

Metabolomics analysis

Zhijun Cao

An integrated analysis of proteomics, peptidomics, metabolomics, and inflammation markers for assessment of pre-analytical variability of human plasma

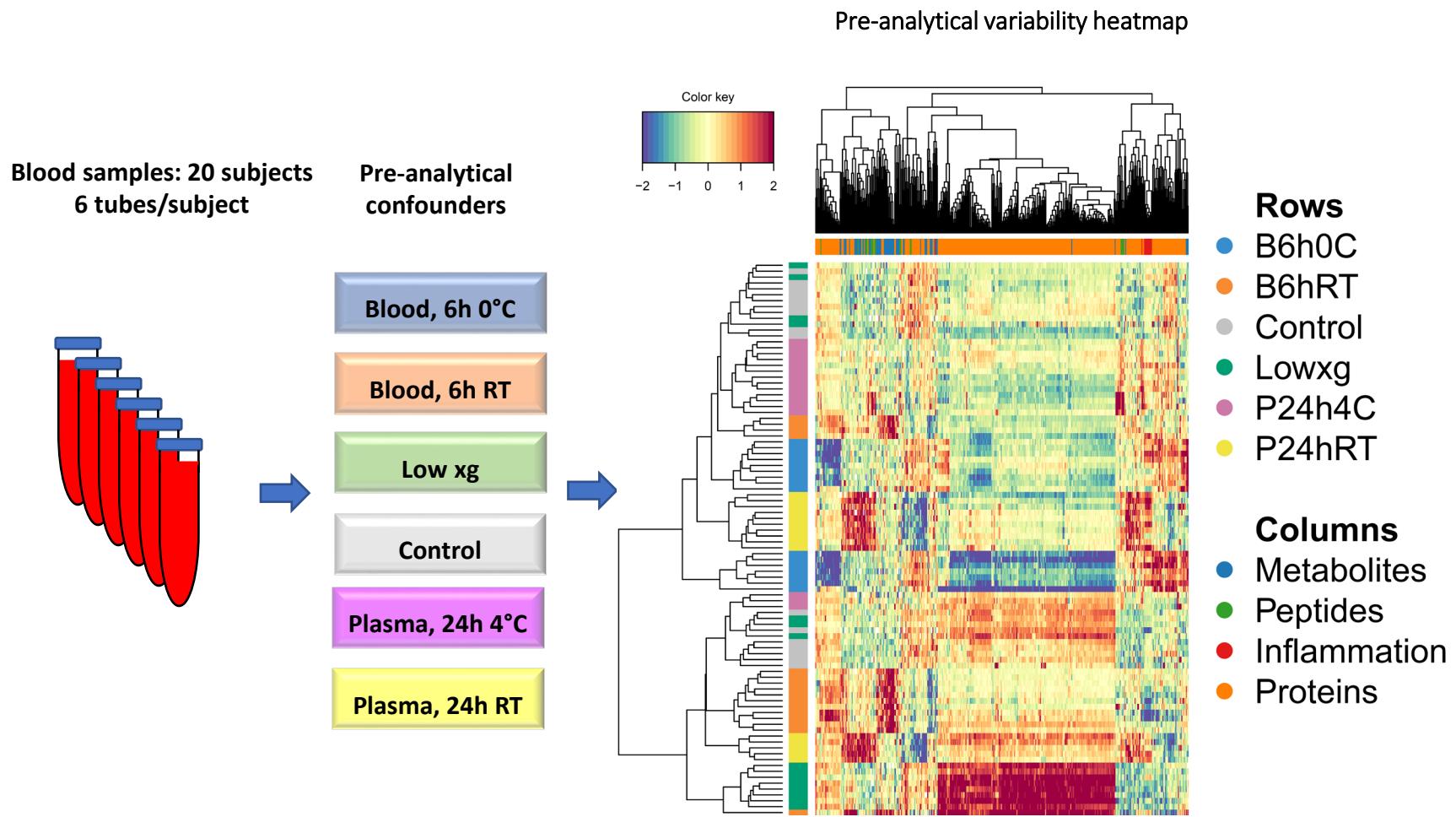
Background

- Differences in sample processing will change the blood samples.
 - *Delay time for sample process*
 - *Storage conditions (temperature and time)*
 - *Centrifugal force*
 - *Freeze-thaw cycles*
- Blood Sample collection and processing needs quality assessment or quality controls.
- Metabolomics and proteomics may be able to discover biomarkers of faulty blood sample collection or processing.

Goals of the study:

- Investigate the impact of pre-analytical variables on proteins, peptides, metabolites and inflammation markers in human plasma
- Discover metabolite, peptide, protein as sample quality markers related to variations in pre-analytical process of clinical plasma samples

Graphic Outline



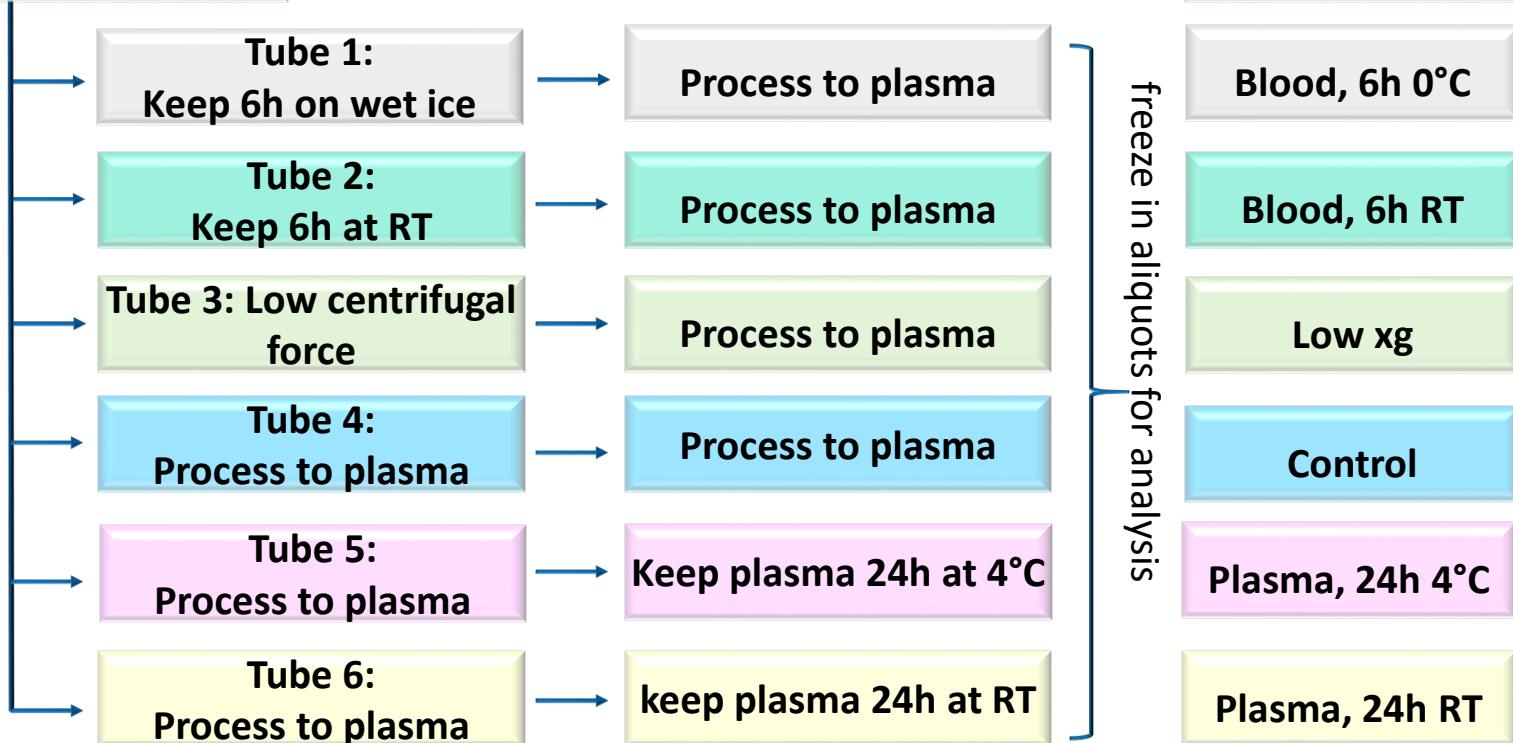
- 20 healthy volunteers
 - Including criteria: M,F, age 18-40, fasting, BMI18-30kg/m²
 - Excluding criteria: acute/chronic diseases, anemia, pregnancy (2nd&3rd trimester), medication with heparin, non-steroidal anti-inflammatory drugs (NSAID), steroid anti-inflammatory drugs (SAID) (within the last 10 days), anti-histamines and selective serotonin re-uptake inhibitors (within the last 4 weeks), No DSB members

Study Design

20 healthy volunteers

Draw 6 x 8-9ml/subject

Tube randomized for each subject

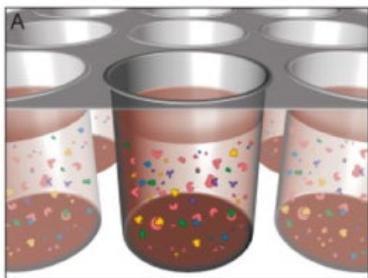


Applied relative centrifugal force was 2500 xg for all processing conditions except Low xg at 1300 xg.

