A LABEL-FREE, MULTI-OMIC STUDY TO QUALITATIVELY AND QUANTITATIVELY CHARACTERIZE THE EFFECTS OF A GLUCOSYLCERAMIDE INHIBITOR ON OBESITY

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INTRODUCTION

Obesity is a growing problem for human health which has reached epidemic proportions affecting over 500 million people worldwide. The condition arises due to abnormal or excessive fat accumulation that can lead to further health implications such as type 2 diabetes, heart and liver disease and potential links to various forms of cancer. Previous studies involving the treatment of mice with glucosylceramide inhibitors such as MZ-21 have shown reduced blood glucose levels and increased insulin sensitivity.[1] In order to gain a greater understanding of the role that such inhibitors may contribute within obese subjects, a multi-omic study involving protein and lipid analysis have been conducted using a label-free LC-HDMS^E (LC-DIA-IM-MS) approach, providing qualitative and quantitative information from a single experiment. The curated datasets were then interrogated using pathway analysis tools, indicating that physiological processes such as hepatic system development, inflammatory response and carbohydrate metabolism are influenced following MZ-21 treatment.

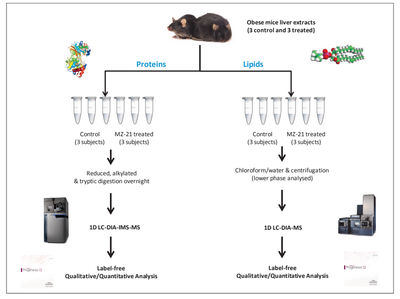


Figure 1. Experimental design study for proteins and lipids extracted from liver tissue.

METHODS

Sample preparation

Proteins and lipids were extracted from liver tissue, which originated from 3 control and 3 treated (MZ-21 inhibitor) obese mice. The protein extracts were prepared with 1% RapiGest SF prior to reduction, alkylation and overnight digestion with trypsin.

Lipids were extracted by homogenizing liver tissue in chloroform-methanol (2:1, v/v) and extracted according to the Bligh and Dyer method.[2] The extracts were centrifuged for phase separation and the lower fraction collected for LC-MS analysis. An overview of the experimental and analytical workflow is provided in Figure 1.

LC-MS conditions

Label-free LC-MS was used for qualitative and quantitative peptide analyses. Experiments were conducted using a 90 min gradient from 5 to 40% acetonitrile (0.1% formic acid) at 300 nL/min using a nanoACQUITY system and a HSS 1.8 µm C18 reversed phase 75 µm x 15 cm nanoscale LC column.

For lipid identification, the LC-MS experiments consisted of a 20 min gradient from 3 to 40% isopropanol:methanol (10mM ammonium formate) at 500 µL/min using a ACQUITY UPLC system. Here, a BEH 1.7 µm C8 reversed phase 2.1 x 10 cm LC column was used.

Proteomic data acquisition utilized data independent analysis (DIA) with a nanoscale LC nanoACQUITY system directly interfaced to a hybrid IMS-oaToF Synapt G2-Si. Lipidomic measurements were conducted using a Xevo G2-S mass spectrometer, operating in DIA. Ion mobility (IM) was used in conjunction with the proteomic acquisition schema, illustrated in Figure 2.

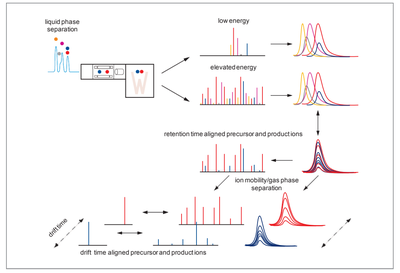


Figure 2. Retention and drift time principle ion mobility enabled data-independent analysis (IM-DIA-MS).

Bioinformatics

The LC-MS peptide data were processed and searched using Progenesis QI for Proteomics, whilst the lipid data was processed and searched with Progenesis QI. In both cases normalized label-free quantification was achieved from the Progenesis software.

Additional statistical analysis was conducted with EZInfo. Pathway and network analysis was also performed with Ingenuity Pathway Analysis (IPA).

RESULTS

Small amounts of the purified liver extracts were analyzed to identify, quantify and investigate the proteomic and lipidomic variance between control and inhibitor treated subjects. PCA was used to identify significant changes between control and inhibitor treated samples, of which an example is shown in Figure 3. Similar clustering patterns are observed for both the protein and lipid data. Over 1200 proteins were identified with 30% differentially expressed. An overview of the experimental metrics can be generated from the Progenesis QI informatics. The QC Metrics for the proteomic data is provided in Figure 4. Proteins exhibiting expression changes with high statistical significance can be readily identified from the volcano plot (Figure 5).

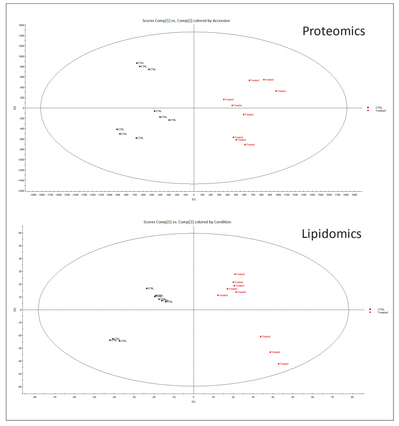


Figure 3. Unsupervised PCA scores plot for proteomic (upper) and lipidomic (lower). Inhibitor treated subjects are highlighted in red whilst controls are black. In both cases the PCA is complimentary with good separation between groups and tight clustering over technical replicates.

