NMR-based Metabolic Profiling of OCD in Horses; Metabolic Profile and Gender Specific Metabolic Response

Jon Spires[1], Daniel Kim[1], Kenith Conover[1], Sarah Ralston[2], Kyla Ortved[3], István Pelczer[1]

[1] *Department of Chemistry, Frick Chemistry Laboratory, Princeton University, Princeton, NJ 08544, USA*
[2] *Professor Emerita and Fulbright Scholar, Howell, NJ*
[3] *Department of Clinical Studies, New Bolton Center, University of Pennsylvania, Kenneth Square, PA*

Introduction

A case-controlled set of serum samples (n=20*2) were analyzed to identify and characterize the metabolic markers of the presence or absence of clinical osteochondrosis dissecans (OCD), focal failure of endochondral ossification, in yearling Standardbred horses, using high-resolution FT-NMR, as well as TD-NMR.

The FT-NMR approach includes collecting both spectra with water suppression only, and also applying relaxation filter to highlight small molecular components, respectively.

The spectra were evaluated with multivariate analysis, including back-calculation of OPLS-DA coefficient plots and STOCSY analysis (in the works, not shown here), with focus on metabolites, which contributed most significantly to the separation of healthy and OCD affected cohorts. For meaningful multivariate statistical models and a valid metabolic interpretation, as demonstrated, judicious exclusions of selected outliers and careful inspection of individual spectra were conducted. Outliers were carefully weeded out to find reasonable consistency and acceptable statistical models.

As an alternative diagnostic potential, time-domain NMR (TD-NMR) was also utilized to identify the relaxation behavior of the water in the samples. The three experiments obviously present different information; the water suppressed spectrum is dominated by the large molecules (lipids, lipoproteins), the relaxation filtered version highlights the small molecule metabolites. The TD-NMR spectra tell about the bulk water's behavior mostly as the function of the protein/lipoprotein/lipid composition, and were processed using inverse Laplace Transform (iLT).

There were correlations distinguishing between OCD affected and healthy with both modalities.

We could also make a novel observation, that had not been detected in previous studies; there were differences between the metabolic responses to the disease for the two genders, both in FT-NMR and TDNMR. Looking at both genders together lead to poor quality results, which improved greatly upon separation of the gender-based subsets.

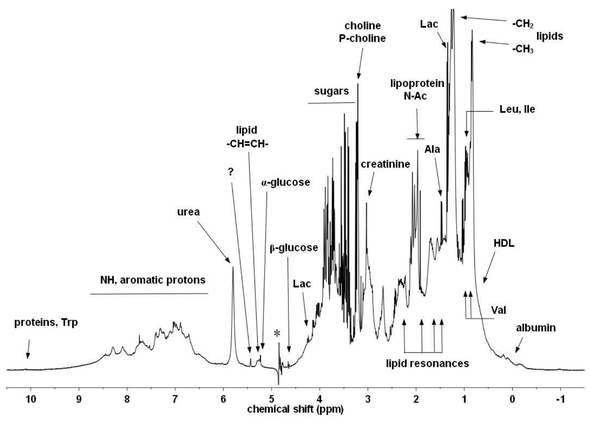

Figure 1: Typical ^{1}H-NMR spectrum of horse blood (serum). Some of the characteristic metabolites and other components are denoted.
(from: Ralston SL, Pappalardo L, Pelczer I, Spears PF; "NMR-based metabonomic analyses of horse serum: detection of metabolic markers of disease" *Rec. Adv. Animal Nutr.*, 18(2011)197-206)

Experimental

Sample Preparation, data acquisition

All serum samples were stored at -80°C, then gradually thawed to ambient temperature (22°C) for recording the NMR spectra on a 500 MHz Bruker Avance-III instrument at 295.2K, equipped with a TCI cryoprobe. Approximately 300 uL of the sample was transferred into a 4 mm OD tube plugged with a teflon plug. No buffer was added. This tube was inserted into a 5 mm OD container tube with ca. 35 uL D_2O, which lined up between the walls to provide the lock signal.

Data processing

NMR spectral data processing and visualization was conducted using MNova, up to v.14.0 (MestreLab Research S.L., Santiago de Compostela, Spain).

Multivariate statistical analysis was done in SIMCA (v.14, Umetrics, Umea, Sweden). All data presented here were mean centered and univariate scaling was used.