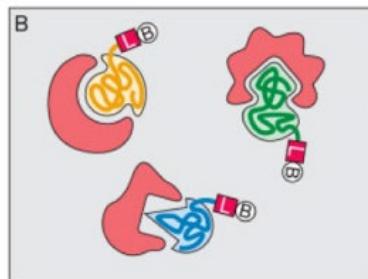
Assays

- **Proteomics (SOMAscan: 1305 proteins)**
- **Metabolomics (GC and LC-MS/MS)**
- **Peptidomics(nanoLC-MS/MS)**
- **Inflammation markers (Bio-Plex: 37-plex)**

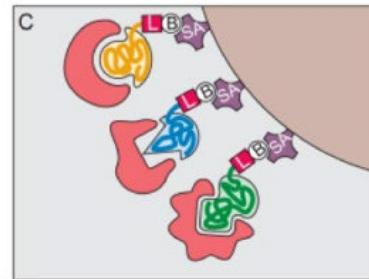
SOMAscan assay:



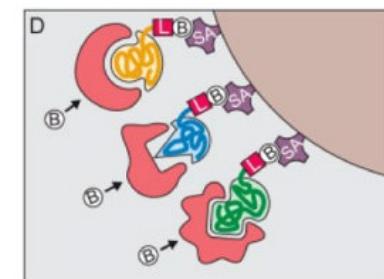
Incubation



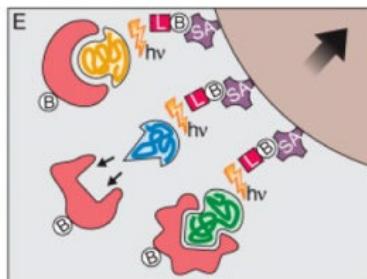
B-L-Aptamer+
protein



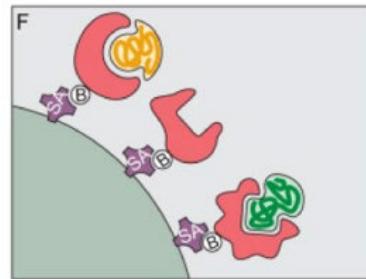
Beads-SA+
B-L-Aptamer+
protein



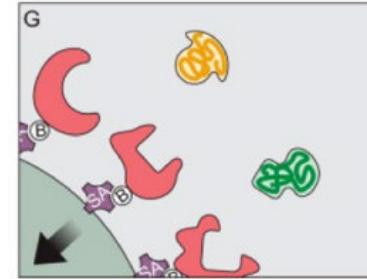
Beads-SA+
B-L-Aptamer+
Protein-NHS-B



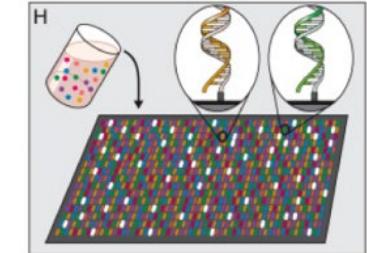
Beads-SA+
B-L...**Aptamer+**
Protein-NHS-B



Beads-SA+
B-NHS-protein+
Aptamer



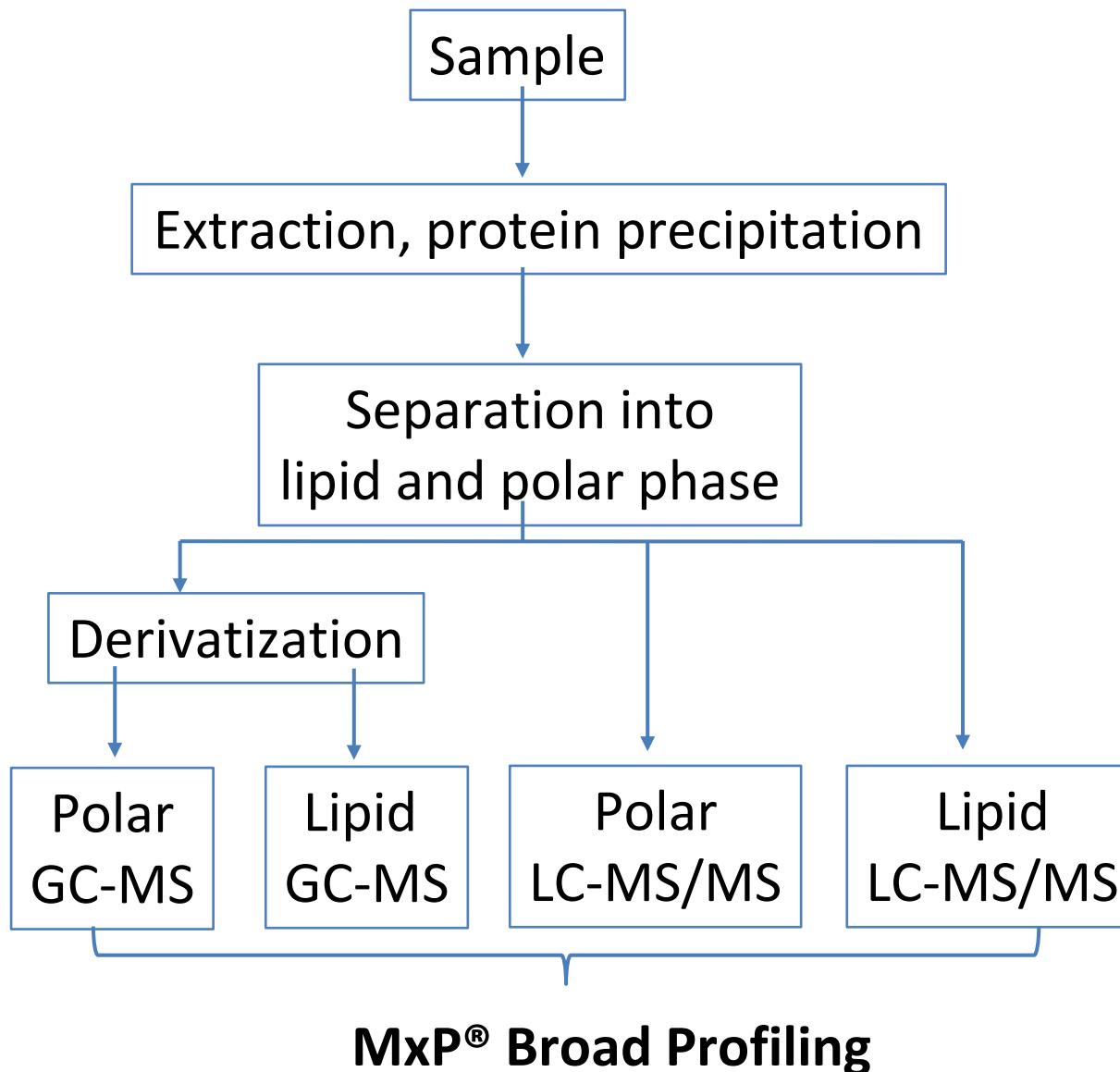
Beads-SA+
B-NHS-protein+...
Aptamer



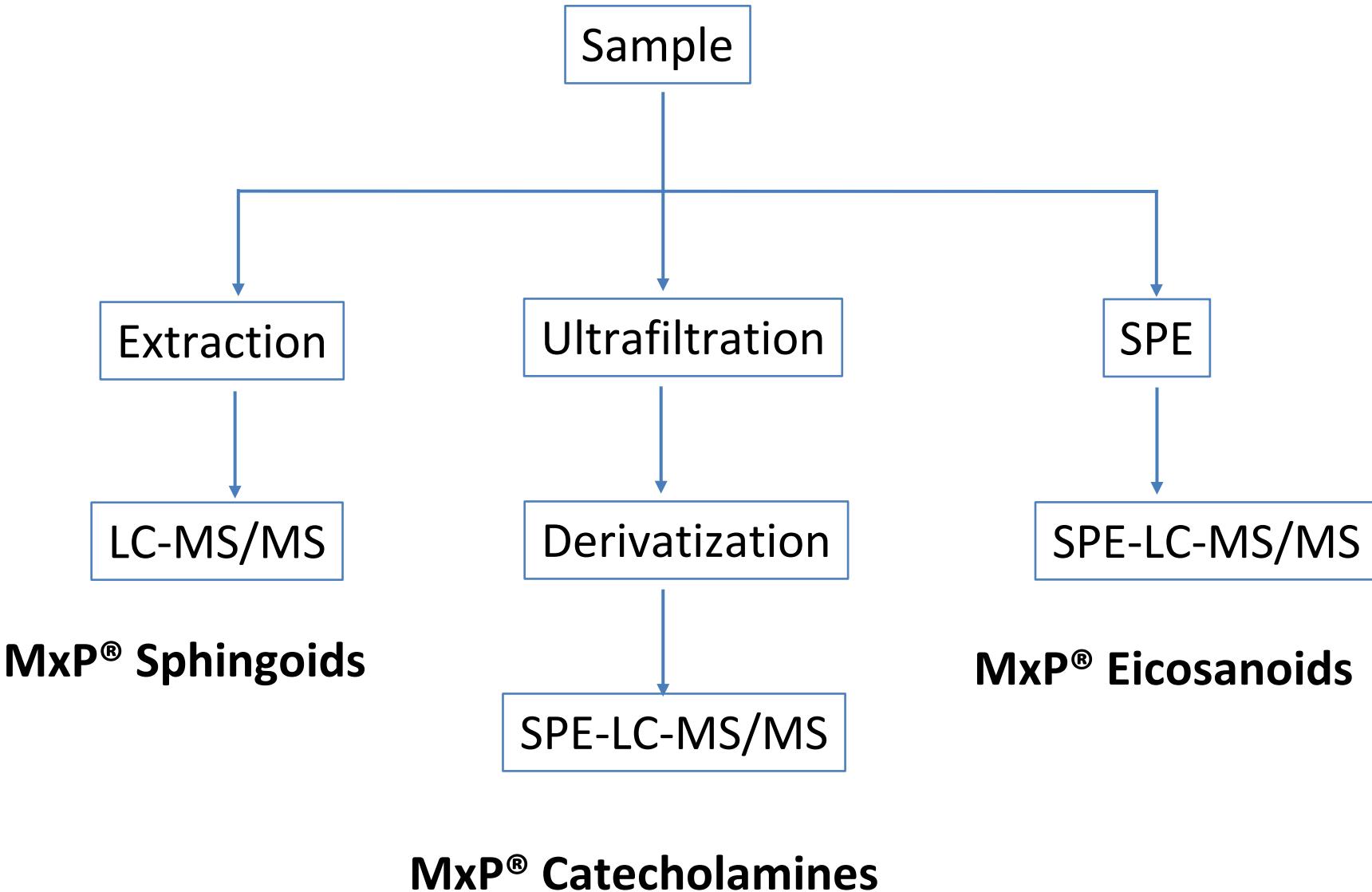
Aptamer: quantified
by Agilent microarray

