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Nota de aplicación

A Multi-omic Approach for the Study of Heart Regeneration Using Zebrafish as a Model Organism

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Abstract

This study uses a multi-omics approach to study zebrafish plasma to provide a greater understanding of the mechanisms involved in heart tissue regeneration following the operation.

Benefits

- Label-free multi-omics approach for qualitative and quantitative information in a single experiment
- Data independent acquisition (DIA) technique combined with ion mobility provides increased peak capacity with high sensitivity for limited volumes of plasma.
- Seamless workflow from LC-MS data processing and database searching to pathway interrogation with Progenesis QI and Progenesis QI for Proteomics

Introduction

Zebrafish (*Danio rerio*) have long been used as model systems for research involving biomedical and preclinical studies. One particular avenue of research has focused on using the zebrafish model for regenerative medicine investigations.[1] Previous studies involving the excision of heart tissue from zebrafish have shown tissue to regenerate in only a few days following amputation. Detailed analysis using genomic and proteomic approaches have identified a number of potential candidates that may be implicated in the regeneration process. This study uses a multi-omics approach to study zebrafish plasma, rather than the excised tissue, to provide a greater understanding of the mechanisms involved in heart tissue regeneration following the operation. Lipidomic and proteomic data have been collected using a label-free LC-HDMS^E (LC-DIA-IM-MS) approach, providing qualitative and quantitative information within a single acquisition. This method of data acquisition provides high sensitivity compared with traditional data dependent techniques and is therefore of particular use for situations were sample availability is limited. Interrogation of the data using a combination of pathway tools have shown high density lipid (HDL) transport, platelet activation, signaling, and aggregation pathways to be implicated following post amputation of heart tissue.

Experimental

Samples

Two groups of male zebrafish were used for the study, consisting of sham operated (anaesthetized with no heart tissue excised) and amputation operated (anaesthetized with heart tissue excised). Subsequently, plasma samples (20 µL) were taken three days after anaesthetization/operation. The method by which plasma was collected has been detailed previously.[2]

Preparation for LC-MS

For proteomic analyses, 7 µL aliquots of plasma per fish were prepared with 1% *Rapi*Gest SF Surfactant prior to reduction, alkylation, and overnight digestion with trypsin.

The remaining plasma was prepared for lipidomic analysis by mixing 1:3 v/v with acetonitrile:isopropanol (50:50)

and stored at -20°C for 20 min. Protein precipitation was completed by centrifuging for 10 min. The supernatant was collected and diluted 1:1 with water in preparation for LC-MS analysis.

LC conditions (proteomics)

LC system:	ACQUITY UPLC M-Class
Column(s):	5 µm Symmetry C_{18} 180 µm x 20 mm 2G trap 1.8 µm HSS T3 C_{18} 75 µm x 150 mm NanoEase analytical
Column temp.:	35 °C
Flow rate:	300 nL/min
Mobile phase:	Water (0.1% formic acid) (A) and acetonitrile (0.1% formic acid) (B)
Gradient:	5% to 40% B in 90 min
Injection volume:	1 µL

LC conditions (lipidomics)3

LC system:	ACQUITY UPLC I-Class
Column:	1.7 µm CSH C_{18} 2.1 mm x 100 mm reversed phase analytical
Column temp.:	55 °C
Flow rate:	400 µL/min

Mobile phase:	10 mM ammonium formate with 0.1% formic acid/acetonitrile:water (60:40) (A) and 10 mM ammonium formate with 0.1% formic acid/isopropanol:acetonitrile (90:10) (B)
Gradient:	40% to 99% B in 18 min
Injection volume:	1 µL

MS conditions (proteomics)

MS system:	SYNAPT G2-S*i*
Ionization mode:	ESI (+) at 3.2 kV
Cone voltage:	30 V
Acquisition mode:	HDMS^E 50 *m/z* to 2000 *m/z* both functions (low and elevated energy)
Acquisition rate:	Low and elevated energy functions at 0.5s
Collision energy:	5 eV (low energy function) and from 19 eV to 45 eV (elevated energy function)
Resolution:	25,000 FWHM
IMS T-wave velocity:	700 m/s
IMS T-Wave pulse height:	40 V

MS conditions (lipidomics)

