METABONOMIC PROFILING OF URINE FROM LYSINURIC PROTEIN INTOLERANCE AFFECTED MICE

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INTRODUCTION

Lysinuric protein intolerance (LPI; MIM 222700) is an autosomal recessive aminoaciduria caused by a defective transport of cationic amino acid (arginine, lysine, ornithine; CAA) at the basolateral membrane of epithelial cells in the intestine and kidney. As a consequence, intestinal absorption and renal reabsorption of CAA are impaired with net loss of these amino acids. Clinical expression of LPI is variable and most frequently includes vomiting, diarrhea, failure to thrive, growth retardation and multiple organ diseases. Metabolic derangement of LPI is related to lower plasma levels of arginine and ornithine which hinder the urea cycle functioning. This leads to hyperammonemia after protein-rich meals and increased orotic aciduria. No pathogenetic mechanisms are known, so far, for many of the main clinical findings such as hepatosplenomegaly, alveolar proteinosis, renal involvement and stunted growth.

LPI is caused by mutations of the solute carrier family 7A member 7 (*SLC7A7*) which belongs to the family of heterodimeric amino acid transporters (HAT) and encodes the y^+LAT-1 protein. This transporter, located at the basolateral membrane of epithelial cells, induces system y^+L activity. The mouse *Slc7a7* gene maps on chromosome 14 and its predicted amino acid sequence is highly homologous to the human protein (90.4% identity; 98.6% similarity).

To elucidate the complex and mostly unknown pathophysiology of LPI, a genetically modified mouse by disruption of *Slc7a7* gene was generated (from Lexicon Genetics Incorporated; The Woodlands, Texas). In the present report we describe the urinary metabolic phenotype (metabonome) of the LPI mouse model by Ultra Performance Liquid Chromatography orthogonal acceleration Time of Flight Mass Spectrometry (UPLC oa-TOF-MS).

METHODS

Metabonomic profiling of urine in knockout mice with lysinuric protein intolerance was performed on 14 samples from three genotypes (homozygous, heterozygous and wild type). For fast, high efficiency separations LC data was acquired using an ultra-performance liquid chromatograph with 1.7 µm column particle size. This was coupled to an electrospray ionization LC-TOF mass spectrometer with enhanced ion optics to maximize spectral resolution and mass accuracy. Novel ion optics facilitate the acquisition of data over a wide dynamic range (up-to four orders) with accurate isotope patterns. Data processing was performed by multivariate statistical analysis utilising a combination of MarkerLynx and SIMCA software.

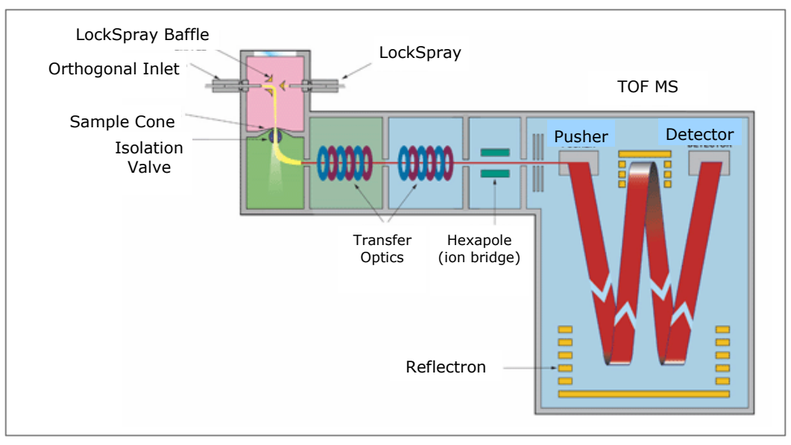

Figure 1. A Schematic of the LCT Premier demonstrating W Optics

Extraction Methods
The urine samples were analysed directly with no pretreatment

UPLC Conditions

Instrumentation	Waters ACQUITY™ UPLC™ System
Column	Waters ACQUITY™ UPLC™ BEH C_{18} 2.1 x 100 mm, 1.7µm
Eluent A	0.1% Formic Acid in Water
Eluent B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.6 mL/min

