discarded due to sample degradation (hemolysis). A single aliquot of serum was analysed for each horse.

There was distinct separation with strong predictability (R^2 (Y) = 0.64, Q^2 = 0.75) between the data sets for the August and September samples (Figure 4) which, based on the loadings plots (not shown), was due primarily to higher sugar peaks and a variety of lipid peaks in the September samples.

However, no clear separations between the OCD and Control groups were evident in the complete data set. Even when the August and September complete spectra were analysed separately, there was no clear separation of the OCD and Control metabonomes. However, when only data from animals with a radiographically verified OCD status (presence or absence) were analysed regardless of the month of sampling, using only the peaks of metabolites that were significant for separation in Experiment 1, the OPLS-DA separation of the OCD and Control groups was clear (R^2 = 0.98) but had poor

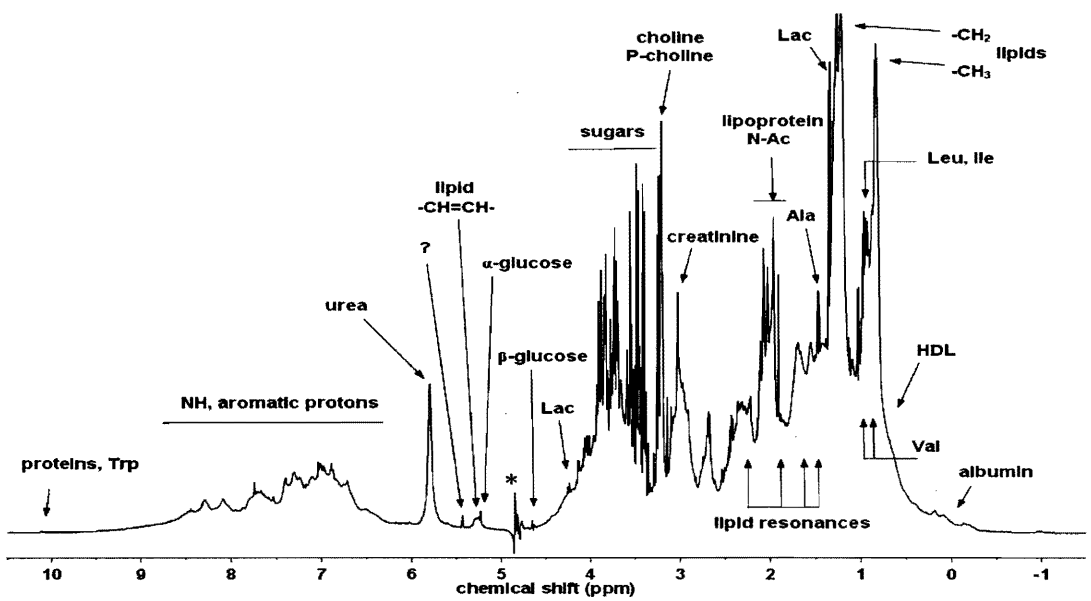

Figure 1. Representative ^{1}H-NMR spectrum of serum with excitation sculpting (ES) water suppression. Selected assignments are shown. The residual water peak is labelled with *. The character "?" indicates an as yet unidentified sugar component that contributed (P < 0.05) to the clustering.

