

AN UNTARGETED METABOLOMICS APPROACH FOR DETERMINING BIOMARKERS INVOLVED IN SPONTANEOUS PRE-TERM BIRTH DELIVERY, USING A LABEL-FREE LC-DIA-MS APPROACH

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OVERVIEW

- Application of an ion mobility workflow for untargeted metabolomics using Vion IMS QTof
- Incorporating ion mobility shows increased peak capacity and specificity for metabolite identification.
- Progenesis QI processed data reveals a variety of lipid species and pathways (i.e. sphingomyelin metabolism) involved in sp-PTB.

INTRODUCTION

Pre-term delivery (sp-PTB) is one of the leading causes of perinatal mortality and there is currently no clinically useful screening test available. Birth before 37 weeks gestation is the single biggest cause of neonatal deaths in the world and the second-most common cause of death in children under five years of age. In particular, early sp-PTB (typically before 34 weeks gestation) is particularly associated with high rates of mortality and morbidity. There are many clinical and biochemical risk factors associated with sp-PTB and it is therefore possible that markers are present in the maternal blood long before the onset of preterm labour. Biomarkers FOR Early Birth (BEFORE) project¹ is focussed on utilising metabolomics for the identification of potential biomarkers, which could be used as early pregnancy screening tests for sp-PTB. Here we describe a discovery metabolomics approach to identify potential markers for sp-PTB.

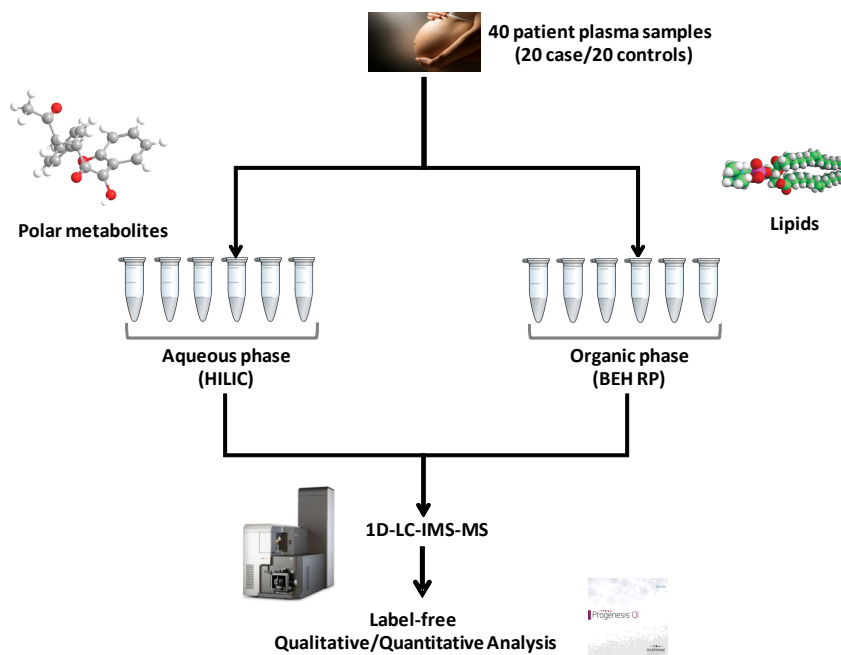


Figure 1. Metabolomic and lipidomic experimental design study from human plasma extracts.

METHODS

Sample preparation

Samples were collected at 15 and 20 weeks of pregnancy as part of the SCOPE (Ireland) study.²

Plasma samples (heparinised) were collected from women (20 weeks) whose pregnancies had reached term gestations (controls, n=20) and women who experienced complications by sp-PTB prior to 34 weeks gestation (case, n=20). Samples were prepared for LC-MS analysis as previously described.³ Briefly, methanol was used for protein precipitation, followed by MTBE extraction for phase separation of lipids and polar metabolites. The lipid layer was aliquoted in fresh vials and left to dry at room temperature overnight prior to being resuspended in IPA:ACN (8:2). QC samples were prepared by pooling equal aliquots of each sample and injected every tenth injection.

LC-MS conditions

Lipids were chromatographically separated using a BEH 1.7 μ m C18 RP 2.1 x 100 mm LC column.

Mobile Phase	Gradient	Flow Rate
A: IPA:acetonitrile (9:1)/ 10 mM ammonium formate	20 min (30-99% B)	0.5 mL/min
B: Acetonitrile:water (6:4)/ 10 mM ammonium formate		

Lipid measurements were conducted using a Vion IMS QToF mass spectrometer operating in positive and negative ESI mode (Figure 2). A data independent acquisition workflow combined with ion mobility (IM-DIA) was used in conjunction with the acquisition schema.