Gradient

Time (min)	%A	%B	Curve
0	100	0	1
1	100	0	6
8	0	100	6
9	100	0	11

Run Time	9 minutes
Column Temp	40°C
Injection Volume	10 µL

MS Conditions

Instrumentation	Waters LCT Premier™
Resolution	>10,000 (FWHM)
Capillary Voltage	2300 V
Cone Voltage	30 V
Desolvation Temp	450°C
Desolvation Gas Flow	800 L/hour
Cone Gas Flow	20 L/hour
Scan time	0.15 sec
Interscan delay	0.01 sec
Mass Range	50-1000 Da
External Calibration	Sodium Formate Clusters
LockMass Reference	Leucine Enkephalin

Software
MassLynx V4.1 with the MarkerLynx Application Manager.
SIMCA-P 11, Umetrics.

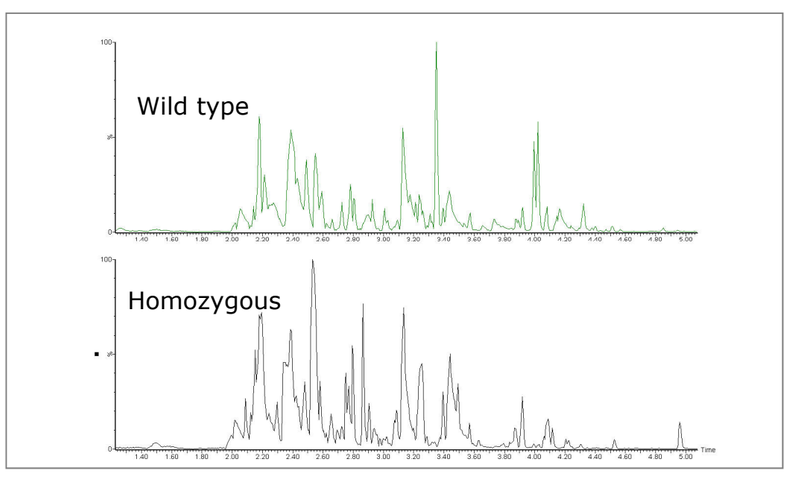

Figure 2. Chromatograms of Wild Type and Homozygous

RESULTS AND DISCUSSION

Acquisition of UPLC oa-TOF-MS data for the three genotypes facilitates the identification of discrete differences between the samples related to the condition LPI. Manual data interpretation whilst possible is extremely time consuming and thus employing an automated approach is advantageous.

The MarkerLynx Application Manager for MassLynx 4.1 is an integrated software package that facilitates processing and reviewing of UPLC oa-TOF-MS metabonomics data. The MarkerLynx browser allows easy viewing of both the derived multivariate statistical data, the original raw UPLC oa-TOF-MS chromatographic data and mass spectral data for dynamic user interaction. MarkerLynx detects peaks in the UPLC oa-TOF-MS data set, lists detected peaks by retention time and their corresponding intensities, allows for removal of xenobiotics and performs principal components analysis (PCA). The scores and loadings plots display the results of the PCA, highlighting the masses of potential biomarkers. The full scan UPLC oa-TOF-MS data was extracted, integrated and aligned within MarkerLynx. This information was used to populate a matrix of objects (samples), with variables being *m/z :* retention time pairs and peak intensities. PCA was then performed.

