

RUTGERS

New Jersey Agricultural
Experiment Station

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

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What is Metabonomics ?

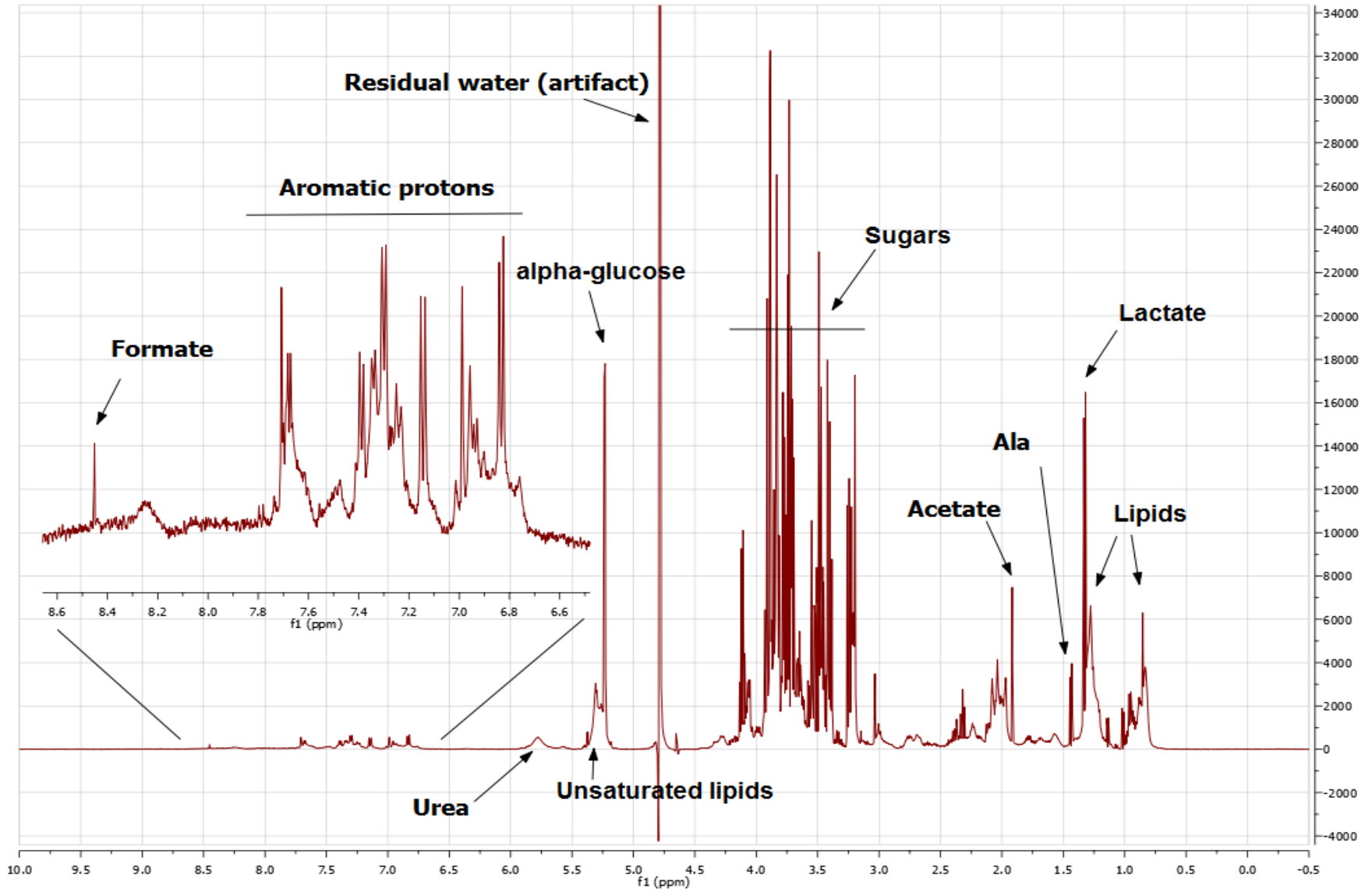
- **The quantitative measurement of dynamic, complex multiparametric metabolite profiles and/or responses of living systems by use of Nuclear Magnetic Spectroscopy (NMR) or Mass Spectroscopy of biofluids** (Lindon and Nicholson, 2007).
 - Used to track complex metabolic changes over time after discrete stimuli such as drug or dextrose challenges are administered.
 - Also effective at identifying metabolic profiles associated with specific disease states, permitting identification of diagnostic metabolic markers and providing better understanding of the underlying disease processes.

Why NMR-based Metabonomics?