TD-NMR

The same 4 mm OD tube samples were inserted into the Bruker mq10 Minispec 10 MHz TD-NMR to measure the composite T2 decay. The analysis, including visualization of the spectrum and finding exact peak locations was done using iLT in MATLAB and in MNova.

Summary, conclusions

We have been studying the metabolic response of OCD condition in horses taking NMR spectra of serum samples of 20 pairs of healthy and OCD affected subjects. FT-NMR data were collected using only water suppression (full spectrum) and relaxation filtering, respectively, resulting in two independent datasets. The same samples were subjected to TD-NMR relaxation measurements as well, where the water response characteristically differs both by disease condition – but also by gender! This novel observation is much more explicit for the full spectra in the FT-NMR set, in accordance with the TD-NMR observation as both reflect the large molecules' behavior at the first place. This observation sheds new light on the differential role of lipids/lipoproteins between genders in horses and definitely deserves further evaluation, and invites re-visiting earlier data as well.

Additional analysis will include back-calculated OPLS-DA coefficient plots to identify the metabolites which significantly contribute to the clustering, and calculating STOCSY (statistical total correlation spectroscopy) traces to find connections in the metabolic pathway network. Both will be done for the complete data as well as the gender-separated subsets.

Regardless of the exceptional good quality of the collected specimens and the consistent and careful data collection, we have experienced difficulties to produce a good statistical model for the OCD affected and healthy cohorts, especially for the full spectra, which carry all the components, large lipids and lipoproteins included.

However, we made a quite surprising observation when preparing a plot of the TD-NMR data for the water peak. As shown on Figure 2. below, there is an interesting gender-specific response to the disease condition, which has opposite bias for colts and fillies.

The opposite behavior of the two genders offers plausible explanation for the poor statistical results of the mixed dataset, where the effects somewhat cancel each other. Therefore we decided to check on the FT-NMR data for the colts and fillies independently.

Indeed, the quality of the multivariate results improved dramatically for both groups, as it is demonstrated in Figure 3. This feature has not been observed during our earlier studies, except another recent dataset while studying diagnostic metabolic signature of laminitis. We do have plans to re-visit some of the old data and check on gender bias.

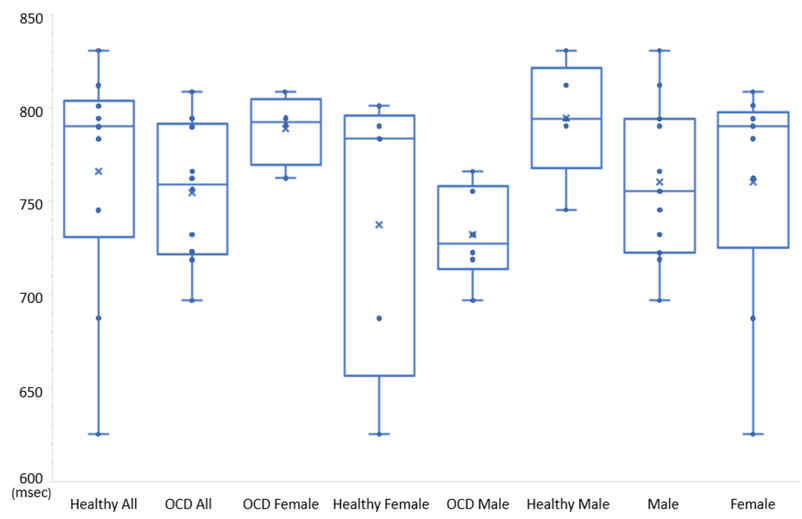

Figure 2: Box-plots of TD-NMR data of the water peak. Pairwise the pairs show the comparison of healthy vs. OCD (*left*), OCD fillies vs. healthy fillies (*second to the right*), OCD colts vs. healthy colts (*third to the right*), and colts vs. fillies (*rightmost*).