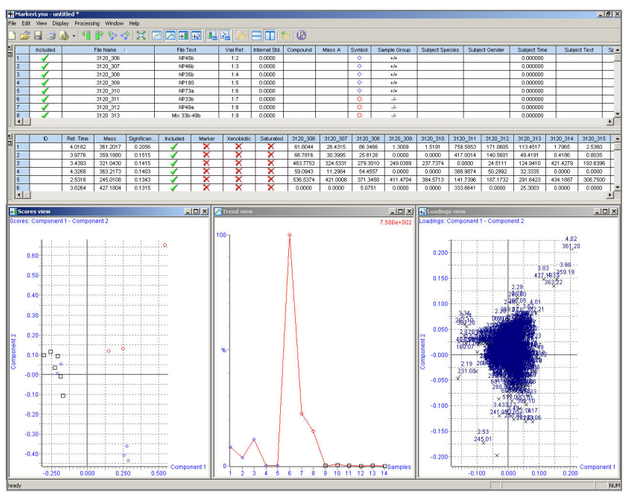

Figure 3. PCA (PC1 vs. PC2) in the MarkerLynx Browser

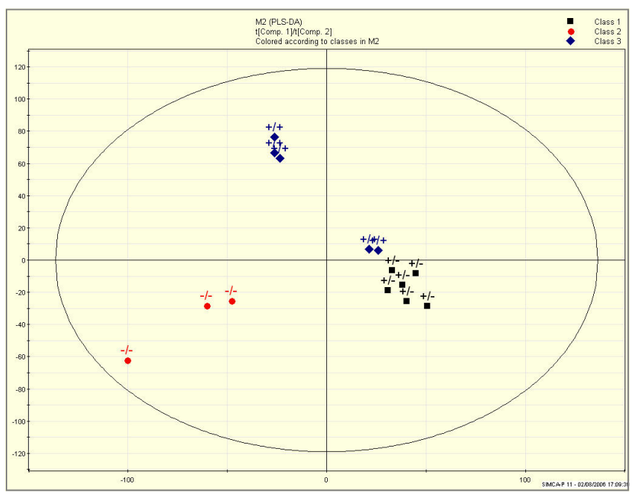

Figure 4. PLS-DA scores plot demonstrating separation of the three genotypes

PCA analysis within MarkerLynx identifies a clear differentiation between wild type and the other classes i.e. homozygous and heterozygous (figure 3). The homozygous and heterozygous classes are overlapping, therefore, an alternative statistical method was utilised to further distinguish the inter class separation.

The dedicated export tool within MarkerLynx was used to transfer the matrix into SIMCA, where alternative statistical methods may be employed. PCA was performed to demonstrate that the results obtained in both software packages were comparable. Further analysis with Partial Least Squared Discriminant Analysis (PLS-DA) was subsequently undertaken and each of the genotypes were successfully separated (figure 4).

Orthogonal Partial Least Squared (OPLS) analysis was performed (figure 5). From this analysis an "S Plot" was generated (figure 6). The "S plot" is a data visualization tool that combines information from the traditional loadings plot and the column plot confidence limits. The result is a filtering out of markers with low confidence. Selection of data points in the >80% confidence regions of the plot is a rapid and powerful method for the identification of *significant* potential markers. The identification of metabonomic differences is therefore greatly simplified, even where a subtle difference is seen in regulation between wild type and homozygous genotypes (figure 7).