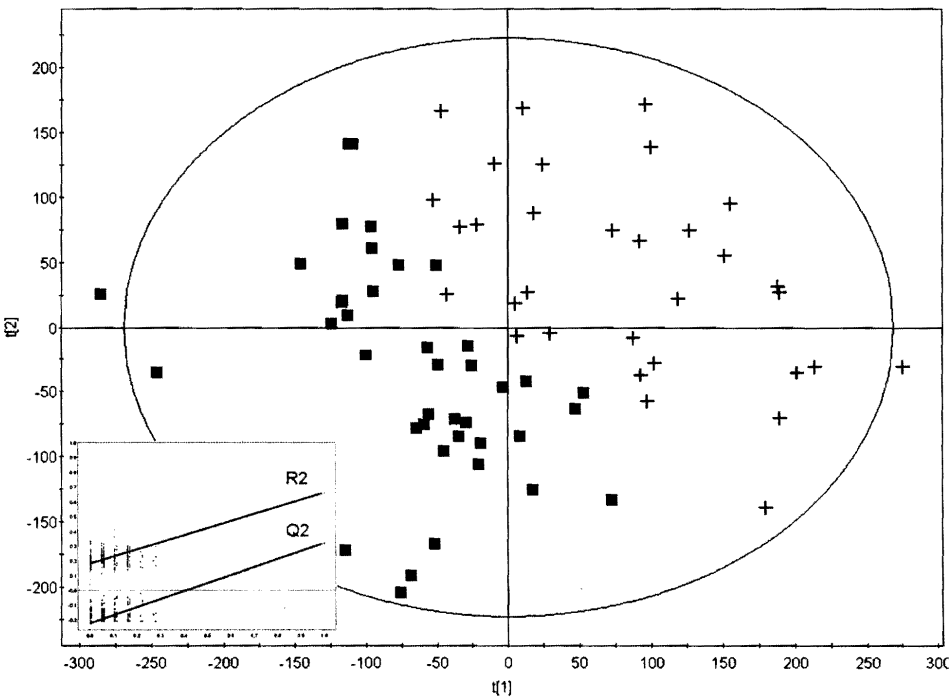

Figure 2. Experiment 1: PLS-DA scores plots of the serum NMR spectra from OCD (■) yearlings vs Controls (+). The strong negative slope for the validation plot of percentage variation explained ($R^2(Y)$) and predictability (Q^2) (see inset) of the metabonomic model with a model $R^2(Y)$ of 0.66 and a Q^2 (cum) = 0.32 indicates a validated strong model.

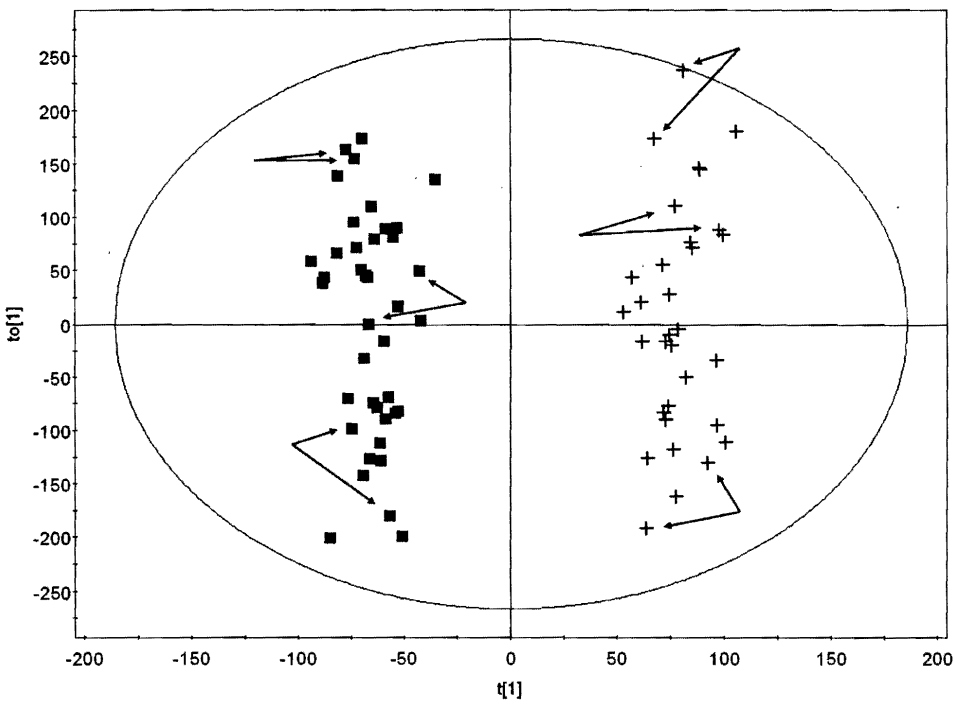

Figure 3. Experiment 1: OPLS-DA scores plots of the serum NMR spectra from OCD (■) yearlings vs Controls (+). With only one component represented, the model has very good quality ($R^2(Y)$ = 0.82 and Q^2 = 0.83). Double arrows highlight selected duplicate aliquots from individual horses, showing the good reproducibility of the metabonomic model.