MS system:	SYNAPT G2-S
Ionization mode:	ESI (+) at 2.0 kV; ESI (-) at 1.0 kV
Cone voltage:	30 V
Acquisition mode:	MS^E 100 *m/z* to 2000 *m/z* both functions (low and elevated energy)
Acquisition rate:	Low and elevated energy functions at 0.2 s
Collision energy:	4 eV (low energy function) and from 15 eV to 40 eV (elevated energy function)
Resolution:	10,000 FWHM

Data management

Progenesis QI

Progenesis QI for Proteomics

EZInfo

Spotfire

Reactome

Bioinformatics

The LC-MS peptide and lipid data were processed and searched using Progenesis QI and Progenesis QI for Proteomics. Progenesis enabled normalized label-free quantification to be achieved along with peptide/compound identifications. Additional multi-variate statistical analysis of the data was performed with EZInfo. Curated datasets were submitted for pathway analysis using Reactome[4,5] and PANTHER Classification System.[6]

Results and Discussion

Statistical analysis of the LC-MS data by means of unsupervised principle component analysis (PCA) for the protein and lipid extracts show clear separation (PC1 and PC2 components) between sham operated and amputated groups (Figure 1). A variety of column chemistries were employed to ensure extensive coverage of the lipidome profile and subsequent processing, using Progenesis QI, resulted in excess of 30,000 features being peak picked. An S-plot constructed from the unsupervised PCA scores with additional filtering of the data ensured only features with a maximum fold change >2, ANOVA (p) ≤0.05 and variable importance in the projection (VIP) values >1 were included for further analysis and database searching using a combination of the Human Metabolite Database (HMDB) and LipidMaps. Example tentative identifications curated against these criteria are shown in Figure 2.

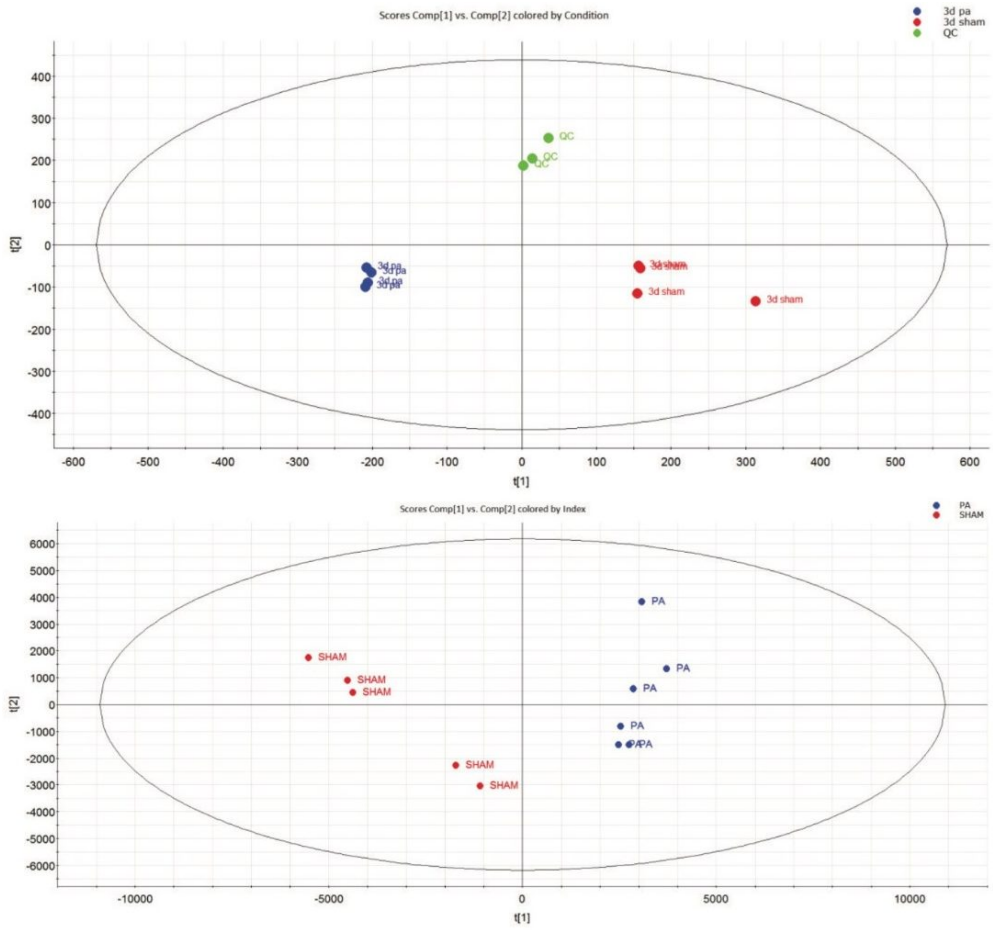

Figure 1. Scores plots resulting from unsupervised principal component analysis for proteomic, lipidomic, and metabonomic datasets.