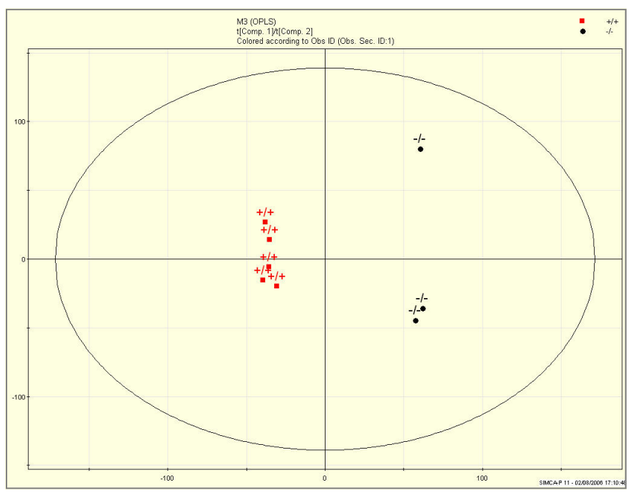

Figure 5. OPLS scores plot

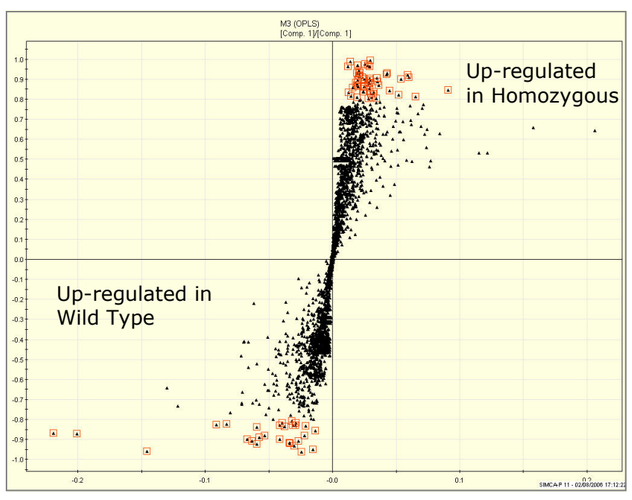

Figure 6. "S Plot" of data >80% Confidence

The metabolic basis underlying the multiorgan involvement of LPI are still unclear. Some of the biochemical LPI findings such as hyperammonemia and increased orotic acid production might be explained by an intracellular CAA depletion in hepatocytes. Indeed, it is more difficult to understand mechanisms underlying important clinical features such as visceromegaly, postnatal growth retardation, early osteoporosis, lung involvement. Therefore, an animal model may represent a crucial tool for obtaining insight into the pathophysiology of this disorder and for testing innovative protocols of treatment.

A mouse was generated in which *Slc7a7* gene function was disrupted, as a consequence, no *Slc7a7* mRNA is present in *null* animals. The homozygous disruption of the *Slc7a7* gene leads to a scarce vitality of *null* pups. Biochemical findings of human LPI such as massive urinary excretions of arginine, ornithine and orotic acid were already present when both animals were kept on the special dietary regimen. Hyperlysinuria appeared only in the acute stage of the disease as seen in urines collected from the *null* male animal. In *Slc7a7 null* mice the defective transport of CAA across cells, organs and placentas maybe influence the regulation of other metabolic pathways as consequence of an altered intracellular CAA homeostasis due to *Slc7a7* ablation.

