

## A Label-Free, Multi-Omic Study to Qualitatively and Quantitatively Characterize the Effects of a Glucosylceramide Inhibitor on Obesity

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### Abstract

A label-free, multi-omics approach provided qualitative and quantitative information in a single experiment to help characterize and investigate the effects of a glucosylceramide inhibitor for treatment of obesity. Progenesis QI and Progenesis QI for Proteomics Software enabled a seamless workflow, from LC-MS data processing and database searching to pathway interrogation, implicating carbohydrate metabolism, and molecular transport networks in the compound's mechanism of action.

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### Introduction

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An increasing problem for human health, obesity is epidemic worldwide, affecting more than 500 million people. Obesity arises because of abnormal or excessive fat accumulation, a condition associated with a number of diseases, such as type 2 diabetes, heart and liver disease, and various cancers. Previous studies involving the treatment of mice with glucosylceramide inhibitors such as MZ-21 show reduced blood glucose levels and increased insulin sensitivity.<sup>1</sup> To better understand the biochemical mechanism of action of such inhibitors in obese subjects, a multi-omic study involving protein and lipid analysis was conducted. Adopting a label-free, LC-HDMS<sup>E</sup> (LC-DIA-IM-MS) approach, the study provided qualitative and quantitative information from a single experiment. The curated data sets, interrogated using pathway-analysis tools, indicated that physiological processes such as hepatic-system development, inflammatory response, and carbohydrate metabolism are influenced following MZ-21 treatment.

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## Experimental

### LC conditions (Proteomics)

LC system:	nanoACQUITY UPLC
Trap column:	nanoACQUITY UPLC Symmetry C <sub>18</sub> , 5 µm, 180 µm x 20 mm (p/n 186007238)
Column:	ACQUITY UPLC M-Class HSS T3, 1.8 µm, 75 µm x 150 mm (p/n 186007473)
Column temperature:	35 °C
Flow rate:	300 nL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	Acetonitrile

Gradient: 3% to 40% B in 90 min

Injection volume: 1  $\mu$ L

## LC conditions (Lipidomics)

LC system: ACQUITY UPLC

Column: ACQUITY UPLC BEH C<sub>8</sub>, 1.7  $\mu$ m, 2.1 mm x 100 mm  
(p/n 186002878)

Column temperature: 65 °C

Flow rate: 500  $\mu$ L/min

Mobile phase A: 10 mM ammonium formate Acetonitrile-water (6:4)

Mobile phase B: 10 mM ammonium formate Isopropanol-  
acetonitrile (9:1)

Gradient: 30% to 99% B in 20 min

Injection volume: 3  $\mu$ L

## MS conditions (Proteomics)

MS system: SYNAPT G2-Si

Ionization mode: ESI (+) at 3.2 kV

Cone voltage: 30 V

Acquisition mode:	HDMS <sup>E</sup> , 50 <i>m/z</i> to 2000 <i>m/z</i> , low-energy and elevated-energy functions
Acquisition rate:	0.5 s, low-energy and elevated-energy functions
Collision energy:	Low-energy function: 5 eV; elevated-energy-function: from 19 eV to 45 eV
Resolution:	25,000 FWHM
IMS T-wave velocity:	700 m/s
IMS T-Wave pulse height:	40 V

## MS conditions (Lipidomics)

MS system:	Xevo G2-S
Ionization mode:	ESI (+) at 2.2 kV; ESI (-) at 1.7 kV
Cone voltage:	30 V
Acquisition mode:	MS <sup>E</sup> , 50 <i>m/z</i> to 2000 <i>m/z</i> , low-energy and elevated-energy functions
Acquisition rate:	0.1 s, low-energy and elevated-energy functions
Collision energy:	Low-energy function: 5 eV; elevated-energy function: from 20 eV to 45 eV
Resolution:	30,000 FWHM

## Data management

Progenesis QI

Progenesis QI for Proteomics

EZInfo (Umetrics, Umea, Sweden)

Spotfire (TIBCO Spotfire, Boston, MA)

Ingenuity Pathway Analysis (Qiagen, Redwood City, CA)

## Sample preparation

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

Lipids were extracted from pre-weighed liver tissue and homogenized in chloroform/methanol (2:1, v/v), in accordance with the Bligh and Dyer method.<sup>2</sup> Centrifugation, for 15 min at 4 °C, yielded phase separation, with the lipid-containing (lower) phase isolated for LC-MS analysis.