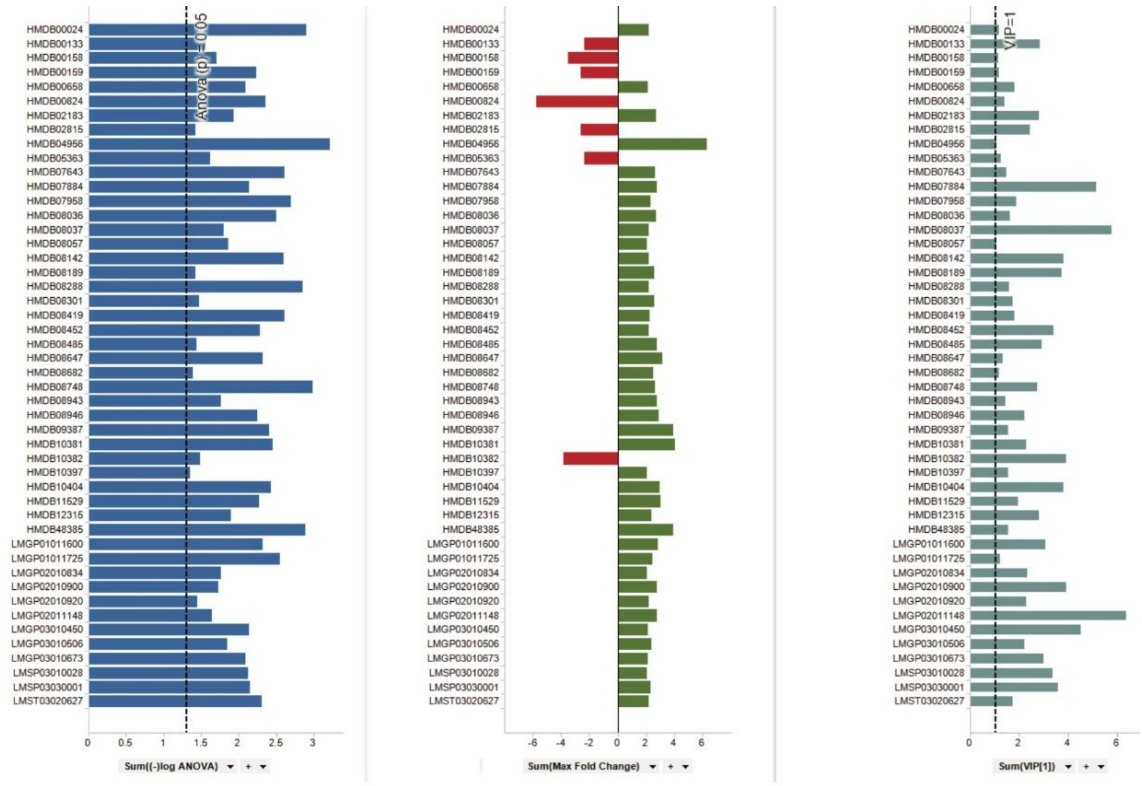

Figure 2. Example metabolites and lipids filtered using ANOVA (p) values ≤0.05 (left), fold change >2 (center), and VIP scores >1 (right). Compounds down regulated are highlighted in red, while those up regulated are shown in green.

The lipid classes identified in the plasma sample extracts which showed differential expression included phosphatidylcholines (PC), triglycerides (TG), lysophosphotidylcholines (LPC), ceramides, diglycerides (DG), phosphatidylserines (PS), and phosphatidylethanolamines (PE). Zebrafish heart tissue is known to fully regenerate within a monthly timeframe. Amputated subjects showed varying abundance plasma profiles for a number of identified lipids over the course of tissue regeneration when compared with their sham counterparts. Figure 3 provides a representative lipid example monitored over a 21 day period.