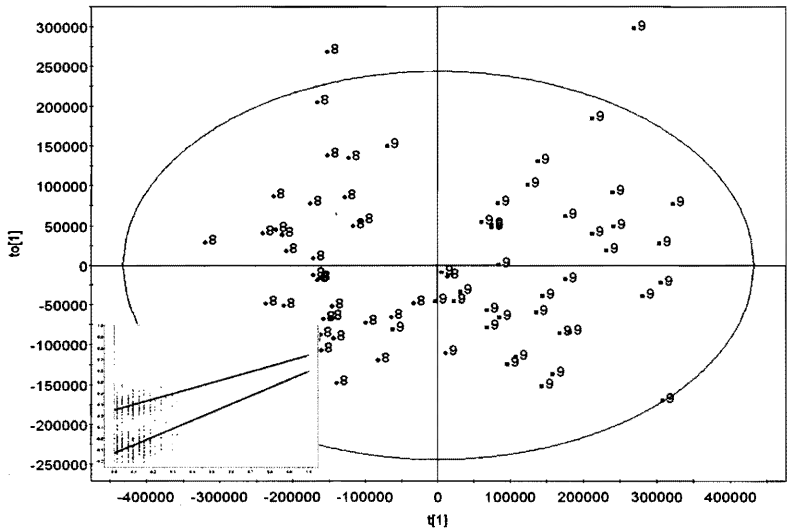

Figure 4. Experiment 2: OPLS-DA scores scatter plot of serum NMR spectra from Standardbred yearlings collected in August (n = 41, "8") and September (n = 39, "9") of 2008 showing separation ($R^2(Y) = 0.64$, $Q^2 = 0.75$). The validation of the PLS-DA model was also excellent for this metabonomic model (see inset).

predictability ($Q^2 < 0.0$).

Experiment 3

Despite the relatively small number of samples (15 for which OCD status had been verified radiographically), separation was highly significant (OPLS-DA; $R^2(Y) = 0.955$, $Q^2 = 0.90$) and well validated when the data for one individual that had been diagnosed as radiographically free of OCD lesions was excluded (Figure 5). However, when that sample was included in the set identified as a control, the $R^2(Y)$ and Q^2 were significantly reduced and the data point appeared in the cluster of the OCD samples (not shown). When its status was changed to OCD, the $R^2(Y)$ and Q^2 were very high (Figure 6). As the individual in question had been sold by the time the apparent discrepancy was discovered, it was not