SIMCA modelling full and relaxation filtered spectra

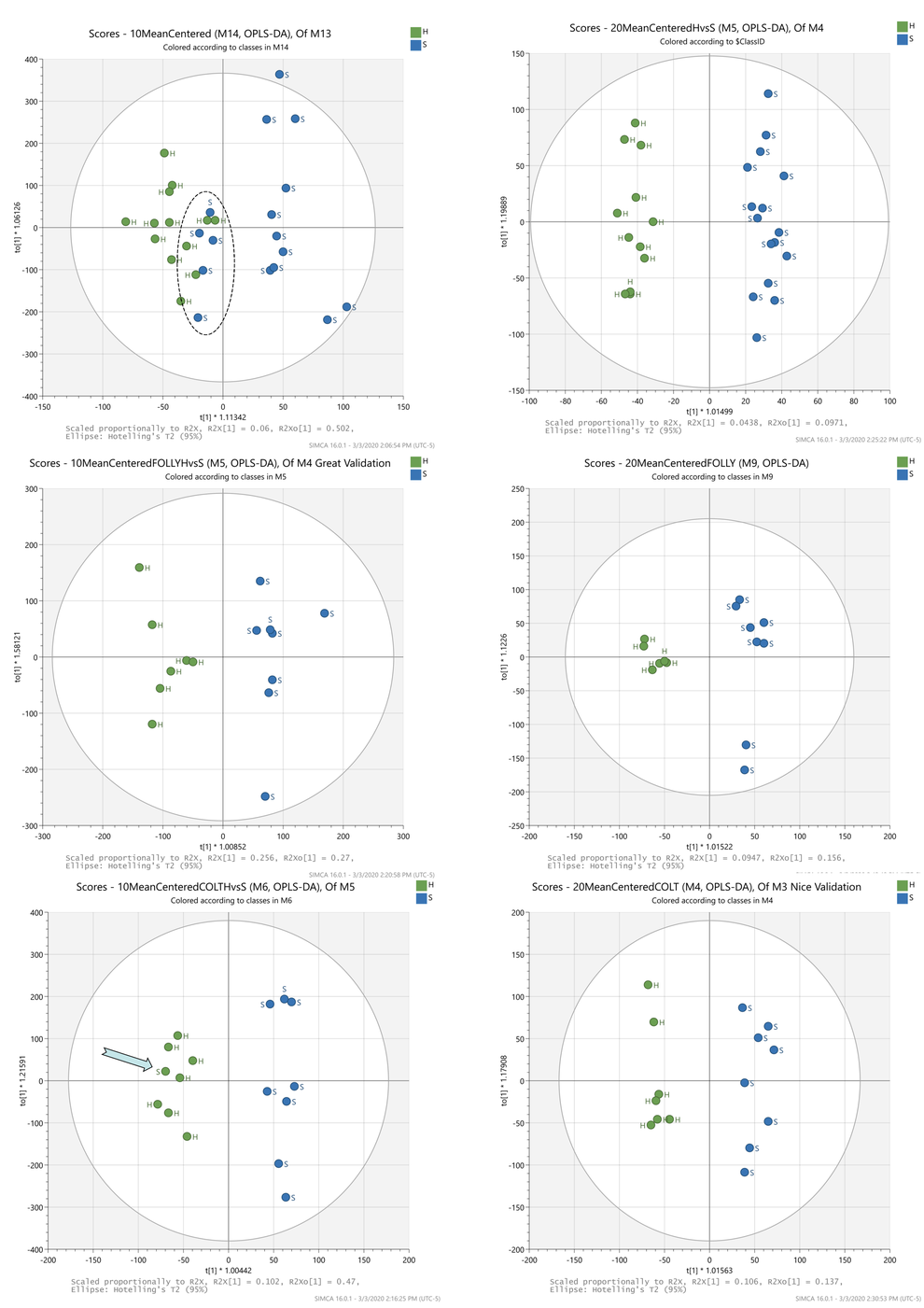

Figure 3: OPLS-DA scores plots for the full spectrum (*left column*) and the relaxation filtered data (*right column*) with no gender specification (*top*), fillies only (*middle*) and colts only (*bottom*), respectively. In the complete set for the full spectrum (*top left*) there are five OCD affected horses which, strangely enough and yet unexplained, cluster with the healthy cohort (*dashed ellipsoid*). When separated by gender, the full spectrum scores plots become very clean with well-separated clusters. However, one horse had to be re-classified from OCD affected to healthy for the colts (shown with an arrow) to reach good statistics. The relaxation filtered data show the same general tendency, although the data are cleanly clustered for the complete set already. This proves the point that the dominant features for gender separation belong to the large molecules (lipids, lipoproteins), I accordance with the TD-NMR data.

Acknowledgements

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Frank Bosco (New Era Enterprises, Inc., Vineland, NJ) is our constant supplier of glassware and willing collaborator for devising new tube constructs (such as the 5/4 mm OD combination).

Umetrics (Umea, Sweden) generously offered 20 SIMCA licenses for use to our students.