## Bioinformatics

The LC-MS peptide and lipid data were processed and then searched using Progenesis QI. Progenesis provided normalized, label-free quantification along with peptide and compound identifications. Additional multivariate analysis of the data was performed using EZInfo statistical analysis software.

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## Results and Discussion

Tissue extracts from the livers of obese mice were analyzed to identify, quantify, and determine protein and lipid variances between the control mice and those treated with MZ-21 inhibitor (Figure 1). Unsupervised, principal-component analysis (PCA) in both data sets clearly shows separation between the control and treated subjects (Figure 2).

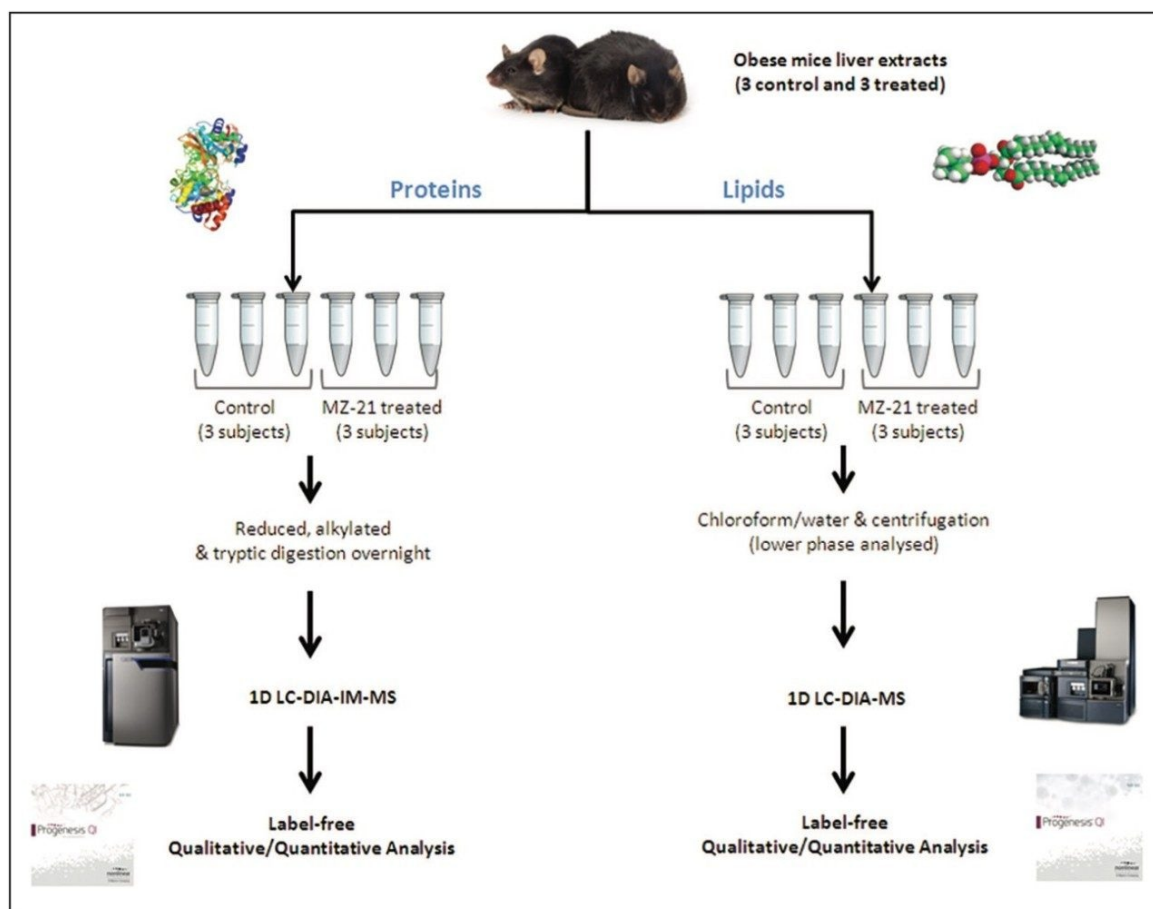
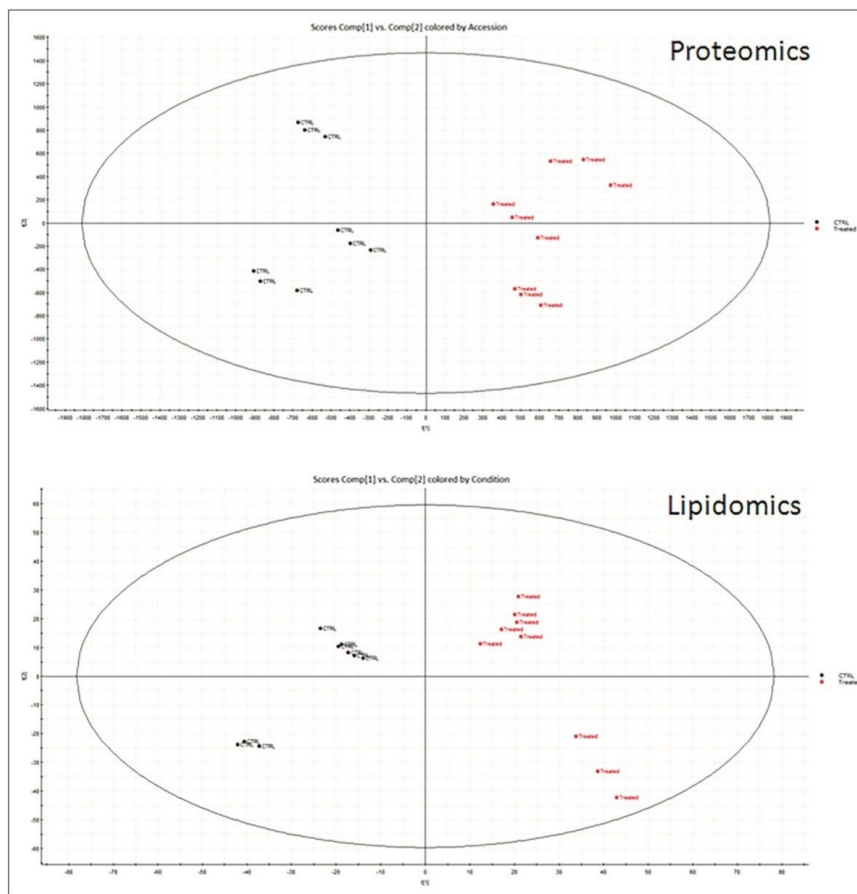
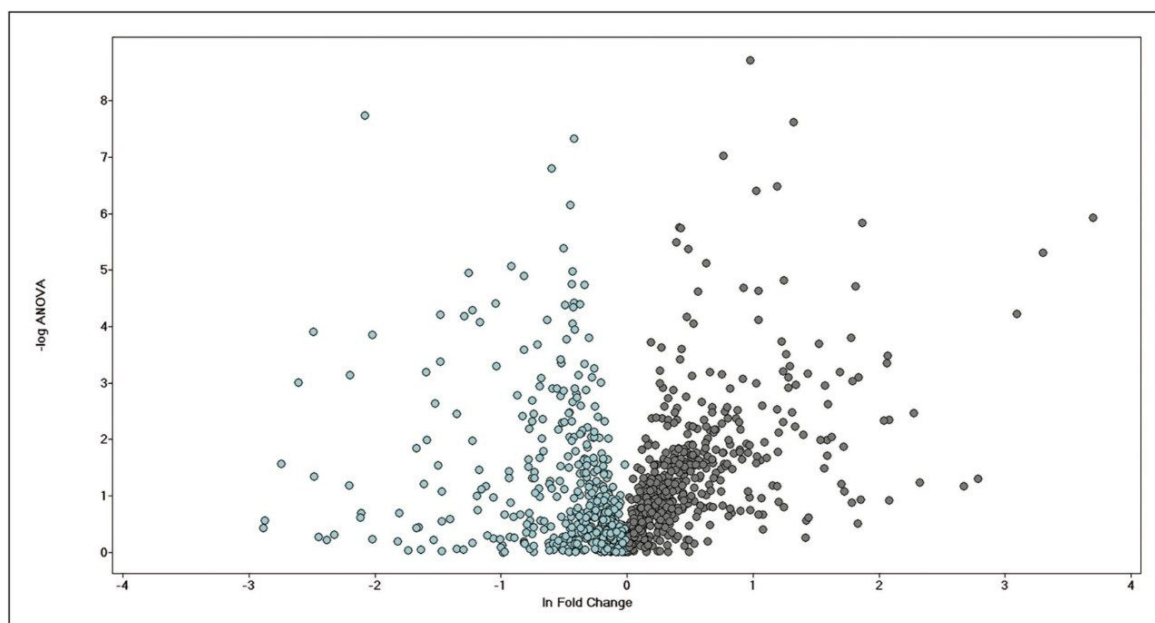


