Imperial College London

Metabolic phenotyping by automated chip-based nanoelectrospray ionization high resolution mass spectrometry

Júlia Dénes[1], Eszter Szabó[2], Steven L. Robinette[1], Manuel Liebeke[1], László Szőnyi[3], Ernst W. Rauterberg[4], Elaine Holmes[1], Zoltán Takáts[1]

[1]Biomolecular Medicine, Department of Surgery and Cancer, Imperial College London, London, UK
[2]Eötvös Lóránd University, Department of Chemistry, Budapest, Hungary
[3]1st Department of Pediatrics, Semmelweis University, Budapest, Hungary
[4]Hesse Child Health Centre, Giessen, Germany

OVERVIEW

- High throughput, untargeted analysis method was developed
- High resolution mass spectrometry and nanoelectrospray techniques were applied
- Multi- and univariate statistics were used for the data analysis
- Tested for different models (newborn screening, urine analysis, bacteria)

INTRODUCTION

LC-MS:

- Two dimensional data: retention time and *m/z*
- No ion suppression effect
- Chromatography is time consuming and factor of uncertainty

FIA-ESI:

- Used generally in newborn screening
- High-throughput analysis method
- Limited amount of information and sensitivity: more MRM channels, loss of sensitivity

Single-stage HR-MS:

- High-throughput analysis
- More relevant biochemical information: without loss of sensitivity!

METHODS

Sample preparation

- 96-well plate format (extraction, filtration)
- Addition of stable isotope labeled internal standards if quantification required

Instrumentation

- High-resolution mass spectrometry: Orbitrap technology (Exactive)
- Chip-based direct infusion: nanoelectrospray technology (Triversa NanoMate)

Measurements

- 1 minute run (30 s negative ion mode, 30 s positive ion mode)
- Full scan mode, *m/z* 50-2000 mass range

Data analysis

- Peak identification by exact mass and MS/MS
- Quantification by isotopic dilution
- Unsupervised multivariate statistical methods
- Univariate statistical methods

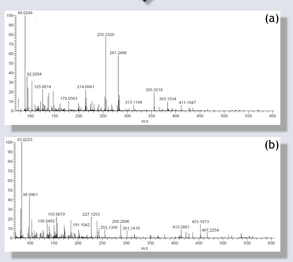


Figure 1. High resolution mass spectra of DBS samples. Healthy newborn sample was measured in (a) negative and (b) positive ion mode.

RESULTS

SCREENING FOR INBORN ERRORS OF METABOLISM[1]:

- **Validation study for 10.000 newborn samples**
- ONE-STEP screening method: tandem MS screening + targeted diagnostics from a single sample
- Detectable metabolites: amino acids, acylcarnitines, organic acids, fatty acids, bile acids, acylglycines, carbohydrates (*m/z* 70-600)
- **Biomarker identification** by univariate statistical methods for the improvement of selectivity and sensitivity, better understanding of metabolic pathways
- **Pattern recognition**, differentiation and classification by multivariate statistical methods such as principal component analysis (PCA) and linear discriminant analysis (LDA)
- Phenylketonuria (PKU) and medium-chain acyl-coenzyme A dehydrogenase deficiency (MCADD) was studied in details; 22 other disorders also investigated
- Extended biomarker list for metabolic diseases
- Characterization of metabolic and non-metabolic conditions (e.g. total parenteral nutrition)
- Reduction of false-positive rate of screening →estimated value: **0.05%**

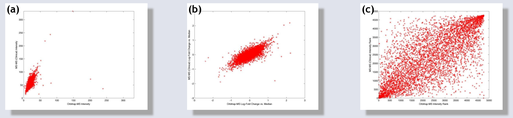


Figure 2. Measurement of phenylalanine by Exactive-MS and tandem MS. Scatter plots showing (a) concentration, (b) log-fold change vs. median, and (c) concentration. Rank indicates that higher linearity between high resolution and MRM measurements is achieved for relative (b and c) rather than absolute (a) quantitation.

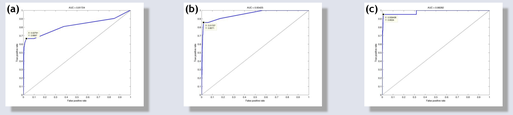


Figure 3. Receiver-Operator Characteristic (ROC) analysis curves for sensitivity and specificity of PKU diagnosis. For (a) top predictive metabolite (phenylalanine) (b) top five metabolites, and (c) all significant metabolites, logistic regression of Exactive-MS concentrations generated a predictor metric of PKU. The sensitivity (true positive rate) and specificity (false positive rate) are analyzed by ROC to show that **increasing the number of metabolites increases the sensitivity** of PKU screening while retaining high specificity.