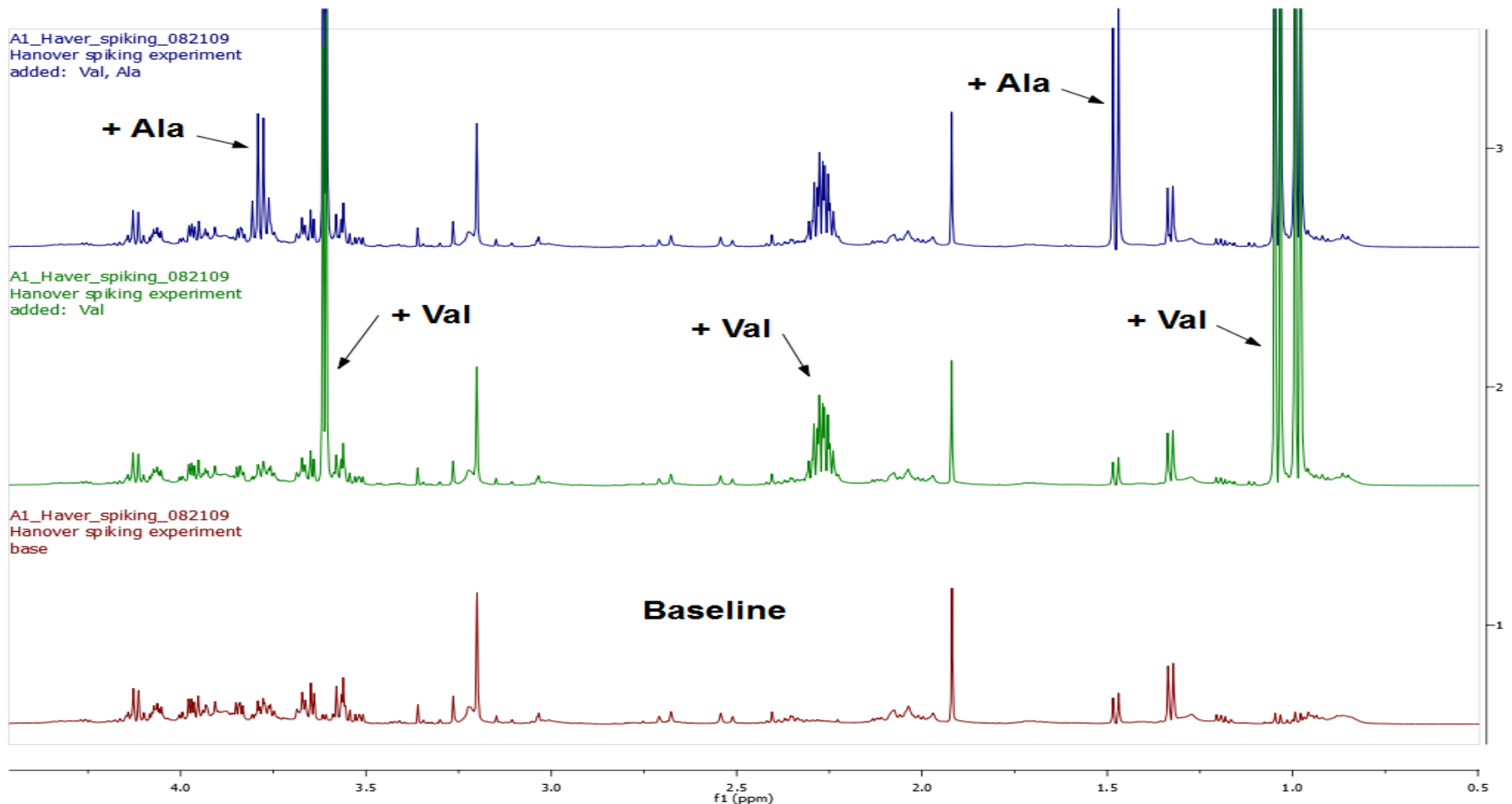
- **Drawbacks to NMR:** Originally low sensitivity relative to MS, but improved by at least 4x by the recent introduction of cryoprobes.
- **Bonuses:** Naturally quantitative (integral of peaks for each metabolite), that requires minimal sample preparation, and robust and highly reproducible spectral analyses.