SA: streptavidin, B: biotin, L: linker, ...: release

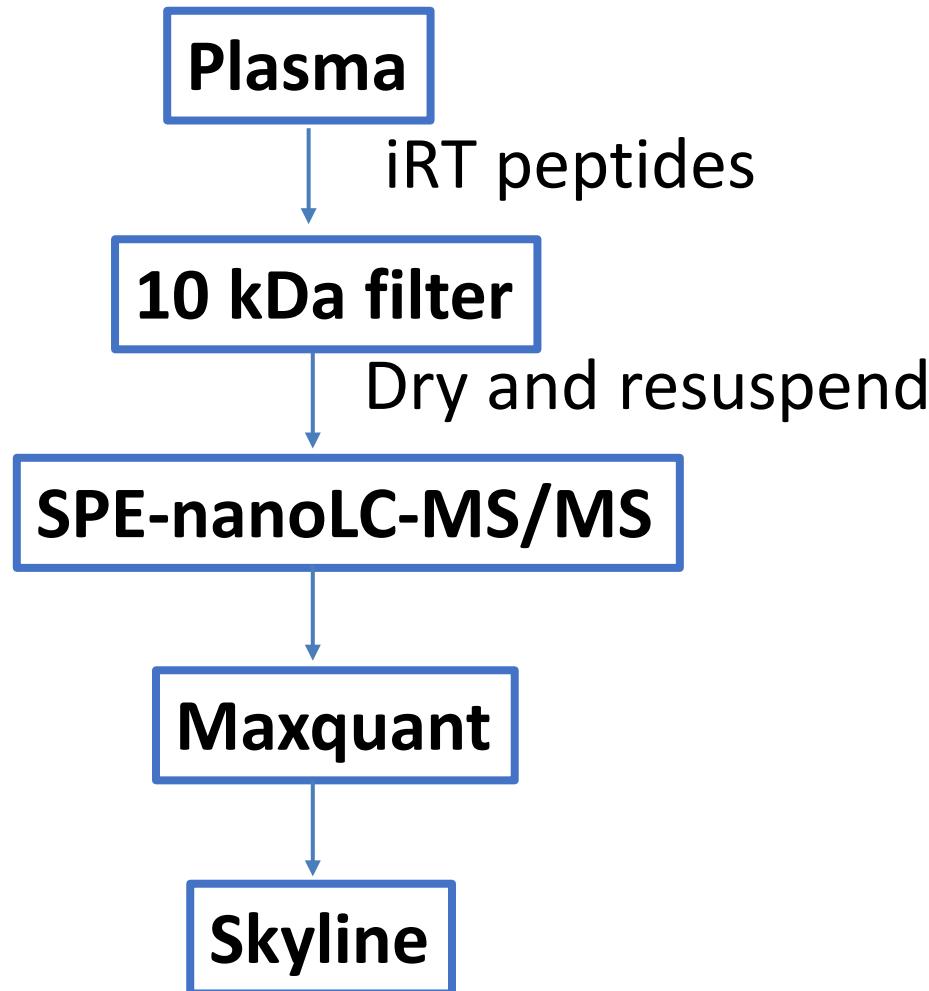
Metabolomics: MxP® Broad Profiling



Targeted MxP® Metabolites Profiling



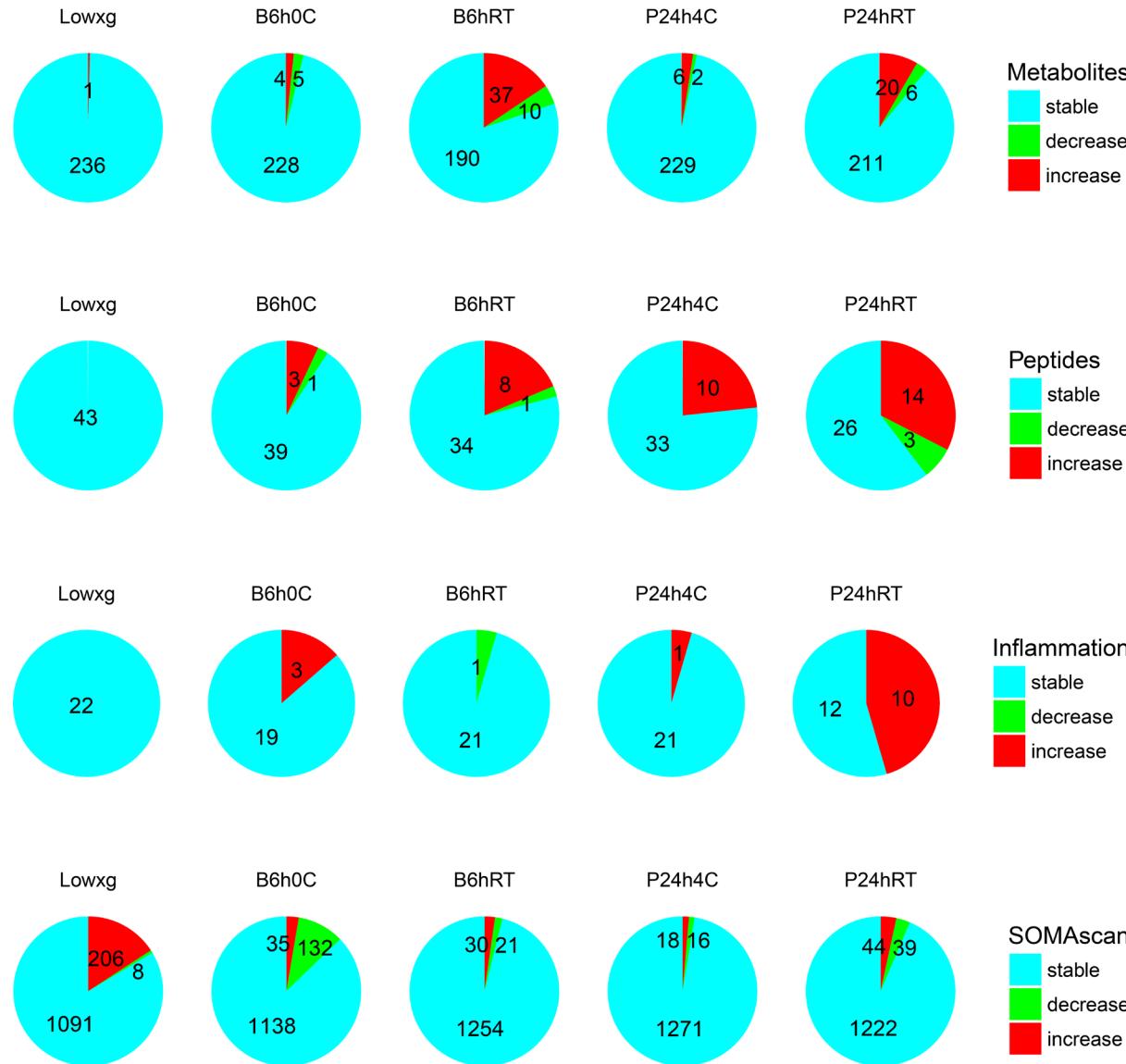
Peptidomics



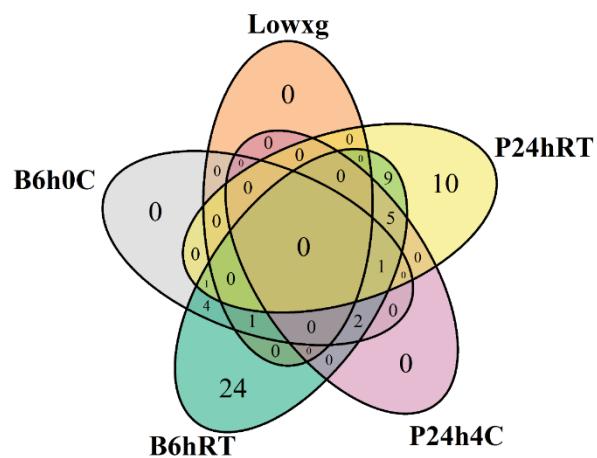
Data Analysis

- Statistical analysis: a repeated measures analysis of variance (ANOVA) following Dunnett's test
- The Benjamini-Hochberg procedure was used to calculate the false discovery rate (FDR).
- Significant change criteria:
 - Fold Change >1.2 for metabolites and proteins
 - > 2 for peptides
 - P value <0.05 , FDR <0.2
- Principal component analysis (PCA)
- Partial least squares discriminant analysis (PLS-DA)
- DIABLO: Data Integration Analysis for Biomarker discovery using a Latent component method for Omics studies

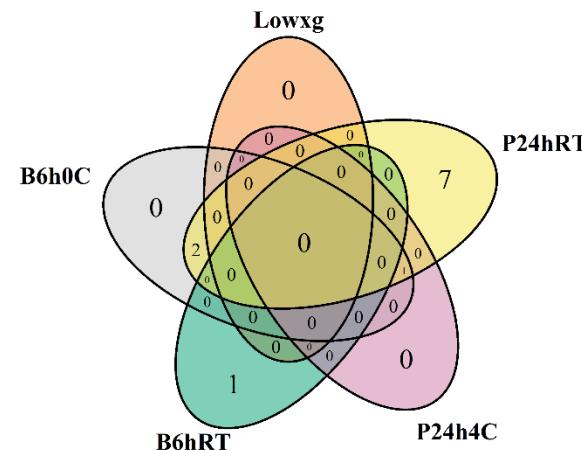
fold cutoff :2 for peptides, 1.2 for others; p<0.05, FDR <0.2