Figure 1. Experimental design and multi-omic analytical and informatic workflows.



*Figure 2. Scores plots resulting from unsupervised principal component analysis for proteomic (upper) and lipidomic (lower) data sets.*

More than 1200 proteins were identified, based on a minimum of 1 peptide per protein and a false discovery rate of 4% (protein level). A volcano plot representing the entire data set readily identifies individual proteins, showing statistically significant ANOVA (p) value changes, indicating protein expression, between the control and treated subjects (Figure 3). Additional filtering of the data ensured that only proteins with a maximum fold change  $>2$  and ANOVA (p) value  $\leq 0.05$  were included for further analysis. Figure 4 represents hierarchal clustering of the filtered proteins, with primary grouping at the technical level and secondary grouping at the sample-group level regulation probability.



*Figure 3. A representative volcano plot of the entire data set signifying statistically relevant proteins, which are expressed between control (grey) and treated (blue) subjects.*



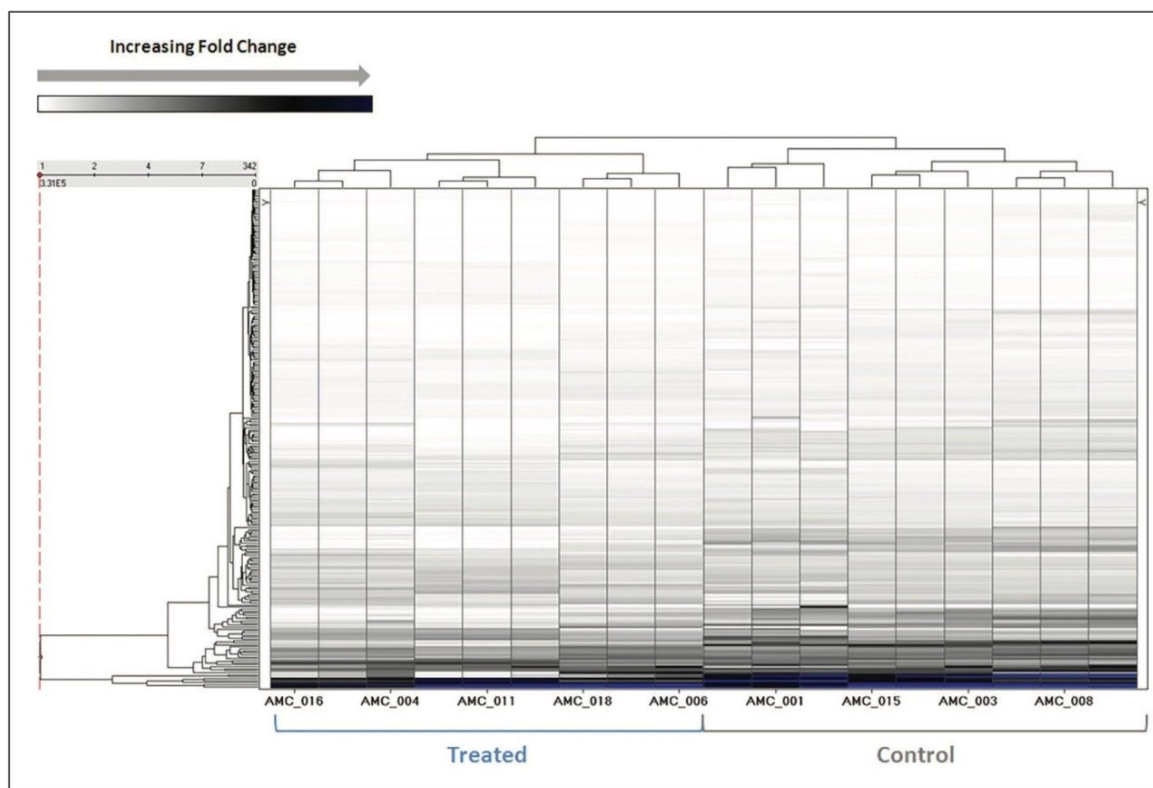


Figure 4. Unsupervised hierarchical clustering of regulated proteins.

The lipidomic workflow results are summarized in Figure 5. An S-plot constructed from the unsupervised PCA scores displays the metabolites' contribution to the model (x axis) and the reliability of the measurement (y axis). Thus the plot highlights significant features subject to database searches for tentative lipid identifications. Example identifications providing the most significant variance are shown. They include phosphatidylcholines (PC), sphingomyelins (SM), triglycerides (TG), and lysophosphatidylcholines (LPC). Stringency to the data was achieved by ensuring that only identifications with mass accuracy ( $<3$  ppm), ANOVA (p) value ( $\leq 5E-5$ ), and fold change ( $>2$ ) were retained for further analysis.