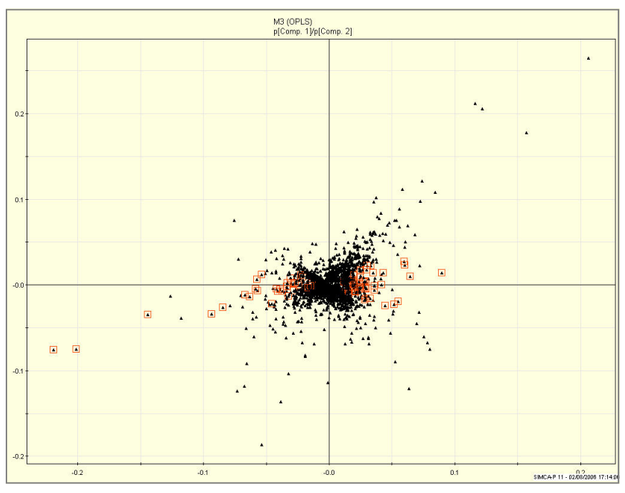

Figure 7. OPLS loadings plot with data >80% Confidence highlighted

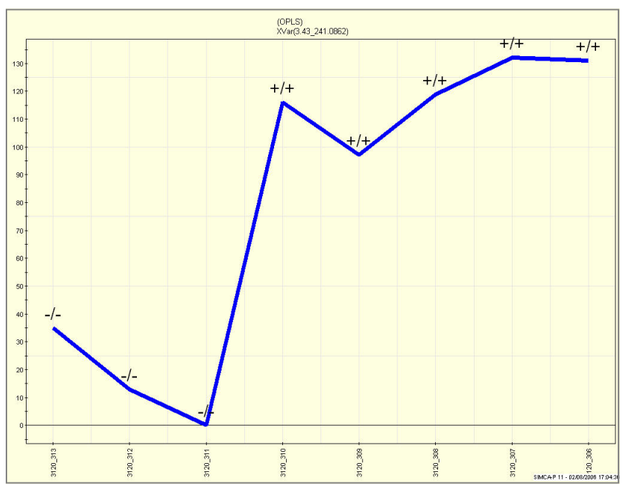

Figure 8. Up regulation of m/z 241.0862 in WT

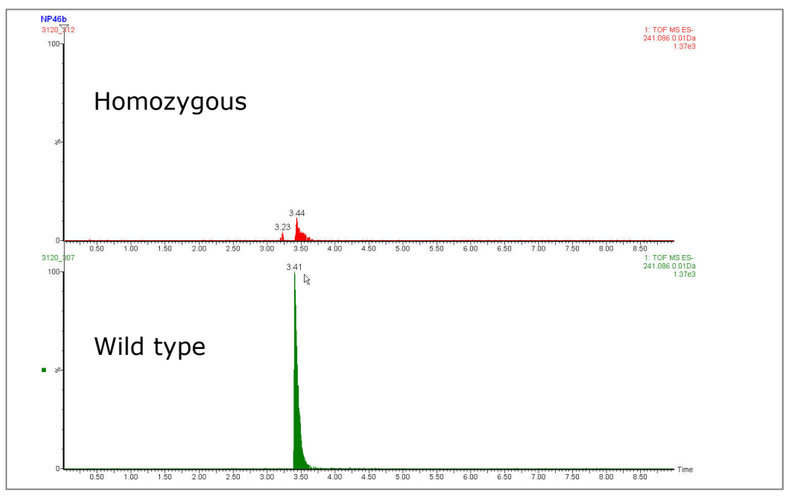

Figure 9. Chromatographic verification of up regulation in the extracted ion chromatograms of m/z 241.0862±0.01 from homozygous and wild type raw data.

CONCLUSION

Our results revealed that the absence of a functional *Slc7a7* mRNA results in the dysregulation of more than 50 urinary metabolites with molecular weight that differ from amino acids. Thus the defective transport of CAA could be linked to these compounds, coming from other metabolic pathways, whose influence and/or maybe are responsible of impaired intracellular homeostasis. Several transporter families mediate uptake of chemicals into intestine, kidney and liver and excretion of chemicals from liver into blood and/or bile. It is not surprising if the defect of *Slc7a7* causes the up- and down regulation of other transporters to produce a compensatory effect on CAA transport into intestine, liver and kidney.

- **The combination of Waters ACQUITY with the Waters LCT Premier provides a robust platform for rapid screening of complex matrix.**
- **MarkerLynx provides an automated approach for rapid deconvolution of UPLC oa-TOF-MS data into a matrix of variables. Various statistical analyses may then be performed on this data.**
- **Advanced multivariate statistical analysis performed using SIMCA-P11 is a powerful and complementary approach to UPLC oa-TOF-MS with MarkerLynx data processing.**
- **By utilising PLS-DA we are able to visualise the differences between the Homozygous, Heterozygous and Wild Type mice.**
- **We are able to provide a comprehensive list of variables/ markers (mass/retention time pairs) that tells the user why the classes are different.**
- **By utilising OPLS we can compare wild type and homozygous classes and extract only the markers which are statistically significant and are within a defined confidence limit.**
- **Metabonomic profiling of the present mouse model offers new insights into the pathophysiology of LPI and, more generally, into mechanisms linking CAA transport with prenatal growth control and the pathogenesis of multiorgan diseases.**

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