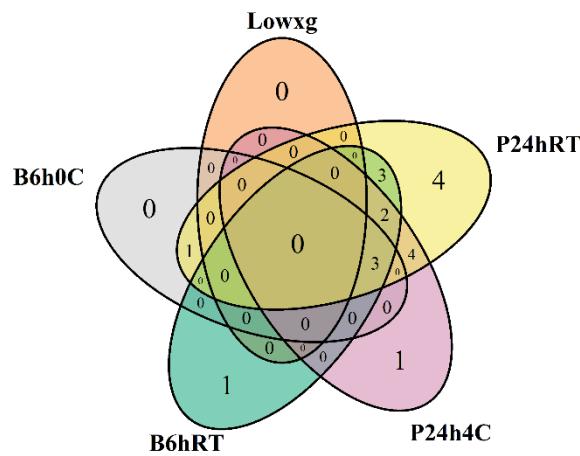
A: metabolites



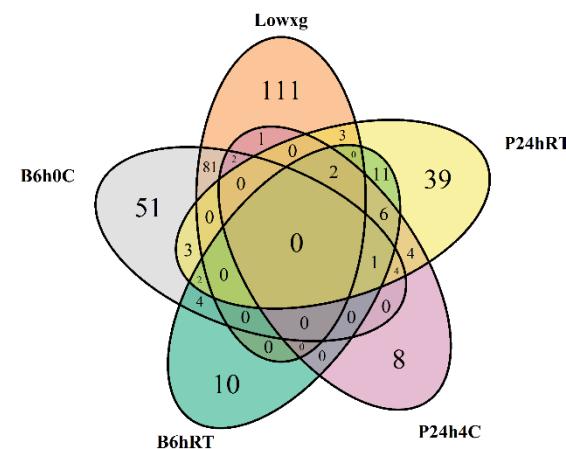
B: peptides



C: inflammation



D: SOMAscan proteins



A : metabolites

B: peptides

PCA analysis

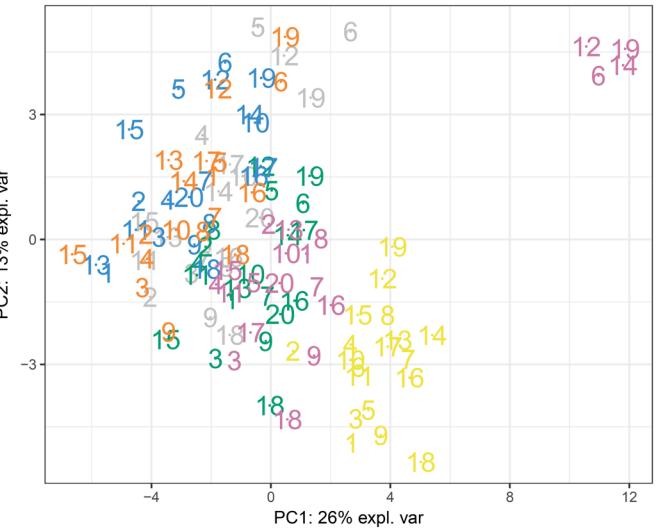
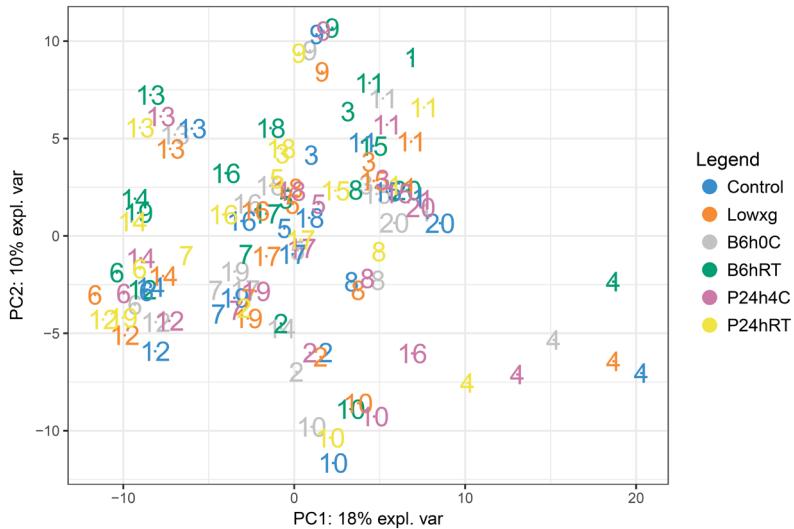
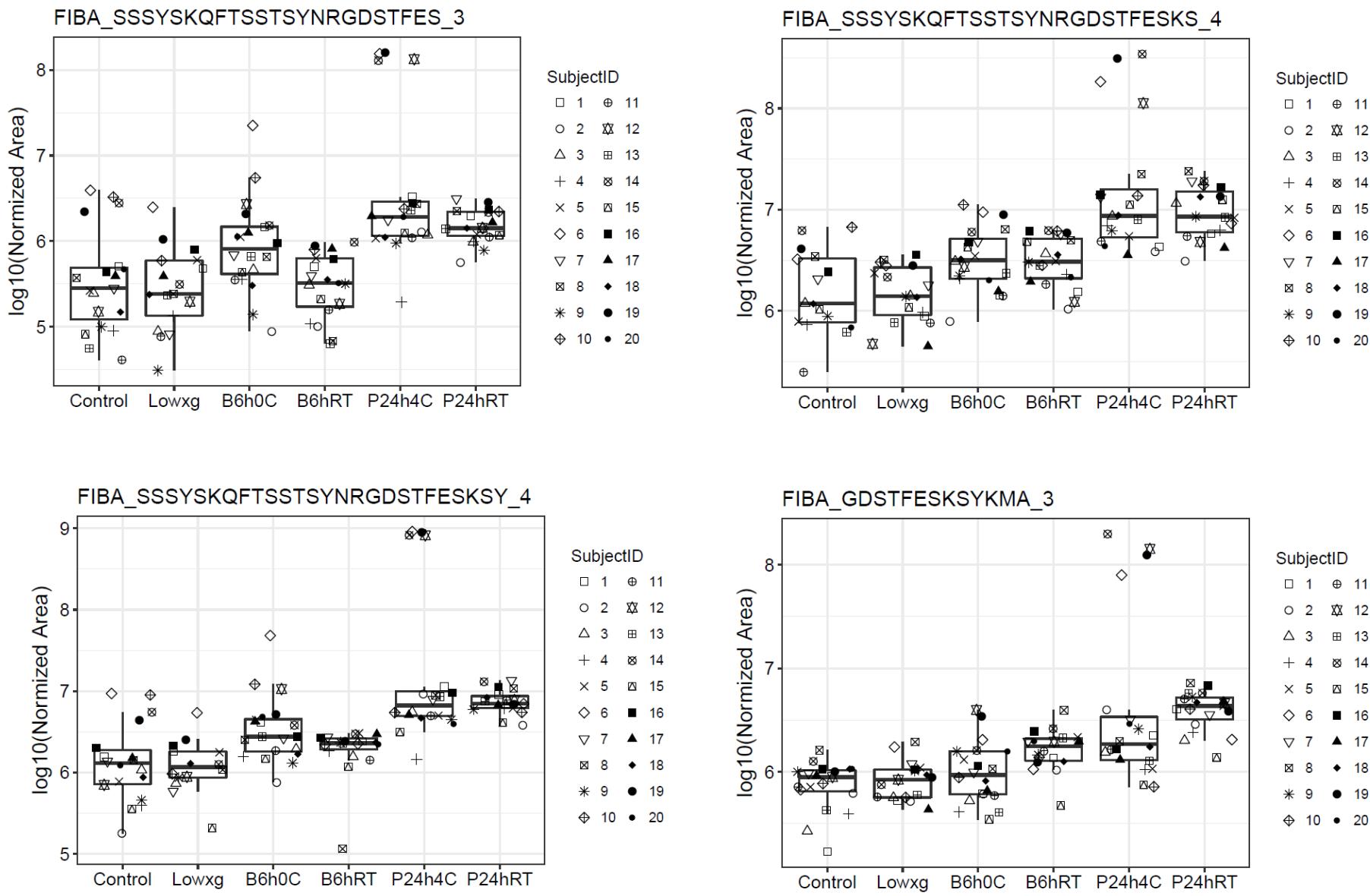