Typical NMR spectrum from horse serum



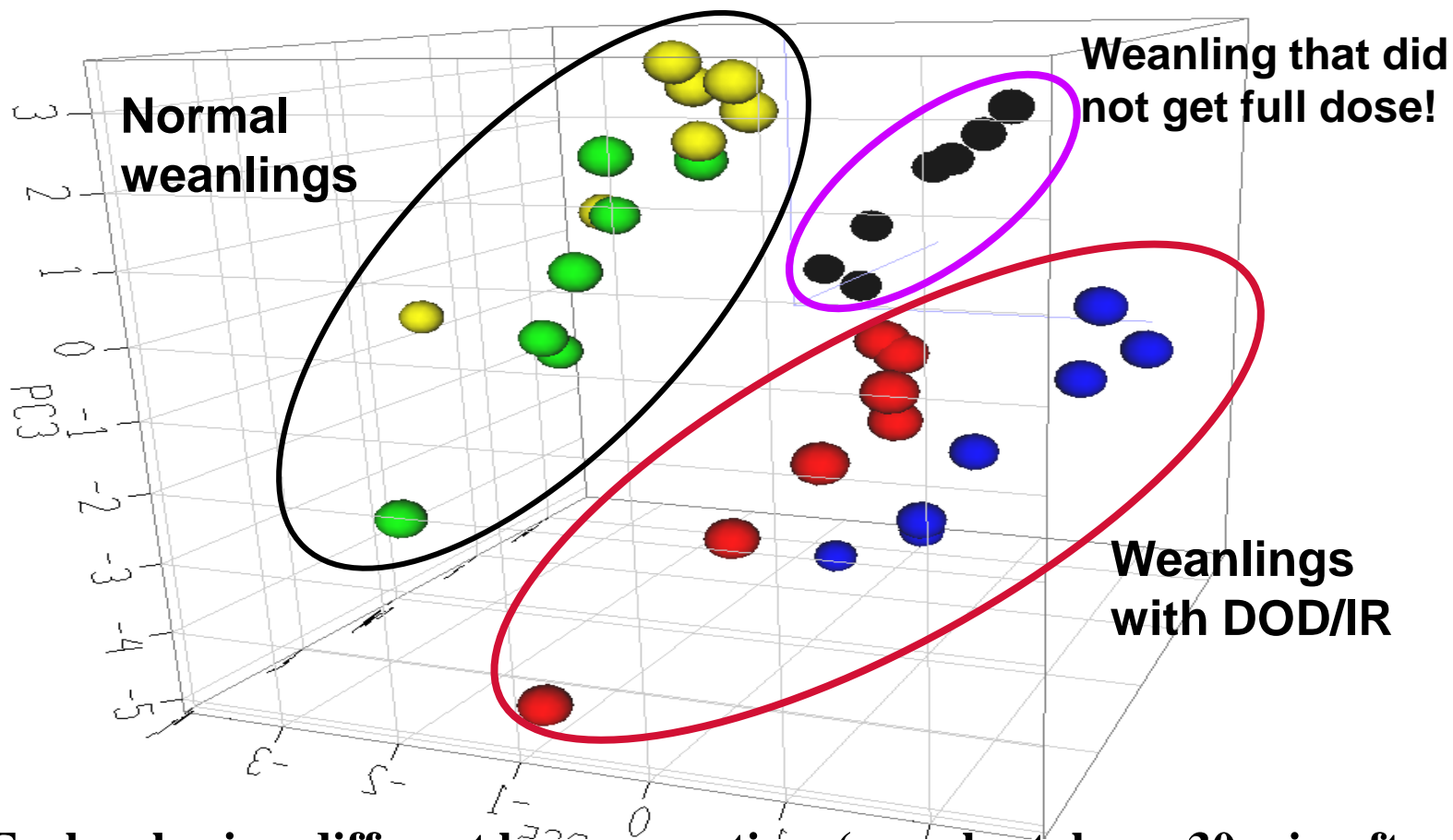
Metabolites associated with specific chemical shift locations have been verified by spiking experiments but not all peaks are identified. There is potentially species specific variation, but none, so far in horses.



- **Multivariate cluster analysis** using:
 - “unsupervised” principle component analysis (PCA-X)
 - “supervised” or “targetted”
 - partial least squares- discriminant analyses (PLS-DA)
 - orthogonal partial least square discriminant analyses (OPLS-DA)
 - Must be cross validated to avoid “overfitting” which would lead to false correlations.
 - R^2 = Proportion of explained variance
 - Q^2 = Proportion of predicted variance
- **Variable Importance Factors (VIP)** are used to identify peaks (metabolites) contributing most significantly to the models’ discrete clusters ($VIP > 2$, $P < 0.05$) .

Metabonomic analysis of weanlings' response to low dose oral dextrose challenge (0.25 gm dextrose/kg) BW

PCA-X plot-“unsupervised” analysis



Each color is a different horse over time (samples taken q30 min after dosing), each dot represents a single serum sample NMR spectrum, the position of which is based on its' metabolic pattern.