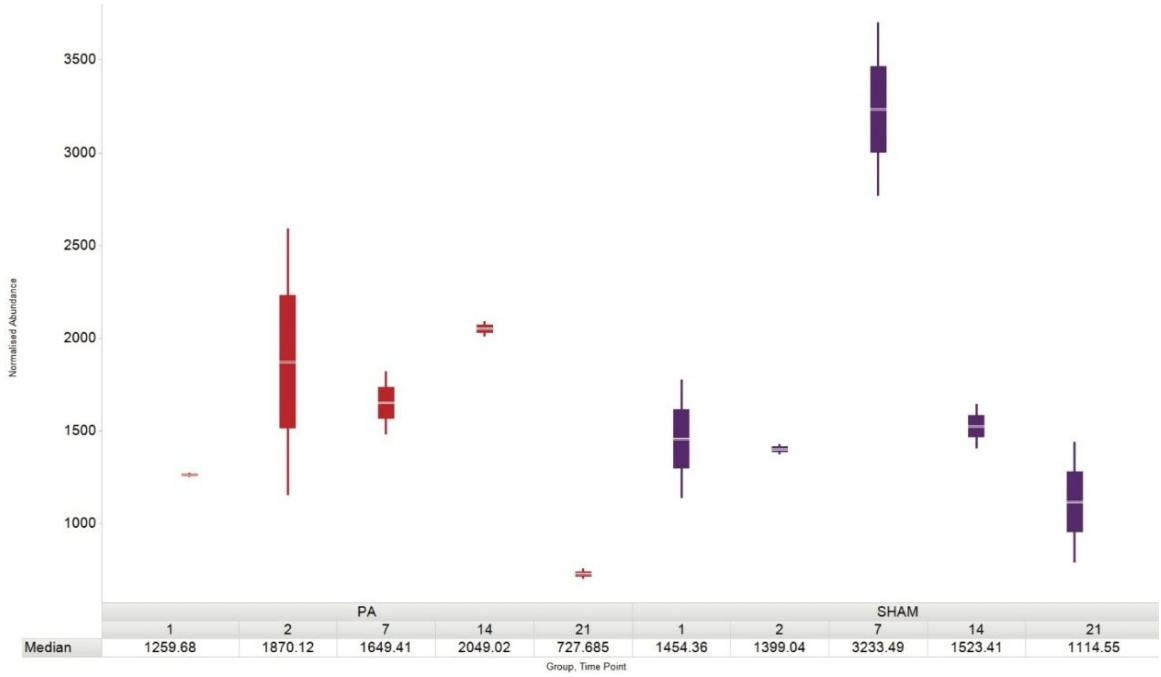

Figure 3. Lipid abundance profiles representing PC (18:2/20:0) over 21 days for PA operated (red) and SHAM operated (SHAM) individuals.

Over 440 proteins were identified and quantified based on a minimum of two peptides per protein and 1% false discovery rate (protein level). A volcano plot corresponding to the protein dataset shows individual identifications which represent changes in expression with statistical significance for amputated operated Zebrafish (Figure 4). Further filtering (ANOVA (p) ≤0.05; fold change >2) resulted in 18% of protein identifications showing significant expression.