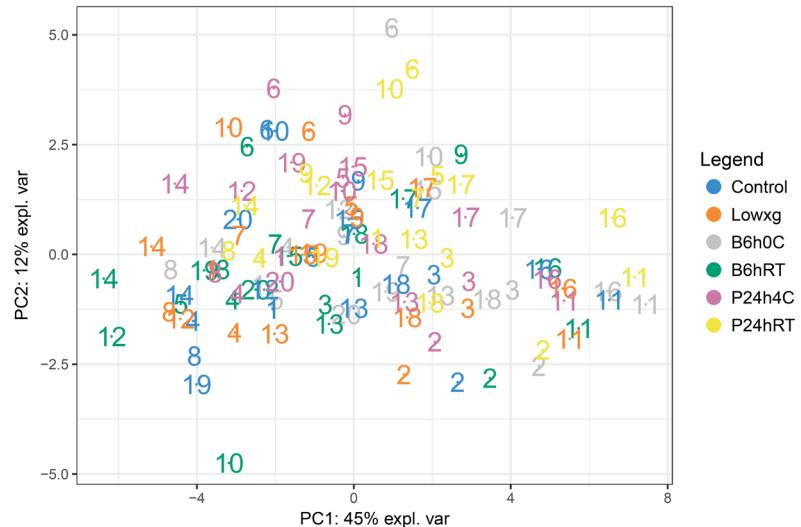
Figure 3



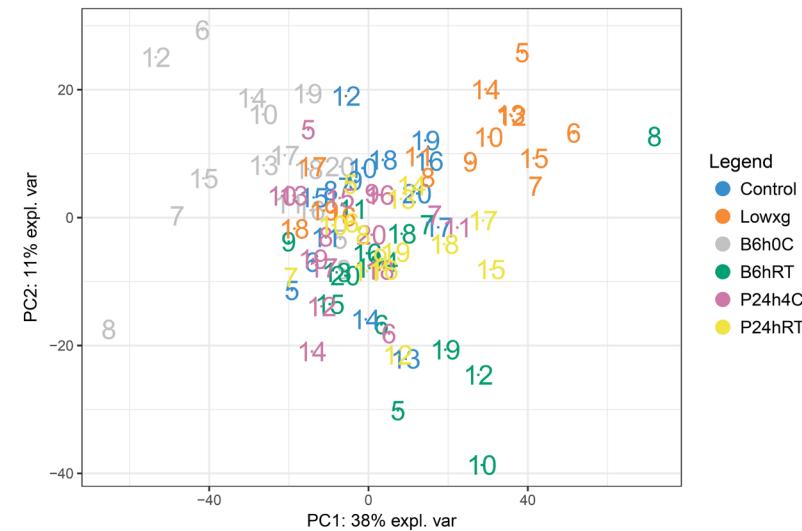
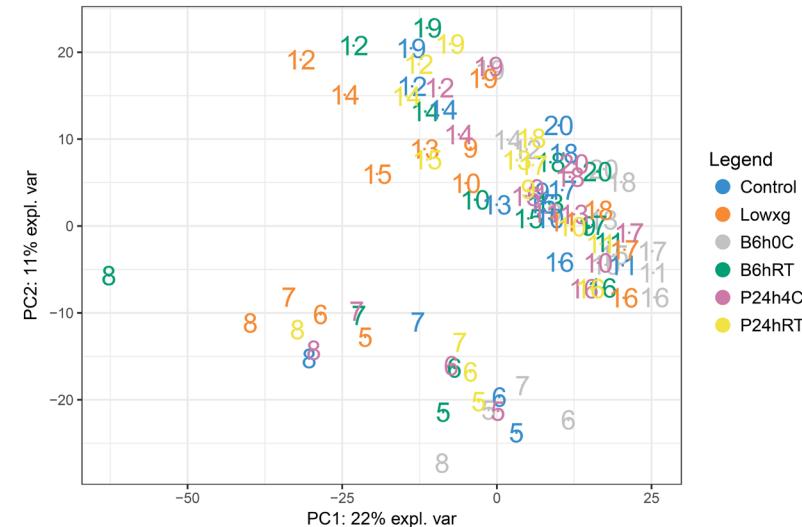
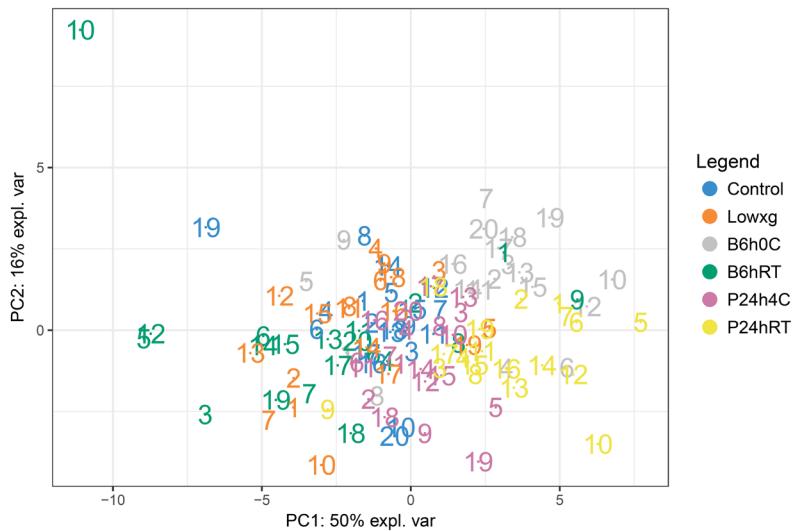
C: inflammation