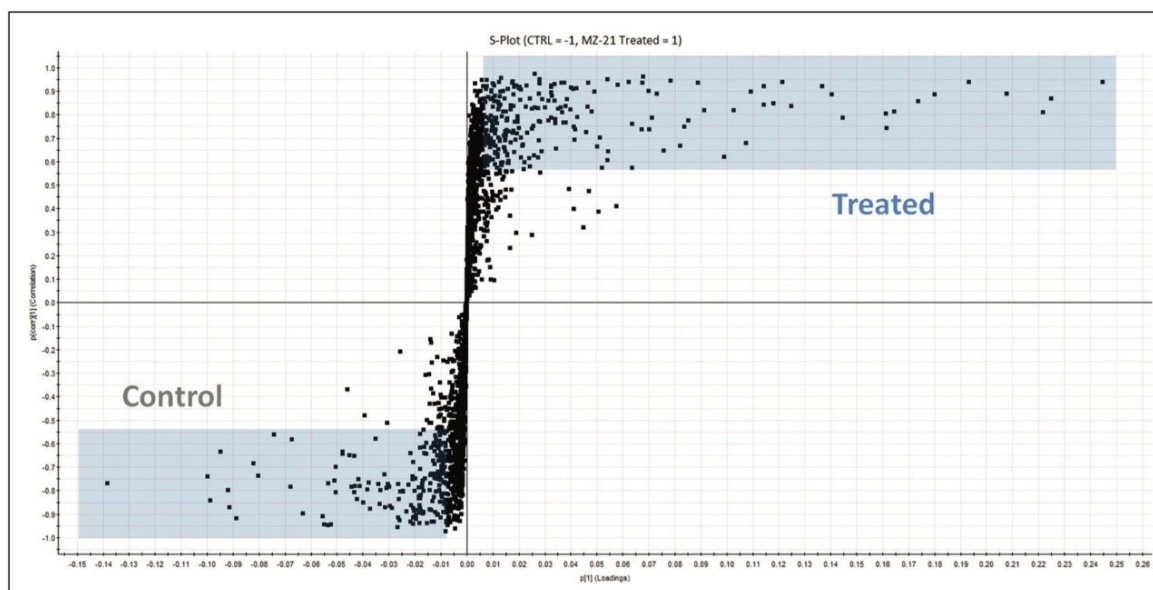


Figure 5. Lipid loadings plot (S-plot) from OPLS-DA analysis of control versus treated subjects. Features contributing to the greatest variance are shaded in blue, with representative identifications provided.

<i>m/z</i>	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

Statistically significant proteins and lipids exhibiting regulation-probability values, identified in both sample groups, were interrogated by means of Ingenuity Pathway Analysis (IPA) software. Various pathways were identified as significant contributors to a range of downstream effects, which include disease and biological