Osteochondrosis Dessicans in Horses

- **Failure of proper endochondral ossification leading to cartilage defects on the articular surface of joints, primarily hock, stifle and fetlock.**
- **Can cause lameness, though degree varies with severity, location and individual horse.**
- **Lesions appear only in the first year of life though often remain clinically “silent” until the horse is put into work.**
- **Can be corrected surgically but the history of OCD lesions reduced the sale price of affected yearlings by an average of over \$9000 relative to similarly bred “clean” horses at the elite at the Harrisburg Sale, 2008 (Zeigler, unpublished).**

- **Multi-factorial causes:**
 - **Major mineral imbalances in any breed.**
 - **High Soluble Carbohydrate intake in Thoroughbreds.**
 - **Correlation with insulin resistance/hyperinsulinemia in Standardbreds, Thoroughbreds, others?**
 - **Heritable predisposition documented in Standardbreds, Swedish Warmbloods, Hanoverians.**
- **The genetic/metabolic defect leading to the heritable predisposition is currently unknown.**

Metabonomic analysis of NMR spectra obtained from Standardbred yearlings with and without history of OCD lesions would reveal a unique metabolic profile associated with the affected horses.

Basically a fishing expedition!

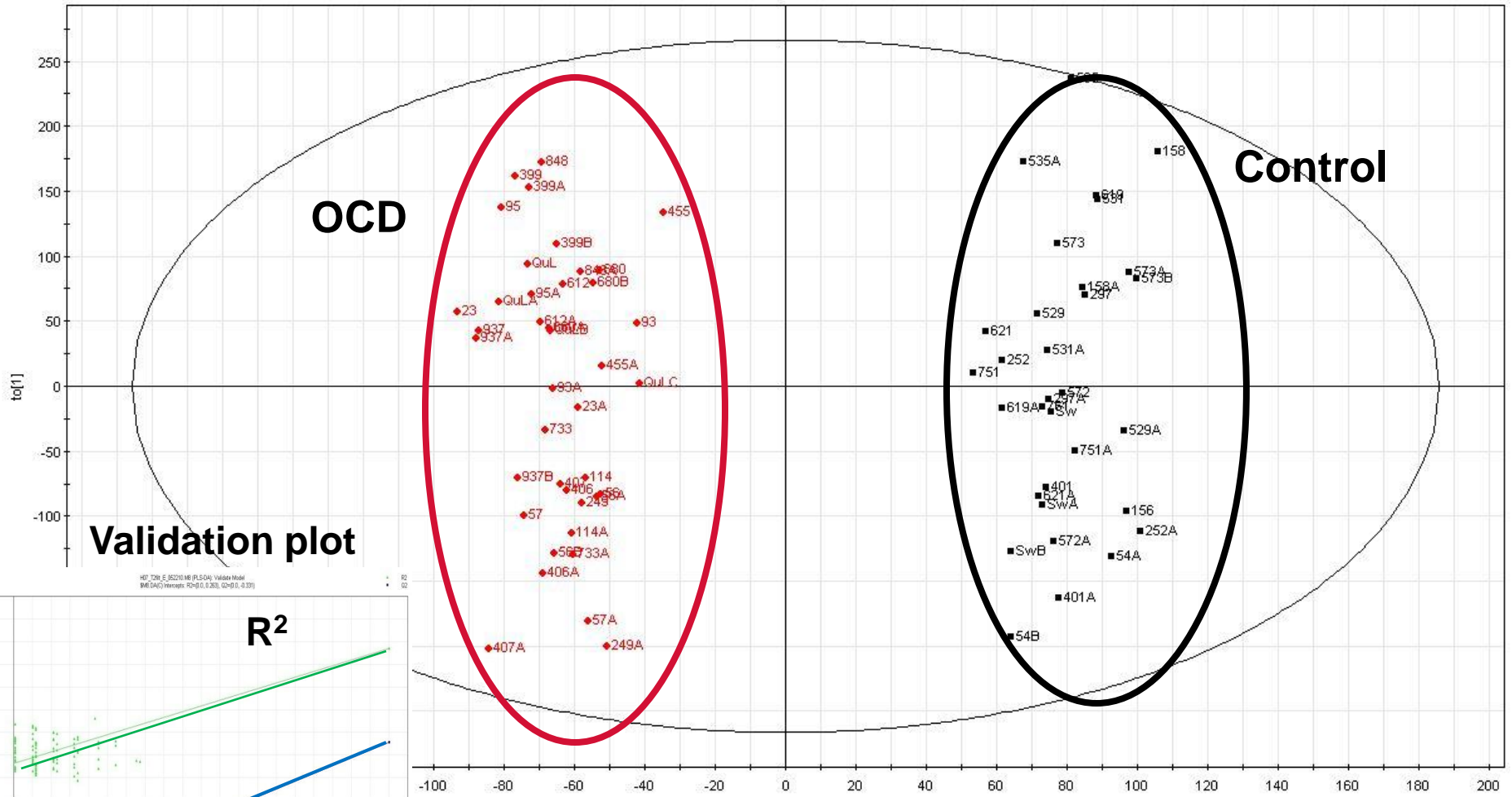
- **20 matched pairs of Standardbred yearlings-one had had hock OCD surgery 2 to 9 months before samples taken, the other had no clinical evidence of OCD.**
- **Paired based on sire, similarly bred dams**
 - **Fed and handled the same**
 - **All blood samples taken on a single day-on ice until spun down 5 hrs later**
 - **Serum for NMR/metabonomic analysis**