Bioinformatics

The LC-MS lipid data were acquired using UNIFI, followed by processing and database searching with Progenesis QI. Normalized label-free quantification and CCS values were achieved from Progenesis QI with additional statistical analysis conducted using EZInfo. Compound searches utilised a combination of Human Metabolite Database (HMDB) and LipidMaps. The data were also interrogated further using MetaboAnalyst³ and pathway mapped with KEGG.

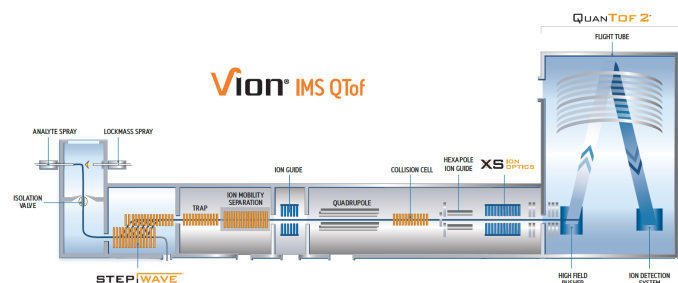


Figure 2. Vion IMS QToF mass spectrometer schematic. CCS measurements were derived using a IM-DIA-MS workflow.

RESULTS

Purified plasma samples were analysed as technical replicates ($n=3$) in a randomised order using Vion IMS Q-ToF. The subsequent data were processed using Progenesis QI with normalisation based on all compounds. The peak picked features were statistically interrogated prior to database searching.

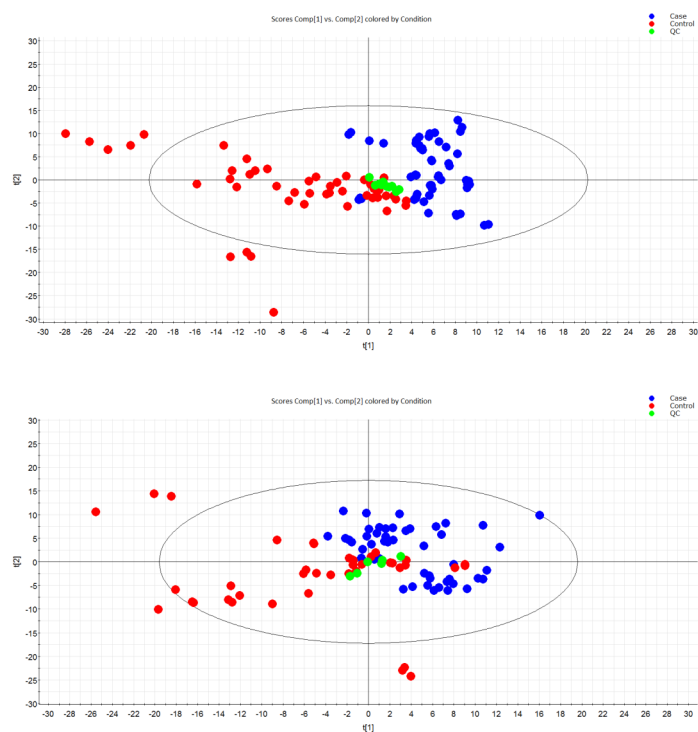


Figure 3. Unsupervised PCA scores analysis. Separation between controls (red) and sp-PTB (blue) cohorts is observed (PC1 vs PC2). Pooled QC samples are centred, highlighting good technical reproducibility.

Unsupervised PCA (based on Pareto scaling) was used to identify significant changes between the two cohorts for positive and negative ion based acquisitions (Figure 3). Separation between controls and sp-PTB is observed. The tight clustering and central positioning of the QC samples illustrates robustness and reproducibility of the analytical method. OPLS-DA allowed for a comparative analysis between the two cohorts prior to compound identification. Database searches were conducted using both HMDB and LipidMaps, with a maximum mass tolerance of 5ppm for both precursor and product ion spectra. Returned identifications were further curated on the basis of %CV <30%, ANOVA (p) <0.05 and fold change >2. Data visualisation tools, such as volcano plots (Figure 4) provide an overview of the data and illustrate identifications matching the filtering criteria.

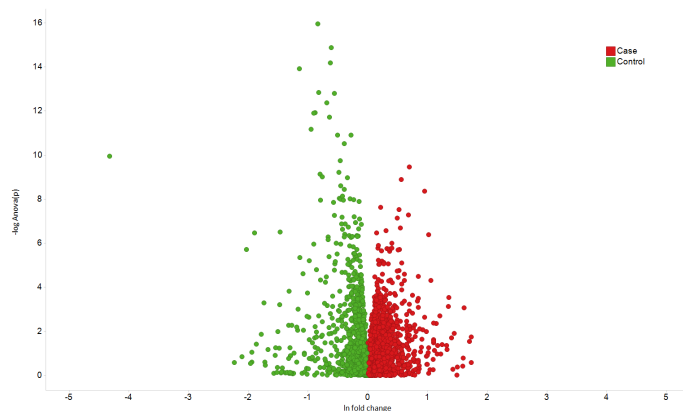


Figure 4. Data visualisation tools for data curation. Volcano plots for example are used to review and identify compounds which meet set threshold criteria based on ANOVA and fold change for the sp-PTB cohort.