D: proteins

PCA analysis

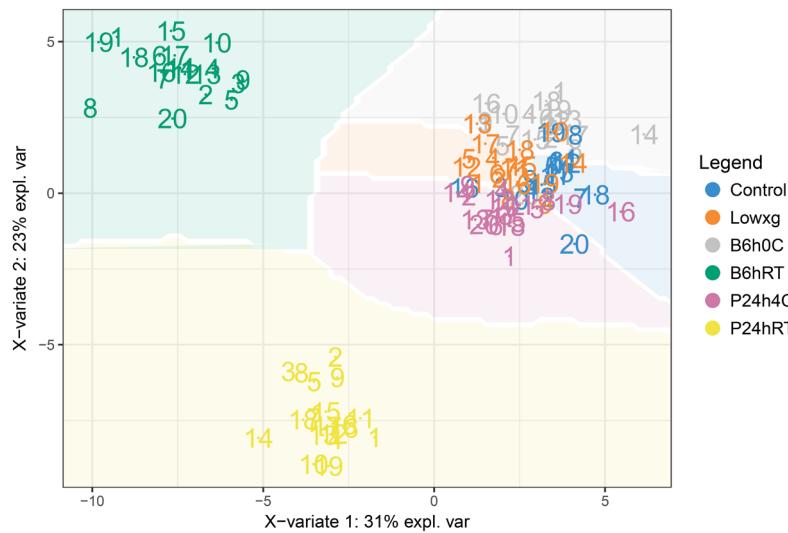


Multilevel PCA analysis

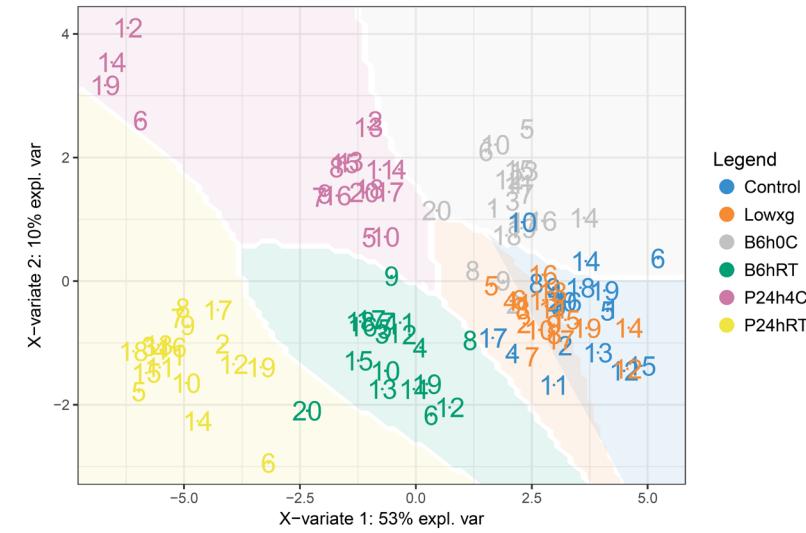


PLS-DA analysis

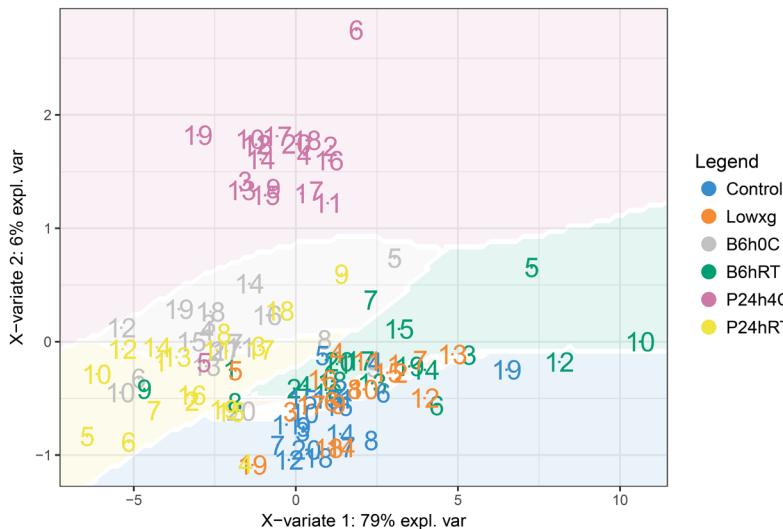
A: metabolites



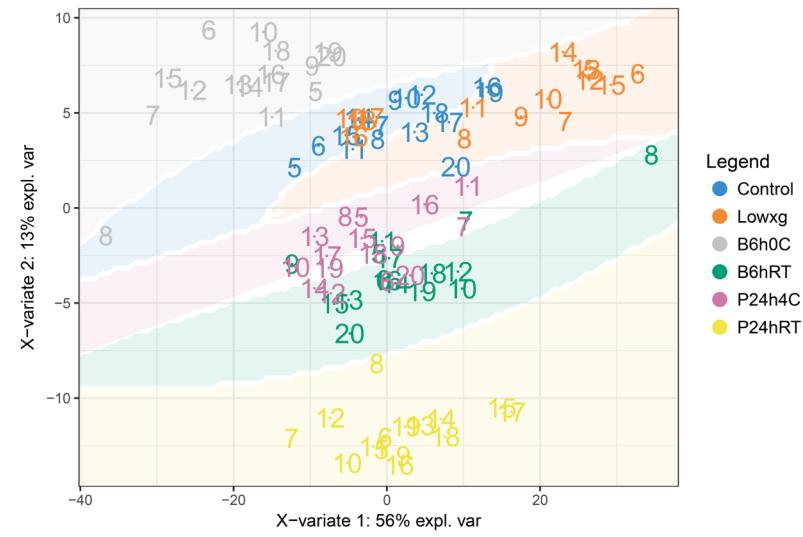
B: peptides

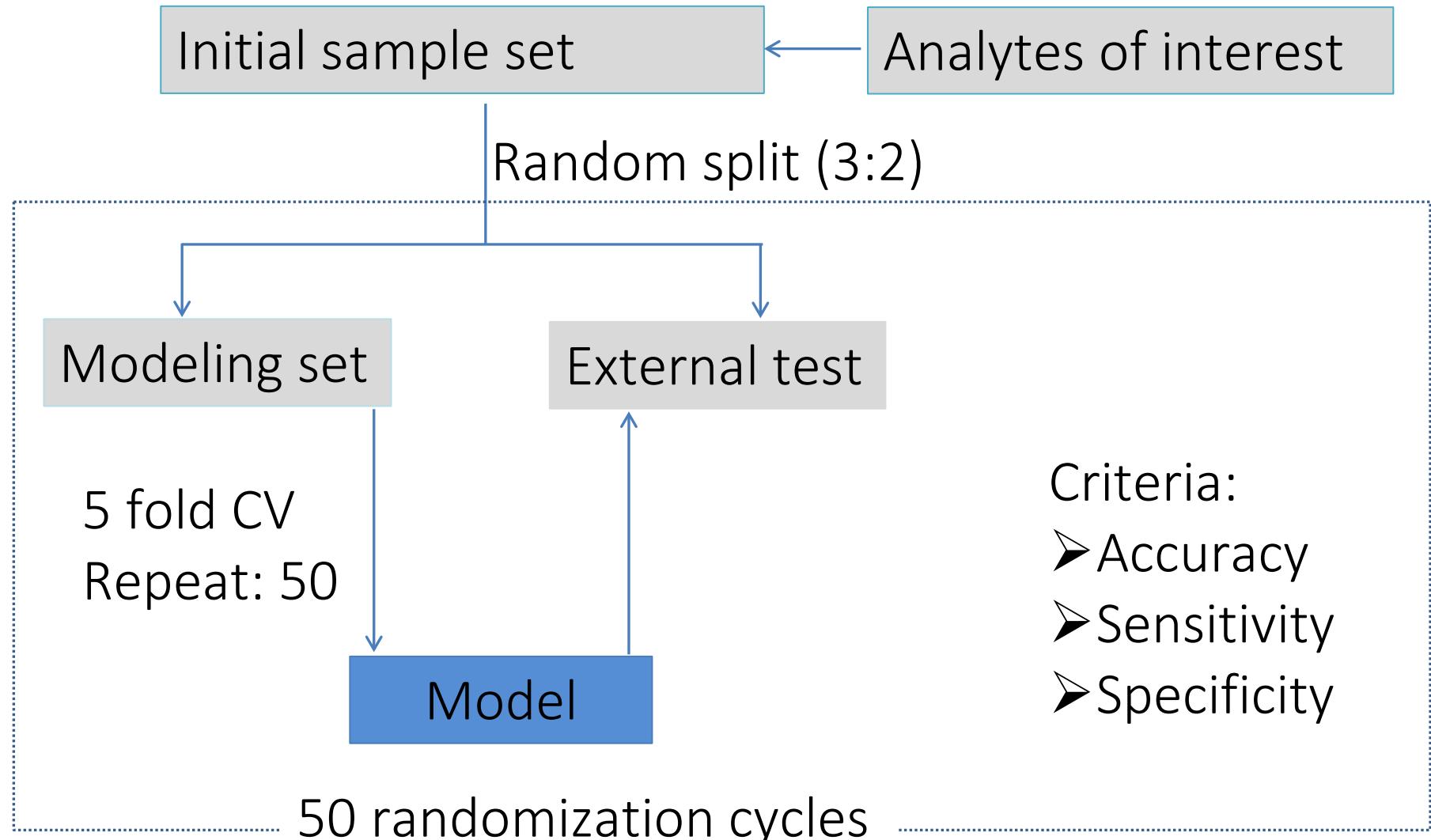


C: inflammation



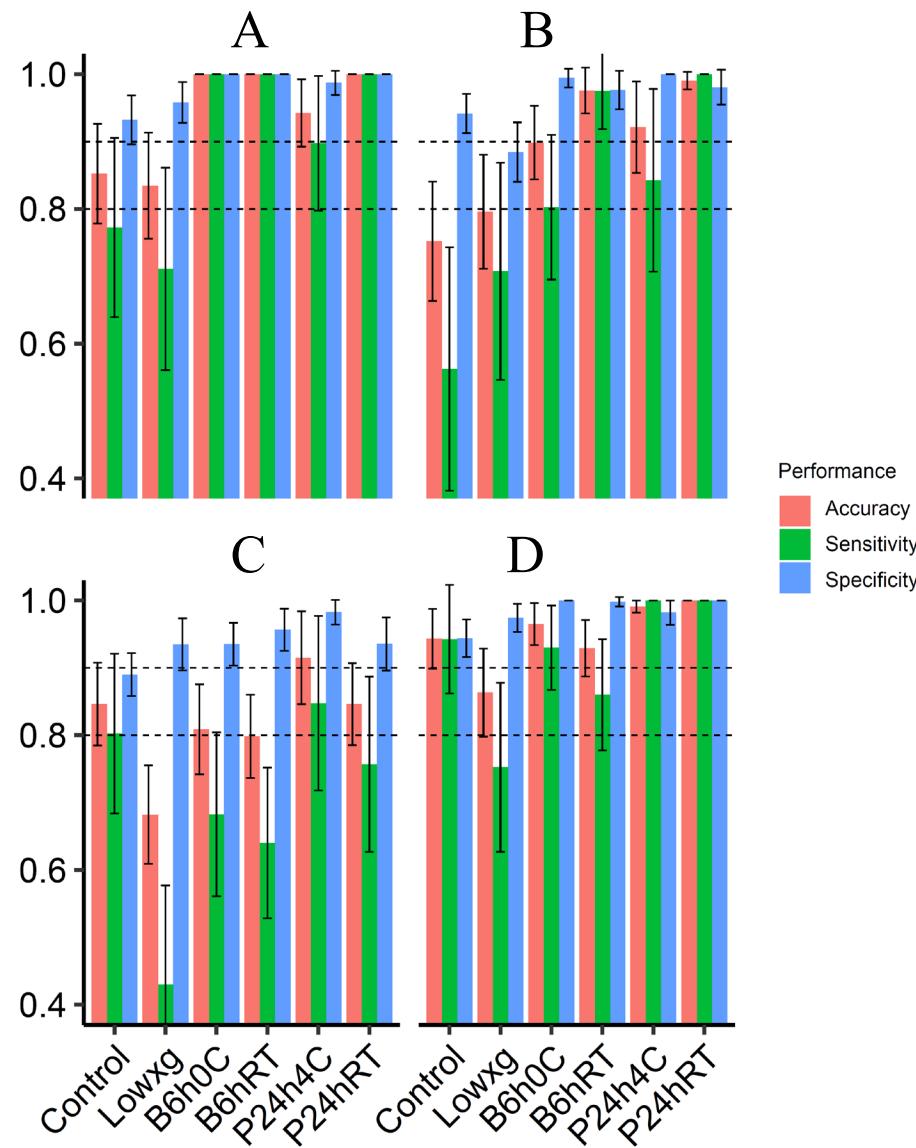
D: proteins





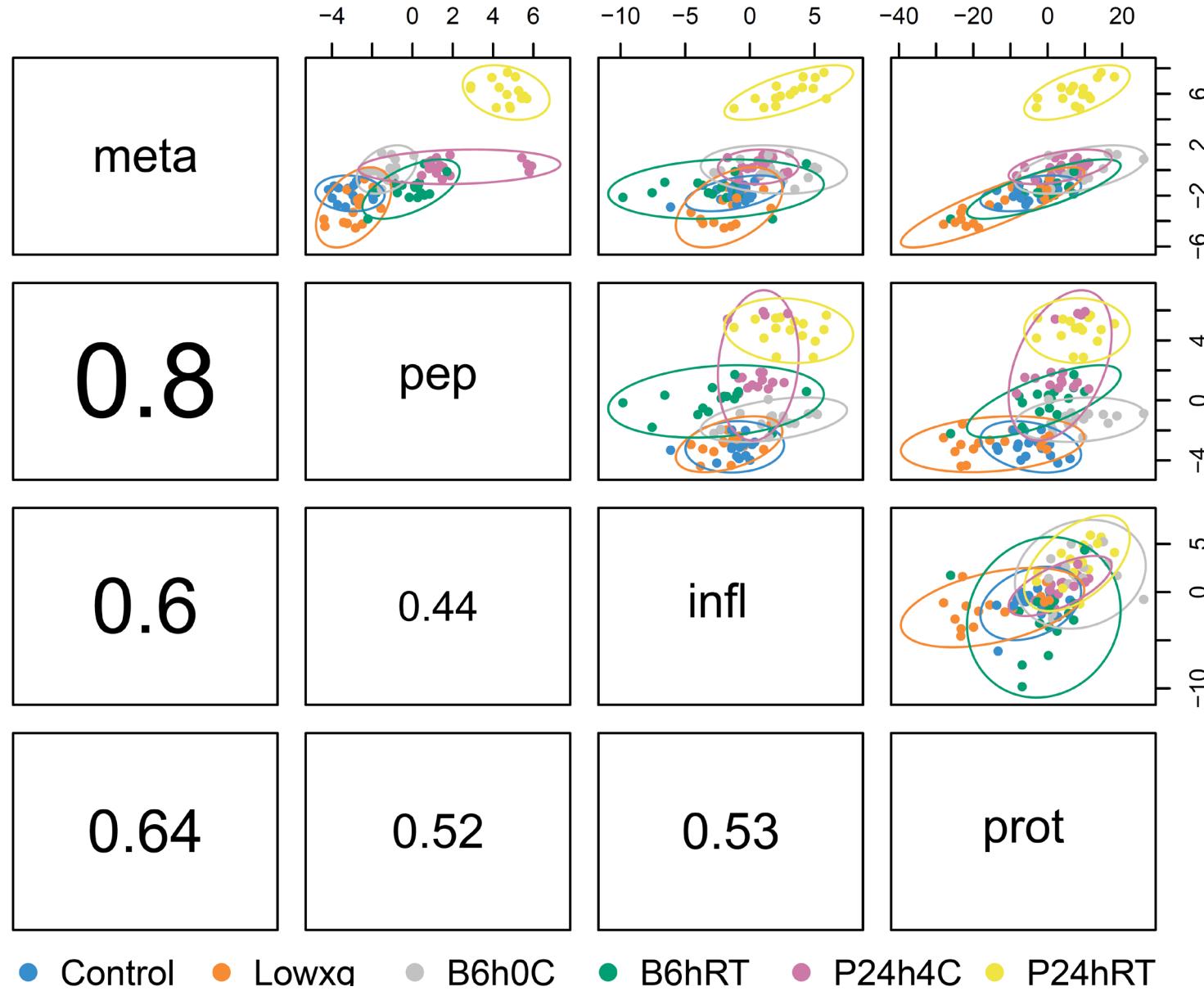
Multi-level PLS-DA modeling process, CV: cross-validation. 20

sPLS-DA models: accuracy, sensitivity and specificity



A: metabolites B: peptides C: inflammation D: SOMAscan proteins

DIABLO analysis (R package: mixOmics)



Conclusions

- Centrifugal force applied to blood processing (e.g., Low xg) has the most significant impact on the plasma proteome as measured by SOMAscan while there is little impacts on other type of molecules.
- Storing plasma at RT for 24h affects the plasma levels of metabolites, peptides and proteins more significantly than storing plasma at a lower temperature.
- Storing blood at 0°C for 6h significantly affects the levels of proteins while storing blood at RT for 6h significantly affects the levels of metabolites and peptides.
- Changes of peptides in plasma due to pre-analytical variable were specific to individual subjects.
- Sample processing at 0-4°C and minimizing incubation time of blood and plasma is generally recommended to achieve reliable results. For proteomics analysis, blood processing to plasma should be done at RT with consistent centrifugal force.
- Metabolites, peptides, and proteins identified as susceptible to pre-analytical variations could be potential quality biomarkers.