2007 Data: OPLS-DA of Principle Component 1

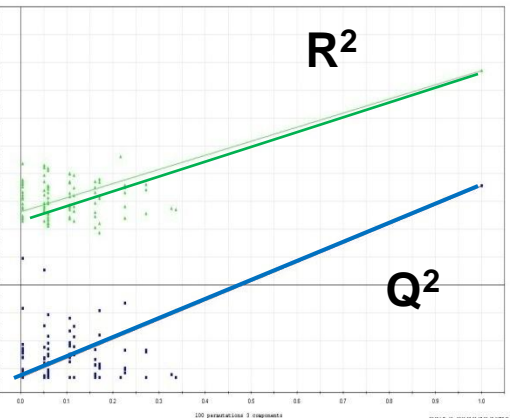
H07_T2fit_E_052210.M7 (OPLS/O2PLS-DA)
t[Comp. 1]/to[XSide Comp. 1]
Colored according to classes in M7

■ C
● O



Validation plot

HP: T01_E_052210.M7 (PLS/O2PLS-DA) Validation Model
RMSECV: 0.0497186, Q2: 0.83



= 0.0497186

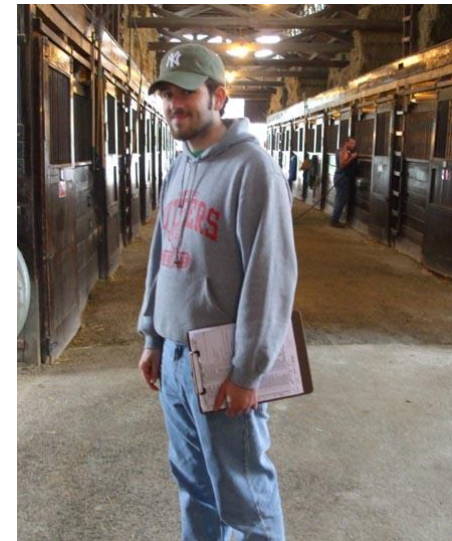
$R^2(Y) = 0.82$ and $Q^2 = 0.83$

- **80 yearling Standardbred horses, most related to 2007 horses.**
 - **22 had had hock OCD lesions corrected surgically 2 to 12 months before samples taken, 22 of the “control” horses had the lack of hock OCD lesions verified radiographically.**
- **Blood was drawn by venipuncture on 2 separate days (41 sampled in Aug, 39 in Sept).**
- **Samples spun immediately and placed on ice then frozen, pending analyses:**
 - **NMR analyses of serum**
 - **Analysis of plasma glucose/insulin concentrations.**
 - **Collection of white blood cells for genomic analyses.**

- **There were NO differences in total plasma glucose or insulin concentrations between the OCD horses and controls, using conventional assays.**
- **White blood cell samples to Dr. Molly McCue, Univ. of Minnesota, for genomic analyses.**