processes such as glycogenolysis and inflammatory and gastrointestinal disorders (Figure 6). A core analysis involving both proteomic and lipidomic data, revealed networks involved with lipid metabolism and molecular transport. Diseases commonly associated with obesity, such as diabetes and inflammation, are significantly identified, and they show a number of key components down-regulated following treatment with glucosylceramide inhibitors (Figure 7).

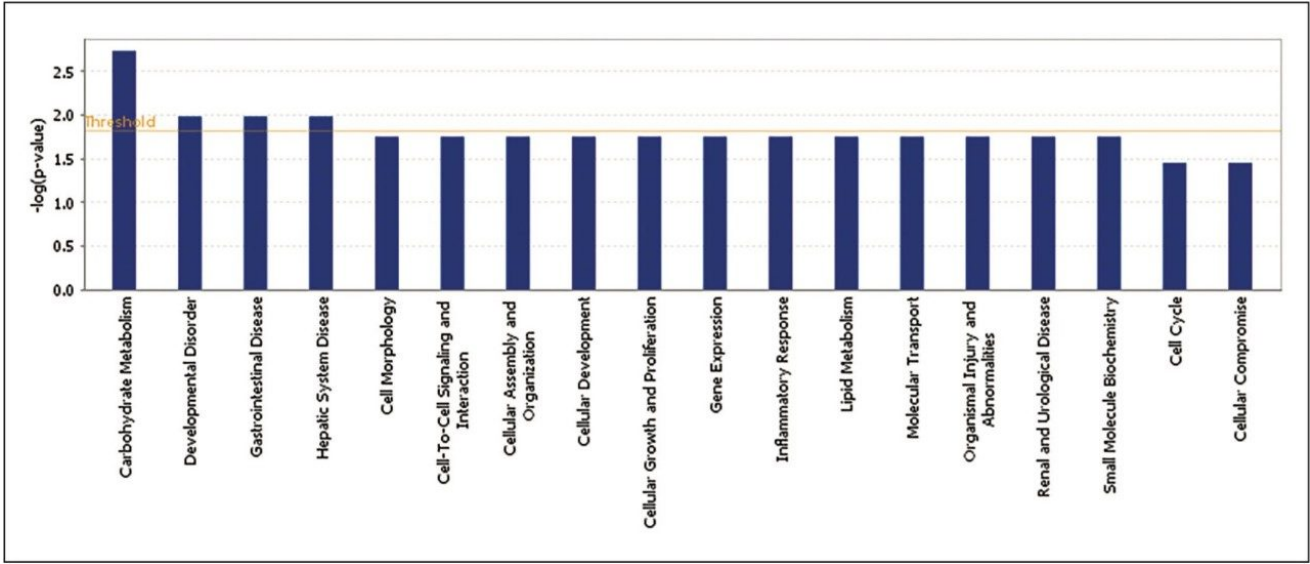


Figure 6. Downstream effects analysis resulting from combined proteomic and lipidomic data sets. Utilizing IPA software, the relevance of each disease or process is measured for its significance using Fisher's Exact Test, with a threshold ( $p$ ) value of 0.01. A z-score algorithm is applied, to minimize the inclusion of any random data.