Acknowledgements

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Evaluation of the Performance of Lipidlyzer Platform and Its Application in the Lipidomics Analysis in Mouse Heart and Liver

Lipids play important roles in cell signaling, energy storage, and as major structural components of cell membranes. To date, little work has been conducted to show the extent of tissue specificity of lipid compositions. Here, the recently acquired Lipidlyzer platform was employed in this pilot study:

- (i) to assess the performance of the Lipidlyzer platform,
- (ii) to explore lipid profiles in liver and cardiac tissue in mice,
- (iii) to examine sex-specific differences in lipids in the liver tissue, and
- (iv) to evaluate biological variances in lipidomes present in animals. In total, 787 lipid species from 13 lipid classes were measured in the liver and heart. Lipidomics data from the Lipidlyzer platform were very reproducible with the coefficient of variations of the quality control (QC) samples, ~10%. The total concentration of the cholesterol esters (CE) lipid class, and specifically CE(16:1) and CE(18:1) species, showed sex differences in the liver. Cardiac tissue had higher levels of phospholipids containing docosahexaenoic acid, which could be related to heart health status and function. Our results demonstrate the usefulness of the Lipidlyzer platform in identifying differences in lipid profile at the tissue level and between male and female mice in specific tissues.

Sample Process

Homogenizing for
40s, repeat: 250 ul
water+ 25mg tissue



25 ul liver homogenate (n=3/sex)
25 ul pooled liver homogenate (n=3)
50 gul pooled heart homogenate (n=3)
50 ul QC sample (n=3)
50 ul QC sample+25 ul QC spike mixture (n=3)



Dilute to 100 ul with water



0.9 ml water, 2ml methanol, 0.9 ml dichloromethane (DCM)



gently vortexed for 5 s

0.1 ml stable internal standard mixtures



centrifuge for 10 min

Bottom organic layer dried under nitrogen flow

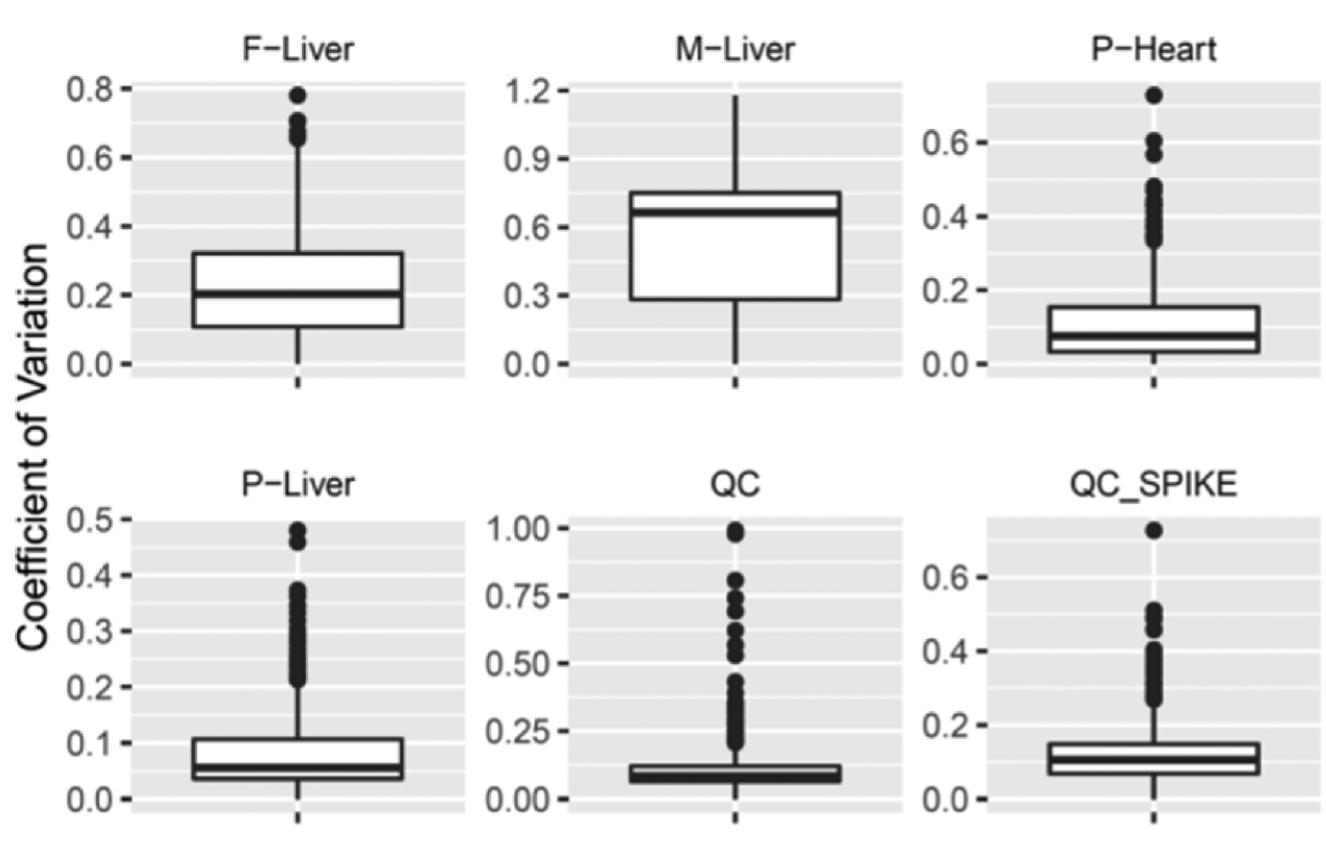


Reconstituted in 0.30 mL of 10 mM ammonium acetate in 1:1 DCM/methanol

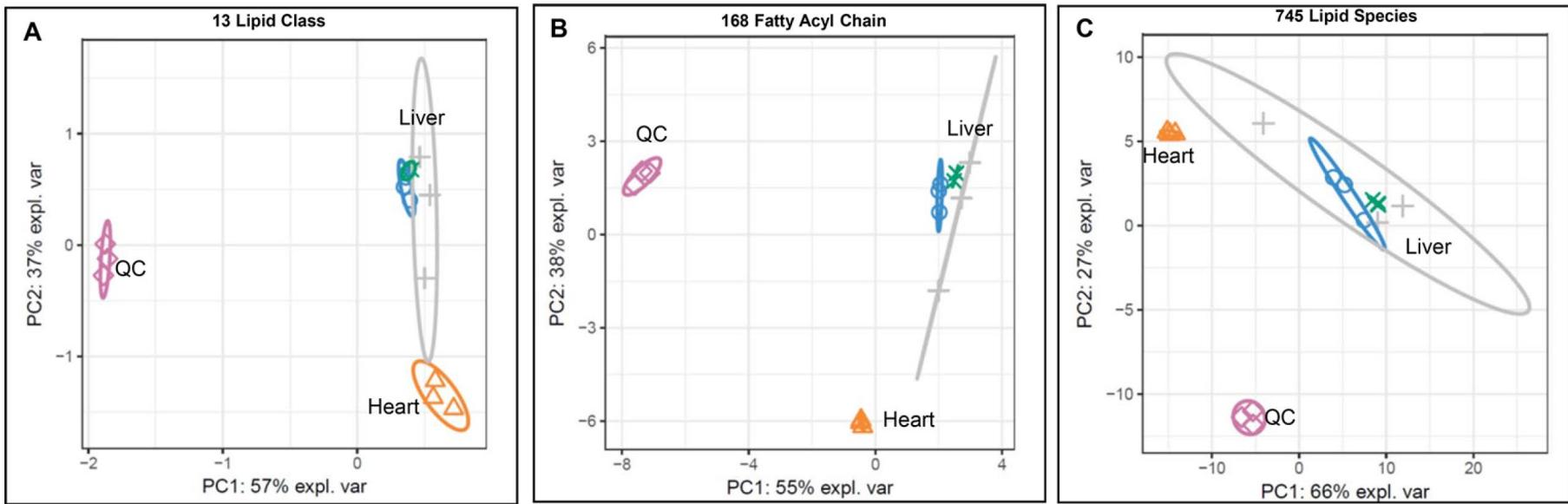
Differential Ion Mobility-MS/MS Analysis

- 50 ul reconstituted sample: directly infuse into QTRAP 5500 MS with SelectION
- Both positive and negative ionization modes
- Flow rate: 7 ul/min