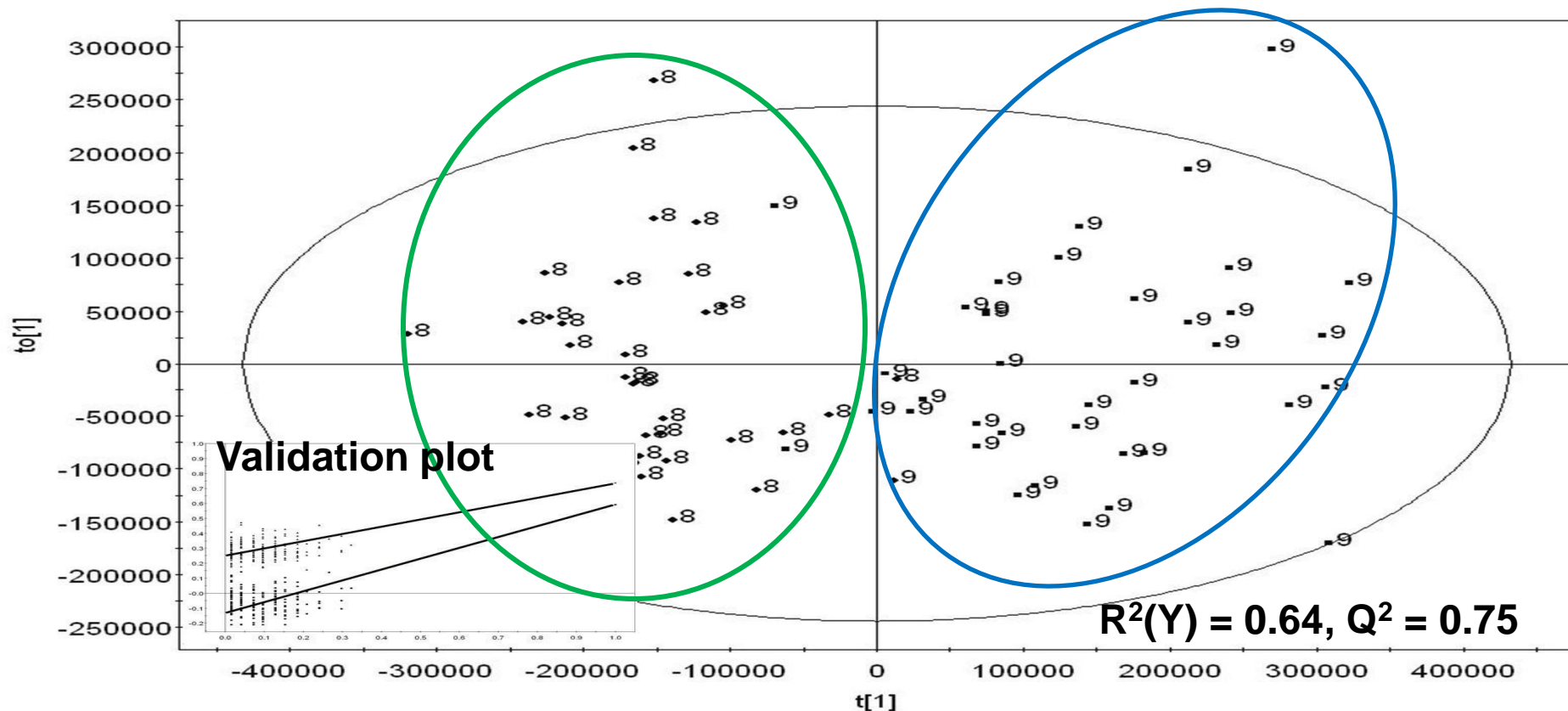
Katie Ziegler



Brian LaBarre

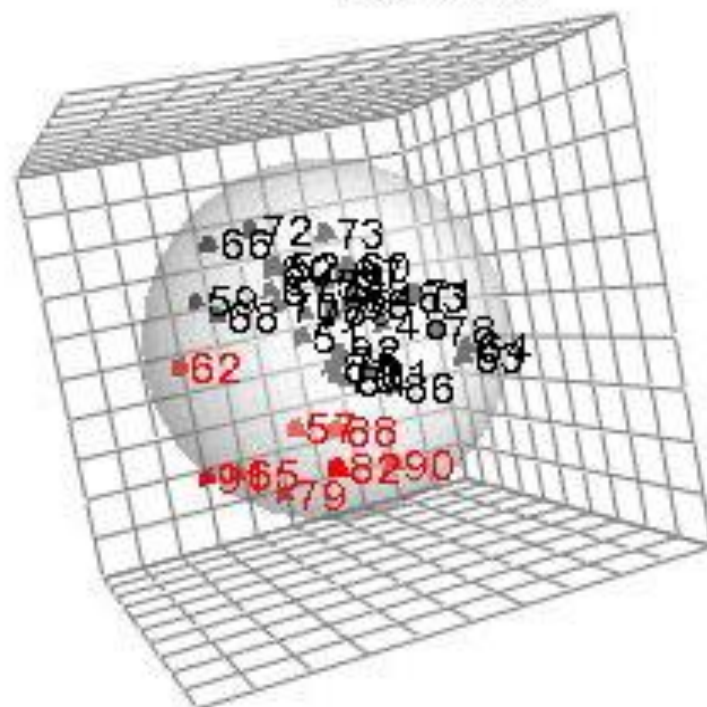
When spectra from all horses were subjected to metabonomic analyses, the clear separation in the plots observed in 2007 was not evident.

However, there was clear separation between August and September groups



However, when just September or August data were evaluated using targeted analysis looking only at metabolites identified as important in 2007, we again got good separation