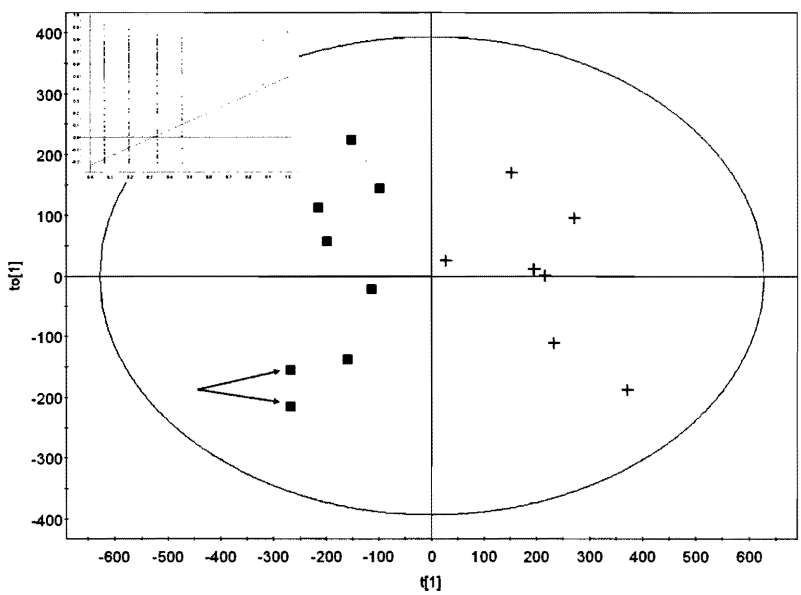

Figure 5. Experiment 3: OPLS-DA scores scatter plot of analysis of serum NMR Spectra from 14 Standardbred foals (1 to 3 months of age) that were radiographically verified as yearlings as having OCD (■) vs the Controls(+). One sample was run in two aliquots and is labelled with a double arrow. With only one component, there was clear separation ($R^2(Y) = 0.955$) and very high predictability ($Q^2 = 0.90$) of the metabonomic model. The inset shows the validation for the PLS-DA model for the same set. The scatter for the validation is larger because of the relatively small dataset.

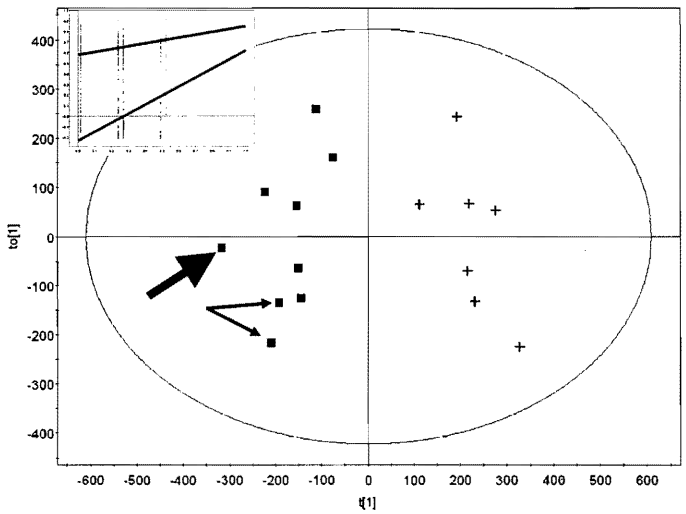

Figure 6. Experiment 3: OPLS-DA scores scatter plot of analysis of serum NMR Spectra from 15 Standardbred foals (1 to 3 months of age) that were radiographically verified as yearlings as having OCD (■) vs the Controls (+). Fourteen foals were the same as used in Figure 5. One individual that had been diagnosed radiographically as free of OCD was included (identified by the single large arrow) as having OCD. When it was included as a Control in the analysis, the R^2 and Q^2 were significantly reduced and its data point was clearly in the OCD cluster. When this horse was identified as having OCD, the metabonomic model had clear separation ($R^2(Y) = 0.8$) and high predictability ($Q^2 = 0.6$).

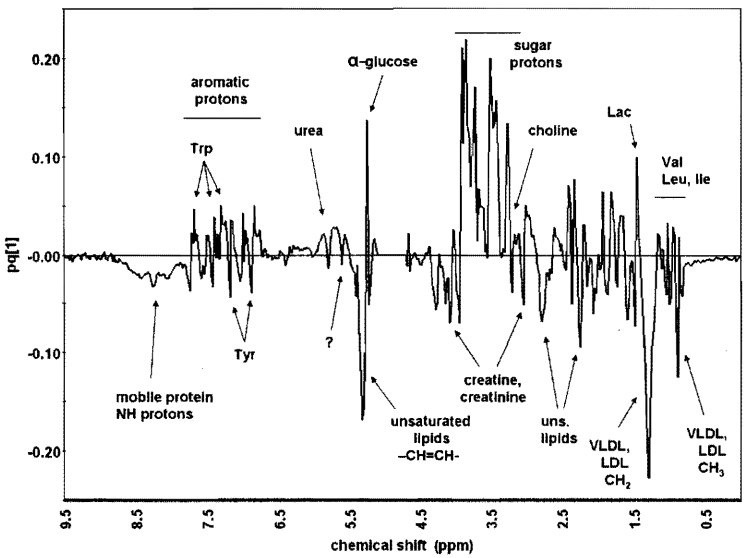

Figure 7. Experiment 3: Loadings/coefficient plot for the OPLS-DA model presented in Figure 6. Peaks for metabolites above the baseline were present in greater amounts in OCD serum than in Control serum ($P < 0.05$) whereas peaks below the baseline were more abundant ($P < 0.05$) in the Controls than in the OCD group.

possible to determine if there had been a misdiagnosis.