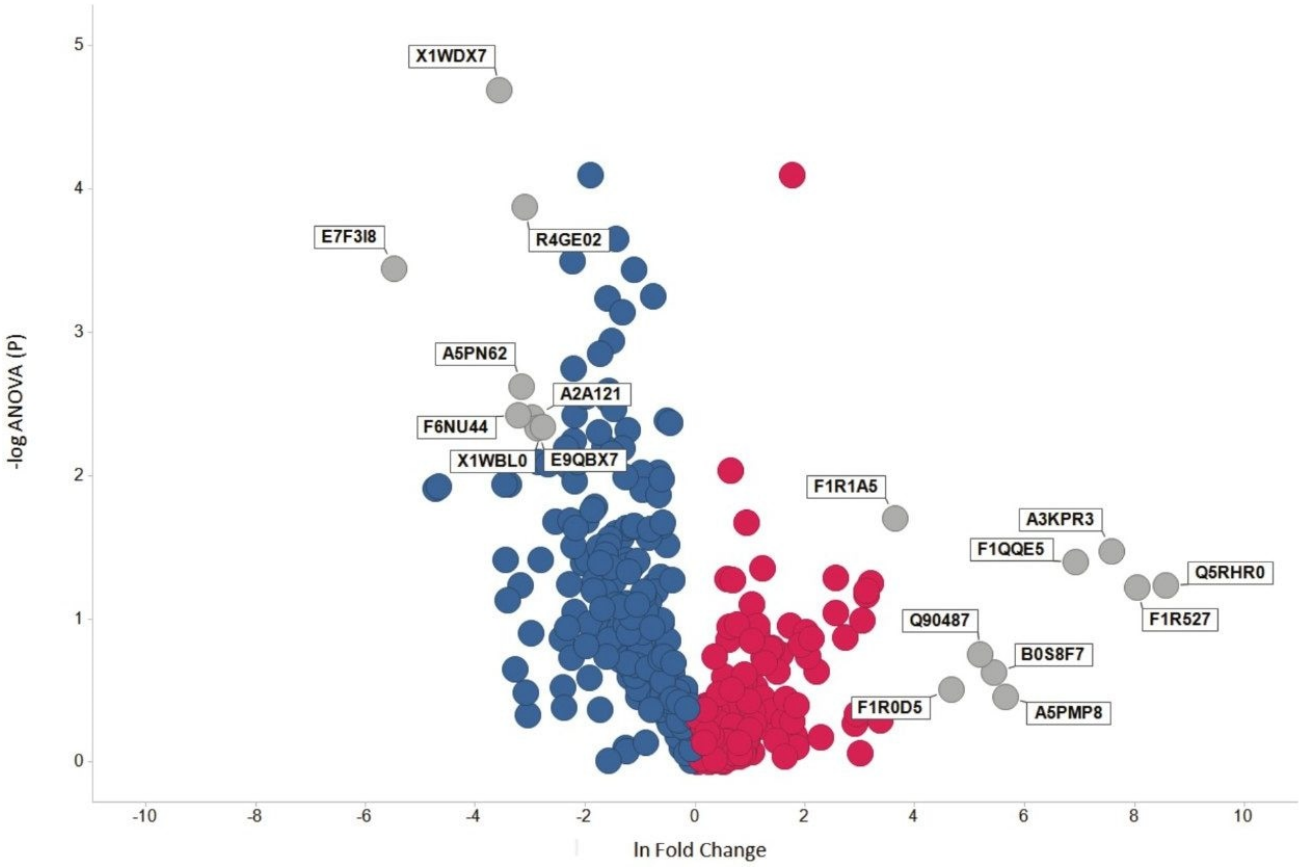

Figure 4. Volcano plot representing regulated proteins for three day post-amputated Zebrafish. Over and under expressed proteins are shown in blue and pink respectively. Protein accessions with high fold change and statistically relevant ANOVA (p) are indicated in grey.

Statistically significant proteins and lipids exhibiting regulation probability values, which were identified in both sham and operated groups were interrogated using a combination of the PANTHER Classification System and Reactome. A variety of biological functions were matched to the data, including metabolic, cellular, regulation, and development processes. These functions were further supplemented with highly correlating pathways such as HDL transport/lipoprotein metabolism, platelet activation, and signaling. Figure 5 shows the identified protein and lipid entries which map to the HDL mediated lipid transport pathway. ATP binding cassette transporter A1 (ABCA1) has been shown to be a key component in providing cardioprotective, anti-inflammatory signaling through interaction with apolipoprotein A (apoA-I). Cascade effects are then initiated allowing cholesterol and phospholipids to be transported to apoA-I thereby generating precursors for HDL particles.[7]