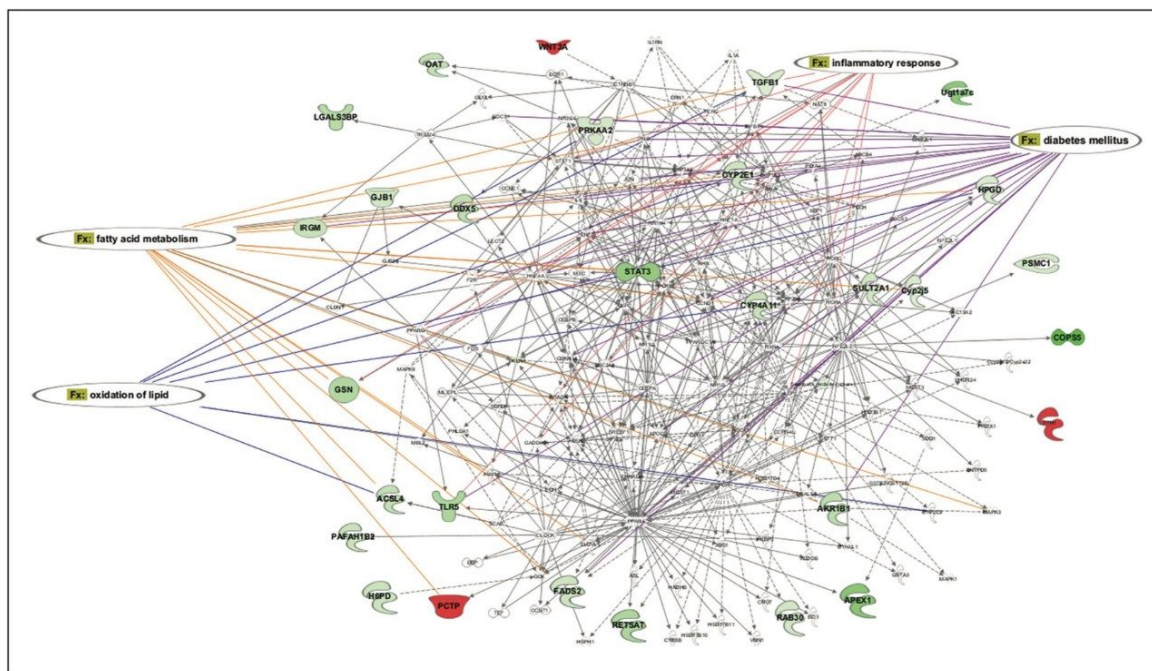


Figure 7. Lipid metabolism and molecular transport network. Groups exhibiting up-regulation are represented in red and down-regulated components in green. The association of certain diseases and functions are highlighted with specific connectors. These connectors include diabetes (purple), lipid oxidation (blue), fatty acid metabolism (orange), and inflammation (red).

## Conclusion

- A multi-omic study using label-free strategies has been applied to the study of obese mice treated with the inhibitor MZ-21.
- Proteomic analysis utilizing LC-HDMS<sup>E</sup> (LC-DIA-IM-MS) identified more than 1200 proteins, of which 30% demonstrate a minimum fold change >2 and ANOVA (p) value ≤0.05.
- Lipid extracts analyzed with LC-MS<sup>E</sup> (LC-DIA-MS) identified phosphatidylcholine, sphingomyelin, triglyceride, and lysophosphatidylcholine classes of lipids.
- The Progenesis Q1/Q1 for proteomics workflows provide complimentary information for further interrogation

with pathway analysis tools such as IPA.

- Statistically curated lipid and protein identifications were subjected to pathway analysis. Lipid metabolism, molecular transport, and networks related to cardiac dysfunction were highlighted as significant.

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## References

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