The loadings/coefficient plot for Experiment 3 (Figure 7) OPLS-DA revealed that, as in previous years, concentrations of alpha glucose and other sugars were lower in the serum of horses with OCD than in the controls, whereas those of certain unsaturated lipids and amino acids, creatinine and choline were higher.

Discussion

This series of experiments shows that NMR-based metabonomic analyses can detect metabolic differences between groups of animals that do or do not develop OCD. However, these analyses are sensitive to a many influences and it is very important that the resultant

models, especially the highly sensitive supervised models generated by OPLS-DA, be rigorously validated and documented for high predictability. For example, the distinct clustering (R^2 = 0.98) of the radiographically verified OCD vs Control yearlings in Experiment 2 was offset by the poor predictability (Q^2 < 0.0) of the model. In the "forced" OPLS-DA analyses in which group identifiers are used a priori, there spurious correlations can arise (Fonville et al., 2010) such that metabolites that are not truly significant factors in the disease metabonome are implicated as important in the statistical model. For example, this was observed when the loadings plots were compared between Experiments 1 and 3, in that a strong urea peak observed in Experiment 1 loadings did not appear in Experiment 3 (data not shown). Comparison of the loadings plots across the data sets may permit identification of metabolites that are consistently associated with OCD, avoiding spurious correlations. This is currently underway using more sophisticated orthogonal signal correction (OSC) analyses that identify the metabolites most correlated with the condition of interest across data sets (Trygg et al., 2007; Fonville et al., 2010). Various manipulations of the data sets, such as suppression of peaks that are known contaminants (such as the alcohol peak in Experiment 1) or known to be non-contributing factors (such as water) also will enable greater refinement of the resultant OCD metabonome. More data with accurate diagnosis and carefully controlled conditions is needed before a truly predictive metabonomic model can be validated for use with random samples.

The validated distinct difference between the August and September groups of yearlings in Experiment 2 was surprising. There were no significant differences between these groups in age, management or sample handling, but the loadings plots revealed apparent differences in the peaks of certain sugars, lipids and urea between the two sample times that may have obscured the previously observed OCD vs Control metabonomic differences when the data sets were combined for statistical analysis. The metabolic origin of the differences between the two sample times is currently being investigated using chemometric analyses (Trygg et al., 2007; Fonville et al., 2010).

The analyses used for the three experiments used relaxation filtered, water suppressed spectra. As plasma and serum contain large proportions of water and the peak for water is large, it can overwhelm and obscure the signals of interest (Hwang et al., 1995; Pelczer, 2005). If the water peak is not suppressed, the baselines of the spectra become distorted and can hide peaks produced from various metabolites. A simple water-suppressed experiment reflects the unbiased, full molecular content of the sample in a quantitative manner. The relaxation-filtered version combines water suppression with a pulse method; this induces controlled loss of signal intensity based on relaxation, which is differential and more efficient for detecting the large and slow-moving molecules in the mixture. As a result, the spectrum highlights the small molecules, whereas large biopolymers, such as proteins, will be highly suppressed (Pelczer, 2005). The direct quantitative information is lost, but variation in relative concentrations is consistent when the same experimental parameters are used for successive samples. In subsequent analyses of these data, we will also investigate the relative importance of large molecules.

Conclusions

NMR-based metabonomic analysis is a sensitive method for identifying differences between distinct groups of individuals. However, the statistical analyses are influenced by a variety of factors such as sample collection techniques, and spurious correlations caused by non-relevant variations need to be identified to maximize the utility of the resultant models. Young Standardbred horses that develop hock OCD differ metabolically from those that do not develop the disease despite similar management and genetic backgrounds. These metabolic differences can be detected using NMR-based metabonomic analysis before lesions appear in nursing foals. However, more analyses are necessary to determine the OCD metabonome that would be useful for diagnosis and prevention of the disease.

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