Unsupervised hierarchial clustering based on a curated list of protein identifications (Figure 6) highlights grouping at the technical level and secondary grouping at the sample level with regulation probability.

The lipidomic workflow results are summarized in Figure 7. Using a contrasting loadings plot, significant lipid features can be found at the extremes and are shaded in blue. These features can subsequently be database searched with Progenesis QI. Example lipids which are found to contribute most significantly to the variance are provided in the corresponding table.

Figure 4. QC Metrics overview of the proteomic data resulting from Progenesis QI for Proteomics.

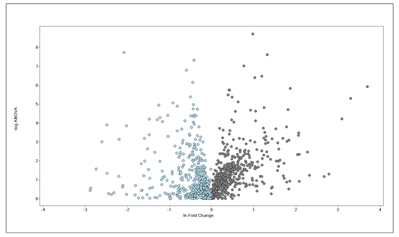


Figure 5. Volcano plot of protein identifications based on ln fold change (x-axis) and –log ANOVA P (y-axis) allows highly probable, differentially expressed proteins to be readily identified for control (grey) and treated (blue) subjects.

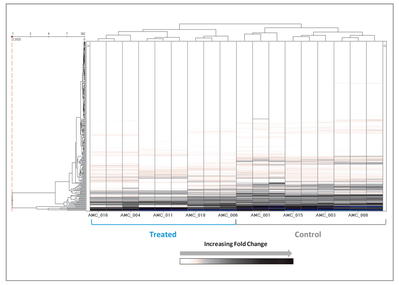
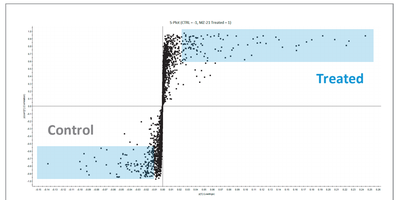


Figure 6. Hierarchical cluster analysis representing regulated proteins with a maximum fold change >2 and ANOVA P-value ≤0.05.



m/z	Identification	ANOVA (P)
568.3397	LPC 22:6	5.21E-06
706.5401	PC 30:0	8.44E-07
759.6359	SM(d18:1/20:0)	2.05E-08
785.6521	SM(d18:1/22:1)	3.75E-06
787.6682	SM(d18:1/22:0)	6.60E-08
811.6658	SM(d18:0/22:0)	3.96E-06
813.6836	SM(d18:1/24:1(15Z))	8.25E-08
836.7703	TG(49:1)	1.84E-08
896.7703	TG(18:1/18:2/18:3)	9.72E-11
912.8012	TG(55:5)	4.14E-07
1590.1432	PC 36:3	4.17E-07

Figure 7. Resulting S-plot from OPLS-DA analysis of control versus treated subjects. Features contributing to the greatest variance are shaded in blue with representative identifications resulting from the shaded areas listed in the accompanying table. Identifications were filtered on the basis of mass accuracy (<3ppm), ANOVA p-value (≤0.00005) and fold change (>2). The main lipid classes identified include lysophosphatidylcholine (LPC), phosphatidylcholine (PC), sphingomyelin (SM) and triglyceride (TG).

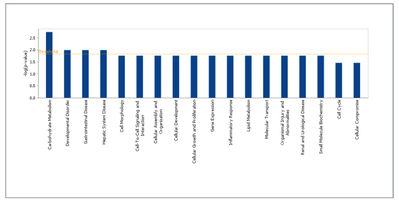


Figure 8. Identified functions and disorders from the pathway analysis based on curated protein and lipid identifications. Statistical significance is provided by means of a Fisher's Exact Test with the threshold set at 1%.

A curated list of proteins and lipids were combined and interrogated for pathway analysis. Pathways were limited to species (mus musculus) and liver tissue. Additional filtering was based on number of components mapped and associated ANOVA P-values. Figure 8 illustrates diseases and biological processes identified, whilst Figure 9 represents the lipid metabolism network.

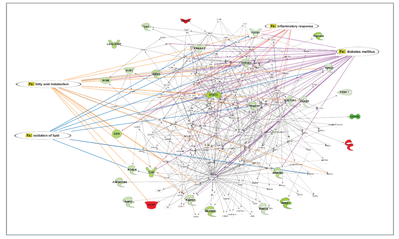


Figure 9. Ingenuity pathway analysis resulting from the combination of curated protein and lipid datasets. These include up (red) and down (green) regulated functions with the intensities depicting fold change variations. Colored connectors highlight associations with diseases such as diabetes (purple), lipid oxidation (blue), fatty acid metabolism (orange) and inflammation (red).

CONCLUSIONS

- **A multi-omic study using DIA, label-free strategies has been applied to the study of obese mice which have been treated with a glucosylceramide inhibitor.**
- **PCA analysis shows both protein and lipid data to be complimentary.**
- **Over 1200 proteins were identified with 30% of the data showing differential expression.**
- **Phosphatidylcholines, sphingomyelins, triglycerides and lysophosphatidylcholines are identified as contributing towards the lipid variance.**
- **Carbohydrate and lipid metabolism were identified as significant pathways. Diseases and functions such as inflammatory responses and diabetes are examples shown as mapping to lipid metabolism.**

References

1. Aerts et al. Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity. Diabetes. 2007, 56, 1341-1349.
2. Bligh et al. A rapid method of total lipid extraction and purification. Can J Biochem., 1959;37;911-917.
3. Li et al. Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. Proteomics. 2009, 9, 1696-719.
4. Richardson et al. A probabilistic framework for peptide and protein quantification from data-dependent and data-independent LC-MS proteomics experiments. OMICS. 2012, 16, 468-82.

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