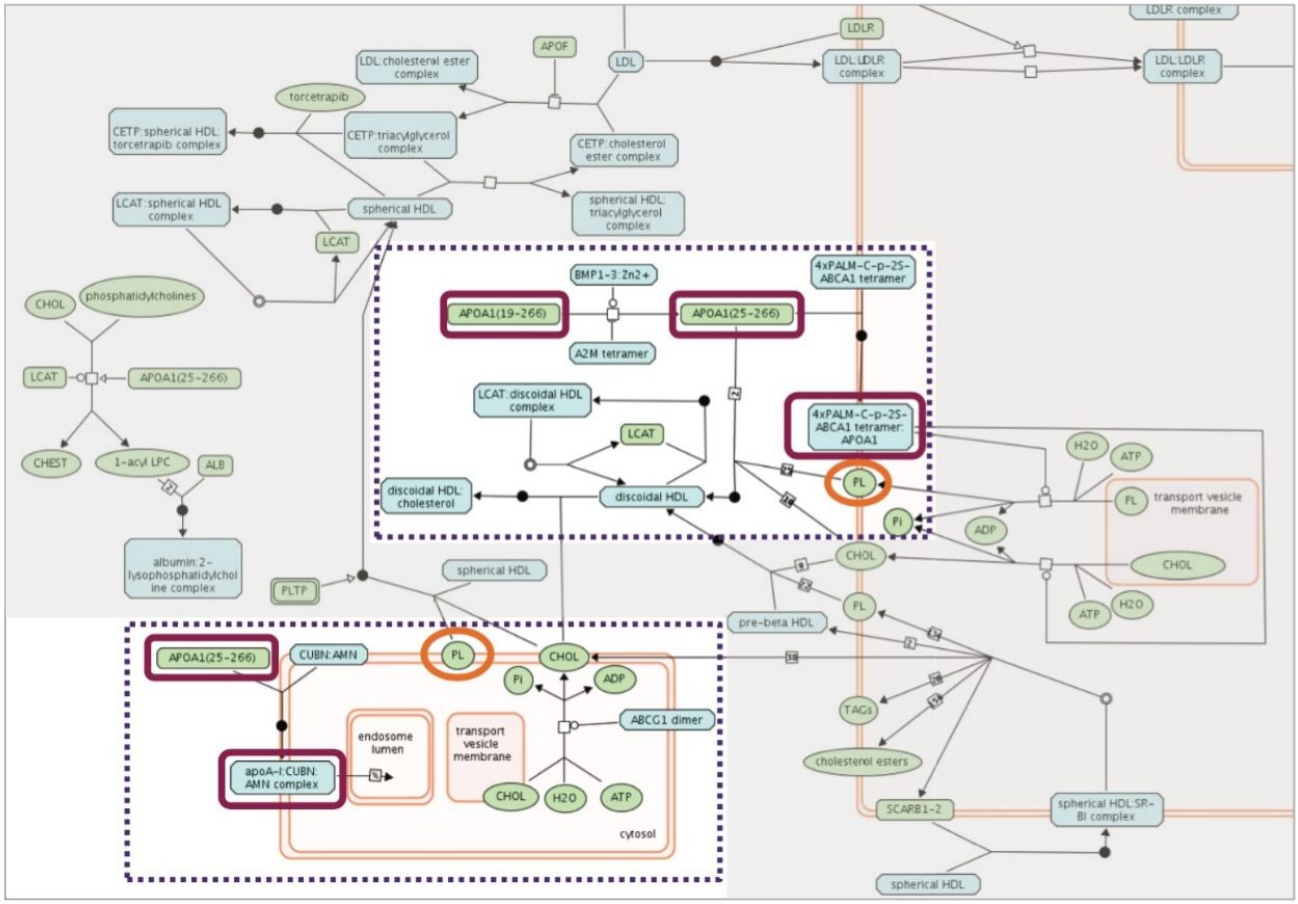

Figure 5. Lipoprotein metabolism pathway example. Magnification of the HDL mediated lipid transportation with specific regions focused around the cytosol and extracellular regions. Proteins identified as over expressed are highlighted (red boxes) while statistically significant phospholipids exhibiting up regulation are also mapped (orange circles).

Conclusion

- A multi-omic study using label-free strategies has been applied to the study of zebrafish to understand the biological processes involved in heart tissue regeneration post amputation.
- Data acquisition using HDMS^E demonstrates utility for experiments which may be sample limited and

therefore require high sensitivity. Implementing ion mobility into the workflow also highlights increased specificity and peak capacity.

- Proteomic analysis utilizing LC-HDMS^E (LC-DIA-IM-MS) identified over 440 proteins, with 18% of identifications fulfilling an FDR threshold of 1% plus having a minimum fold change >2 and ANOVA (p) value ≤0.05.
- Metabonomics analyses revealed a variety of lipid classes implicated in the process of heart tissue regeneration. LysoPC and DG examples clearly show changes in expression following surgery with stable levels being achieved by day 21.
- Statistically curated lipid and protein identifications resulting from Progenesis QI/QI for Proteomics searches were subjected to pathway analysis. Lipid metabolism, platelet activation, signalling, and aggregation were highlighted as significant.

References

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