Incorporating ion mobility as part of the acquisition schema provides a number of distinct advantages such as enhanced specificity and increased confidence scoring for identifications returned. Figure 5 demonstrates increased peak capacity when ion mobility is implemented. A comparison of MS^E and HDMS^E data which is representative of the entire cohort, results in an additional 71% features when acquired with HDMS^E. On completion of database searching, HDMS^E generated 41% more curated identifications.

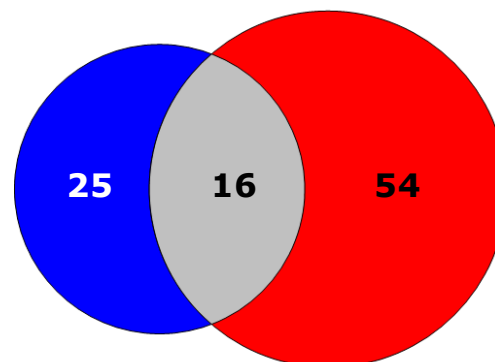
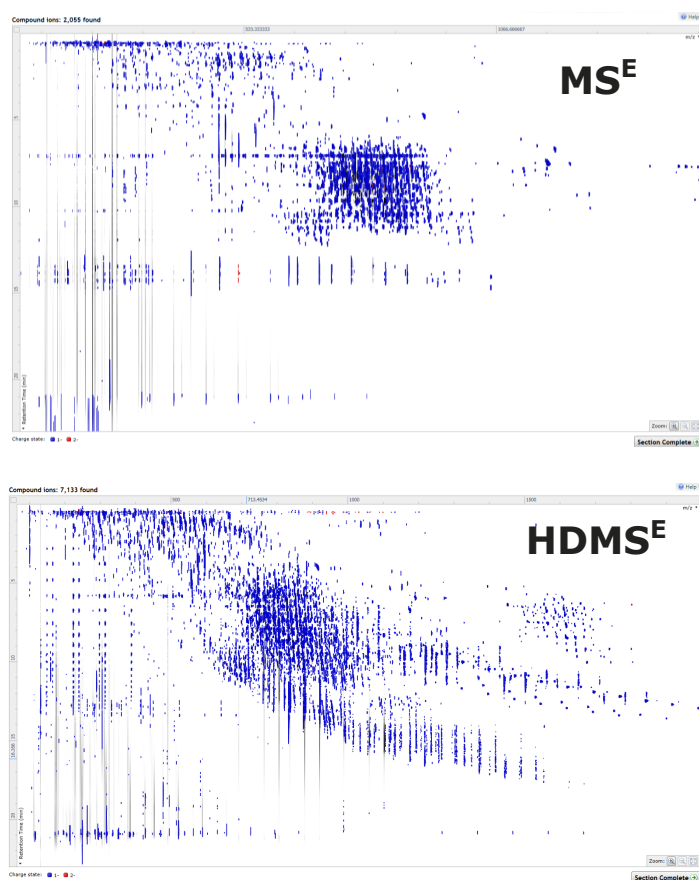


Figure 5. DIA (MS^E) and IM-DIA ($HDMS^E$) negative ion data processed using Progenesis QI. The aggregate maps to the left-hand side represent peak picked features for MS^E (2055 features) and $HDMS^E$ (7133 features). Database searching both datasets results in 41 and 70 curated identifications for MS^E and $HDMS^E$ respectively.

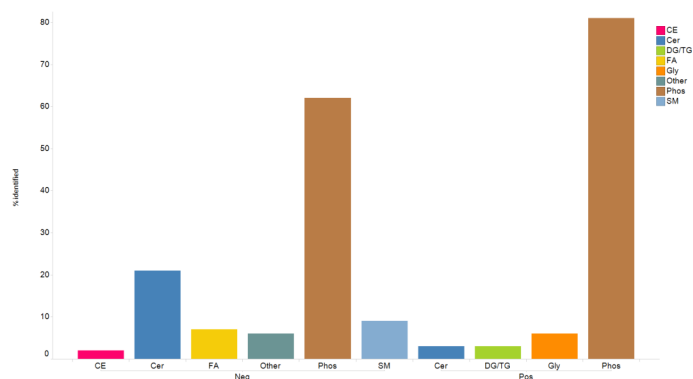


Figure 6. Representative lipid classes identified using $HDMS^E$ (positive and negative mode).