September, 2008 (n=39) Black=Control
Red=OCD



0

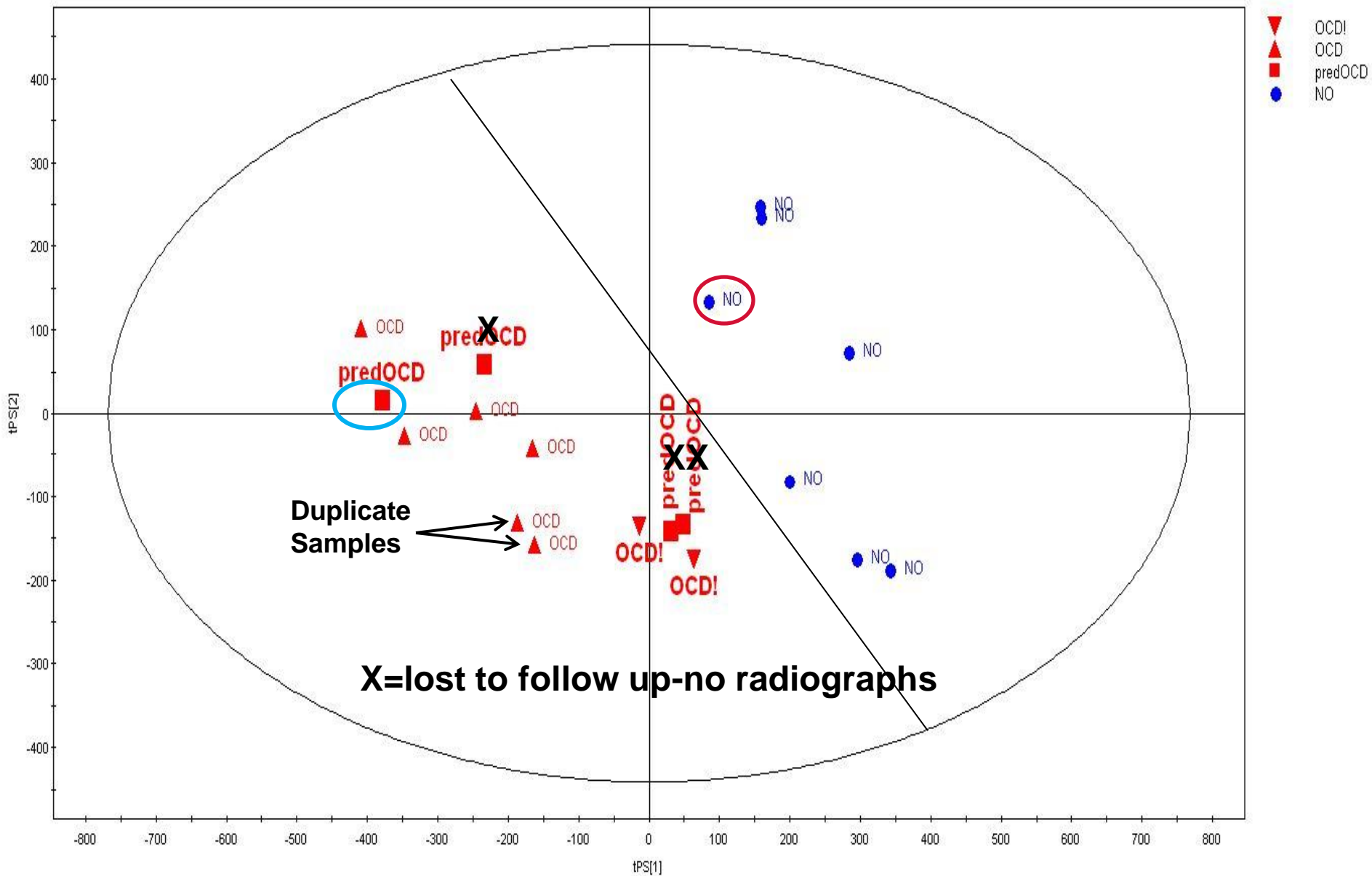
1

Ellipse: Hotelling T2 (0.95)