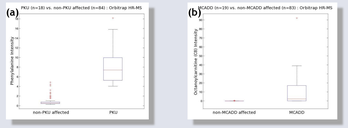


Figure 4. Boxplots of characteristic biomarkers for (a) PKU and (b) MCADD. Left side of the graph: non-affected samples, right side of the graph: true positive samples.

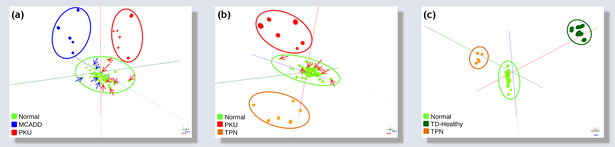


Figure 6. Specified PCA/LDA models for different diseases and conditions of the model set. 61 normal newborn sample, 6 **PKU**, 7 false-positive PKU (↑), 5 **MCADD**, 5 false-positive MCADD (↑), 6 total parenteral nutrition (TPN), 11 healthy, thermally damaged (TD).

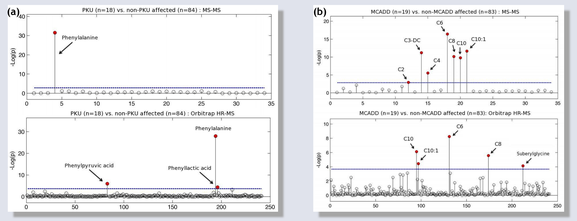


Figure 5. (a) PKU and (b) MCADD biomarker discovery by tandem MS and Exactive. Manhattan plots show the results of an unpaired, two-sided t-test with Sidak multiple hypothesis testing correction for true positive vs. non-affected samples.

URINE ANALYSIS:

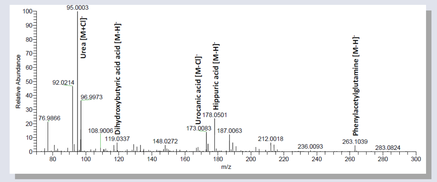
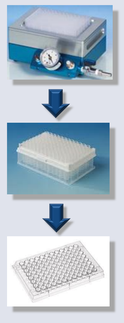


Figure 7. High resolution, negative ion mode spectrum of unmodified urine of a healthy adult volunteer.

- Typical urine metabolites identified
- Sample preparation: dilution or urease treatment
- Quantification by isotope dilution
- Future plan: analysis of different diseases (e.g. diabetes, cystinuria)

ANALYSIS OF BACTERIUM LYSATES:



- Cell suspension was sonicated, washed with organic solvent mixture and filtered
- Total sample preparation time: **5 min for 96 samples!**
- Differentiation of strains and species by small mass range pattern (*m/z* 50-600) and phospholipid mass range pattern (*m/z* 600-100)

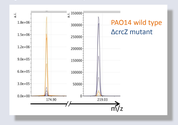


Figure 8. Spectral differences between wild type and mutant.

CONCLUSIONS

Summary:

- ✓ Identified metabolite peaks in different types of samples in negative ion mode: ~400
- ✓ Identified metabolite peaks in different types of samples in positive ion mode: ~250
- ✓ New biomarkers were found for PKU and MCADD in DBS samples, collection of positive samples still in progress
- ✓ Disease and condition differentiation

Outlook:

- Application of the method for other disorders (e.g. galactosemia, lysosomal storage disorders)
- Analysis of more urine and bacteria samples

Cooperation partners:

- Hesse Screening Centre (Giessen, Germany)
- 1st Department of Pediatrics (Budapest, Hungary)
- Mayo Clinic (Rochester, Minnesota, USA)
- National Institute for Health Data and Disease Control (Copenhagen, Denmark)
- The Children's Hospital Sheffield (Sheffield, UK)
- Department of Pediatrics, University of Szeged (Szeged, Hungary)
- Great Ormond Street Hospital for Children (London, UK)

REFERENCES

1. J. Denes, E. Szabo, S. Robinette, I. Szatmari, L. Szonyi, J.G. Kreuder, E.W. Rauterberg, and Z. Takats, Metabonomics of newborn screening dried blood spot samples - a novel approach in the screening and diagnostics of inborn errors of metabolism, *Analytical Chemistry,* **2012**, *84*, 10113-10120.

ACKNOWLEDGEMENTS

- **Exactive** was generously provided by **Thermo Fisher Scientific**
- **TriVersa NanoMate** was generously provided by **Advion**
- Hessisches Ministerium für Wissenschaft und Kunst (LOEWE Schwerpunkt “AmbiProbe”)
- European Research Council under Starting Grant scheme (Contract No: 210356)
- NIHR Biomedical Research Centre