An overview of the lipid classes identified (Figure 6) shows that the major classes are well represented, with a high number of identifications for phospholipids, ceramides and sphingomyelins.

Following curation of the data, a number of lipid species are highlighted as potential markers. Figure 7 provides an example phospholipid marker demonstrating down regulation within the sp-PTB cohort. Trend analysis shows consistency across technical and biological replicates, which are within two standard deviations for both controls and sp-PTB.

Significant lipid identifications were collated and pathway mapped using KEGG, highlighting a number of key pathways and networks. One such pathway of high statistical significance was the sphingomyelin metabolism pathway (Figure 8).

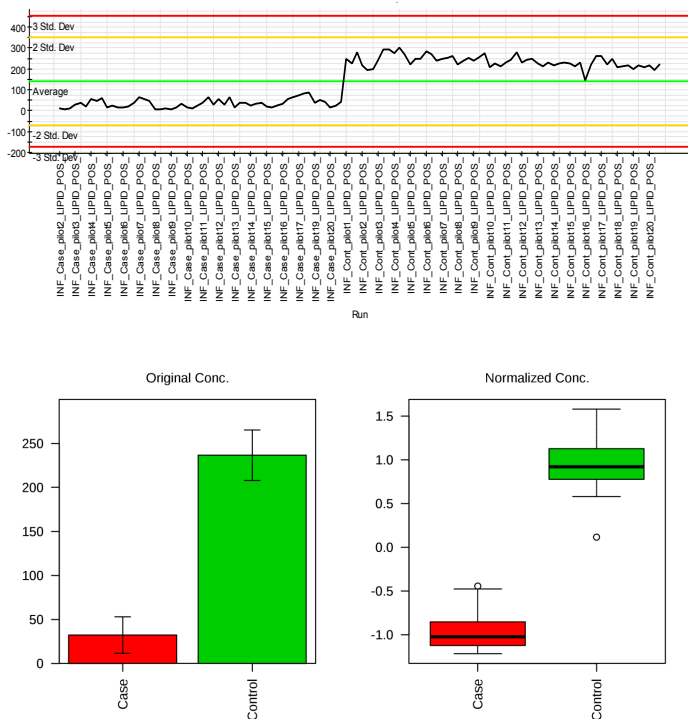


Figure 7. Representative trend profile for a key phospholipid (upper plot), showing significant down regulation in sp-PTB subjects. Box and whisker plots (lower plots) indicate minimal variance and few outliers within each group.

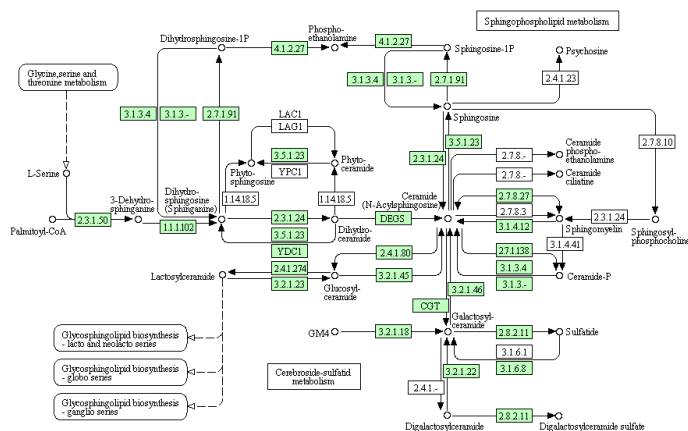


Figure 8. Sphingomyelin metabolism identified as a key pathway for sp-PTB patients (p -value = $7.986 \text{ E-}5$; FDR = 0.006). Identified lipids positively matched to pathway identifiers are highlighted in green.

CONCLUSION

- A discovery metabolomic workflow utilizing Vion IMS QToF for pre-term birth candidates has provided key lipid biomarkers for further analysis.
- A variety of lipid classes have been identified, with phospholipids, sphingomyelins and ceramides as the most significant.
- Comparative MS^E and HDMS^E analyses, shows increased peak capacity when ion mobility is implemented into the acquisition schema. This results in a higher number of confident identifications being returned.
- Key pathways are identified as being implicated in sp-PTB, which include sphingomyelin metabolism as a significant contributor.

References

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3. Matyash *et al.* Lipid extraction by methyl-*tert*-butyl ether for high-throughput lipidomics. *J Lipid Res.* 49 (5): 1137-46.
4. Xia *et al.* MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucl. Acids Res.* (DOI: 10.1093/nar/qkv380).