$R^2_{cum}=0.98$

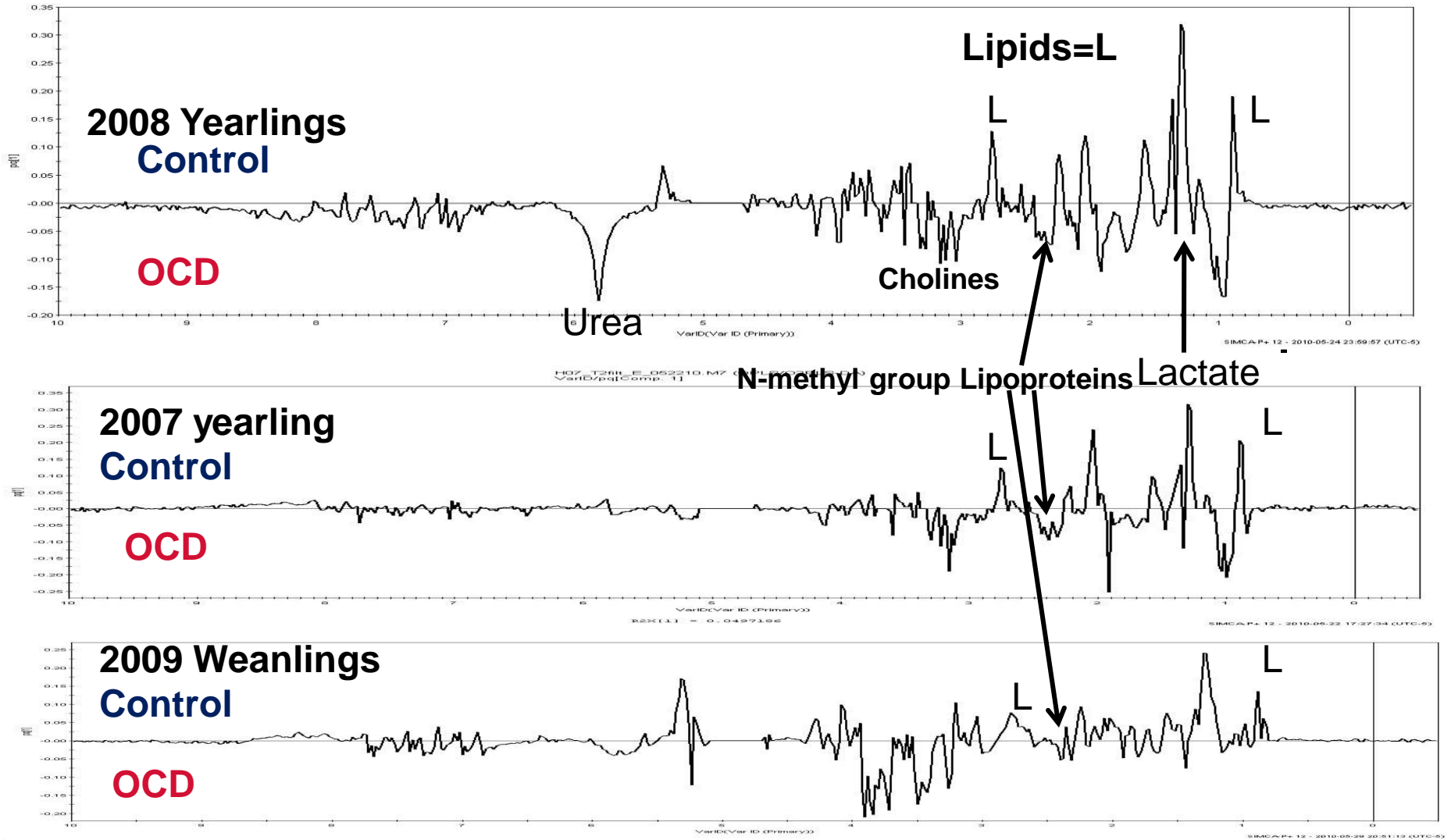
- **18 nursing foals from mares that had previously produced foals with OCD at the same venue were sampled in May, 2009.**
- **None had clinical signs of orthopedic disease or had had surgical interventions at the time of sampling.**
- **Samples stored and processed as described previously.**
- **Follow up radiographs taken 12 to 18 months later on 15 of the horses**

The new model was predictive in all but 2 cases



Loadings Plots from SIMCA P+ OPLS-DA '07, '08 and '09

Loadings plots identify the metabolites that differed systematically between groups in each year



What does this Mean?

- **There are consistent metabolic differences in young Standardbred horses that get OCD versus closely related horses that don't, despite common management/environment, both as nursing foals before lesions are detected and at 18+ months of age when they are no longer at risk of developing new lesions.**
- **The metabolic pathway(s) involved may be related to alterations in lipid, glucose and/or amino acid metabolism. It is NOT just glucose/insulin!**
- **The consistent differences in metabolic profiles may help in detection of the genetic defect that will:**
 - **Provide better understanding of the fundamental biochemistry leading to the development of OCD**
 - **Allow early ID of horses at risk**
 - **Development of feeding strategies to prevent it**

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Katherine Zeigler, George H. Cook Honors '09
Brian LaBarre, RU grad '10
Sarah Cimbol, Livingston High School Honors '10

And above all

**Dr. Istvan Pelczer,
Princeton University**



Questions?



Sample Processing

- **Serum samples stored at -80°C pending ^1H NMR analysis.**
- **Samples run on a Bruker Avance-II 500 MHz spectrometer equipped with proton-optimized cryoprobe, using excitation sculpting(ES) water suppression schemes.**
- **A 1 millimeter deuterated water insert was used in the probe for signal lock to stabilize the instrument without altering the sample.**

- **NMR data processing (apodization, Fourier transformation, phase and baseline correction, calibration and binning by 0.02ppm) done in MNova**
(MestreLab Research, Santiago de Compostela, Spain)
- **Spectra calibrated and aligned to the anomeric proton of alpha glucose (5.233 ppm).**
(Hong *et al.*, Magn. Reson. Chem., in press)
- **Normalization and suppression of unwanted signals (ie: water and possible contaminants like ethanol) were done in Excel.**