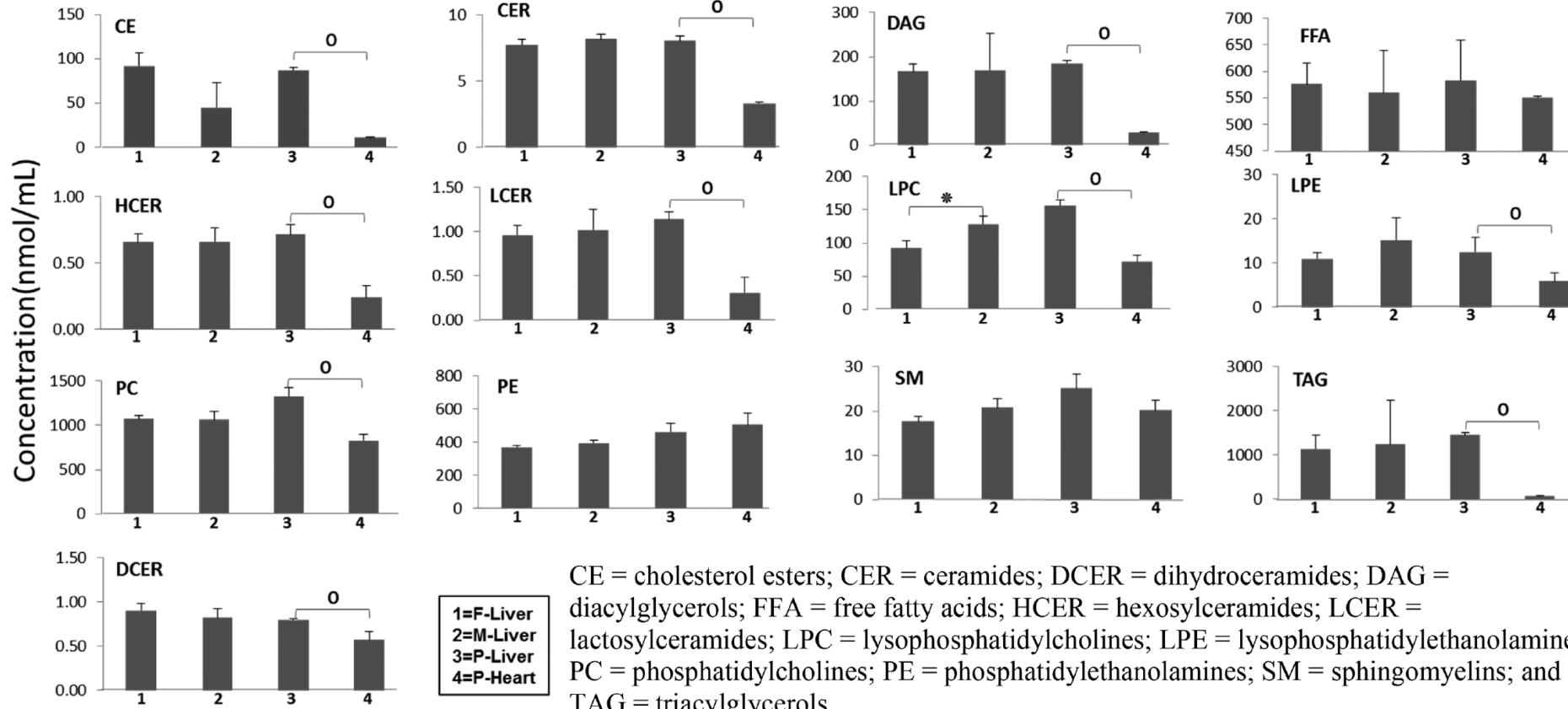
- Discard four outliers out of the 20
- Average the 16 remaining scans to estimate the concentration of each detected lipid



Box-plot of the calculated covariance of the detected lipid species from female liver (F-Liver, n = 3), male liver (M-Liver, n = 3), pooled heart (P-Heart, n = 3), pooled liver (P-Liver, n = 3), QC, and QC spike samples.



PCA scores plots based on 13 lipid classes (A), 168 fatty acyl chain (B), and 745 lipid species (C) data.

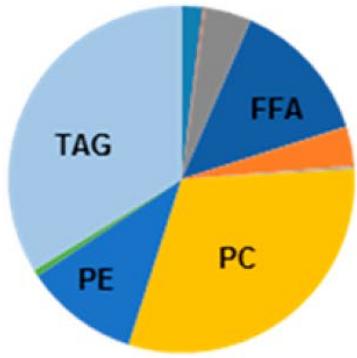


CE = cholesterol esters; CER = ceramides; DCER = dihydroceramides; DAG = diacylglycerols; FFA = free fatty acids; HCER = hexosylceramides; LCER = lactosylceramides; LPC = lysophosphatidylcholines; LPE = lysophosphatidylethanolamines; PC = phosphatidylcholines; PE = phosphatidylethanolamines; SM = sphingomyelins; and TAG = triacylglycerols.

Concentrations (nmol/mL) across female and male liver, pooled liver, and heart per lipid class. Note: p < 0.05 for gender significant difference, significant difference p < 0.05 between pooled liver and pooled heart.

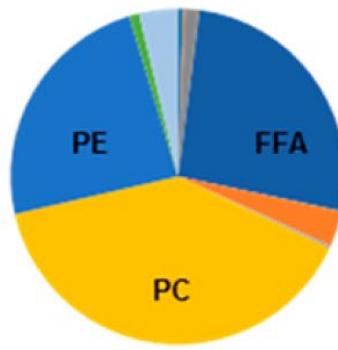
- CE
- CER
- DAG
- DCER
- FFA
- HCER
- LCER
- LPC
- LPE
- PC
- PE
- SM
- TAG

Liver



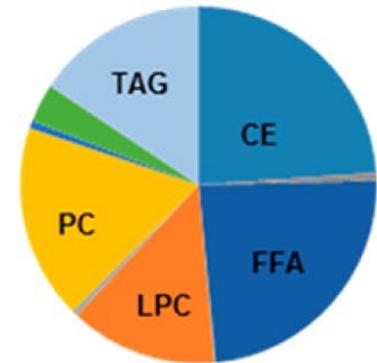
TAG+PC>64%

Heart



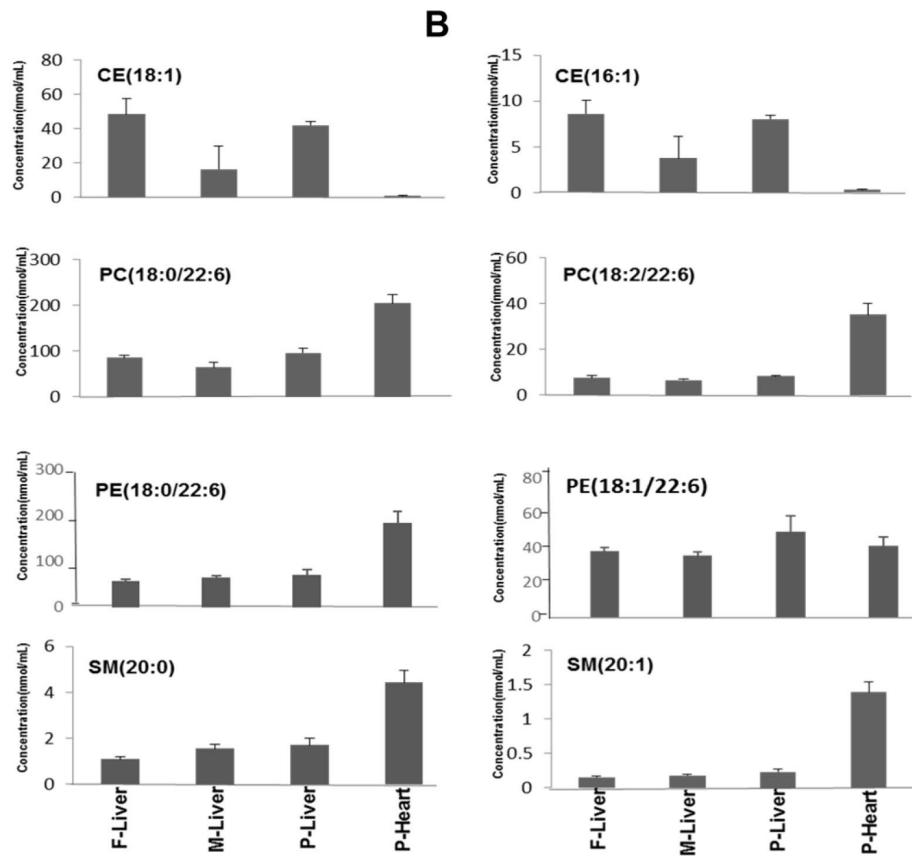
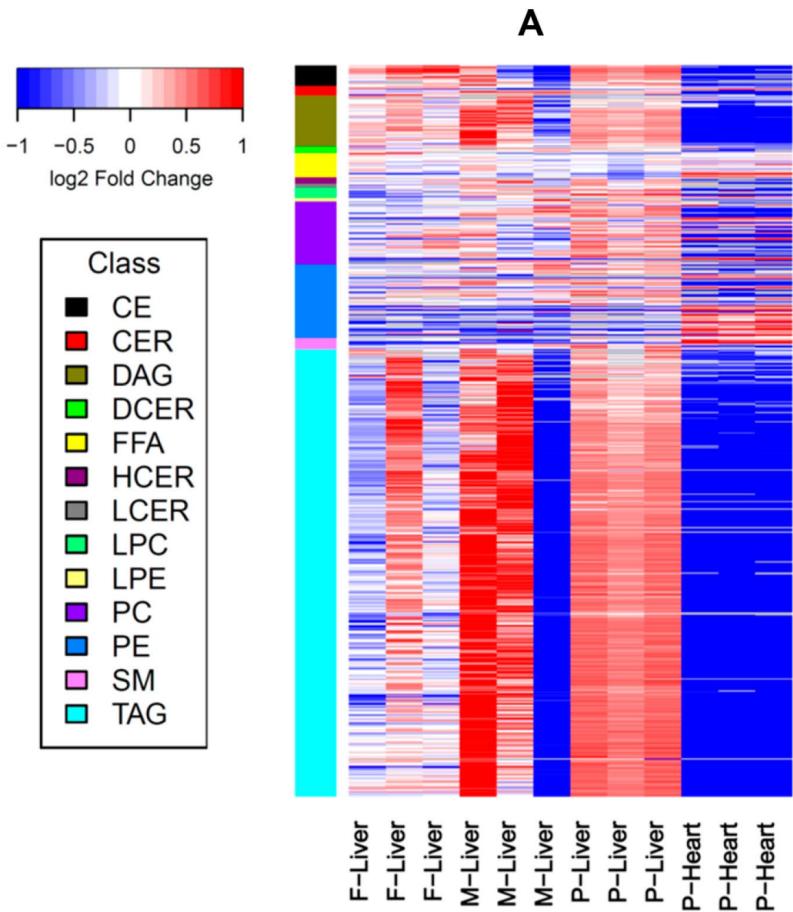
PC+PE+FFA>89%

QC

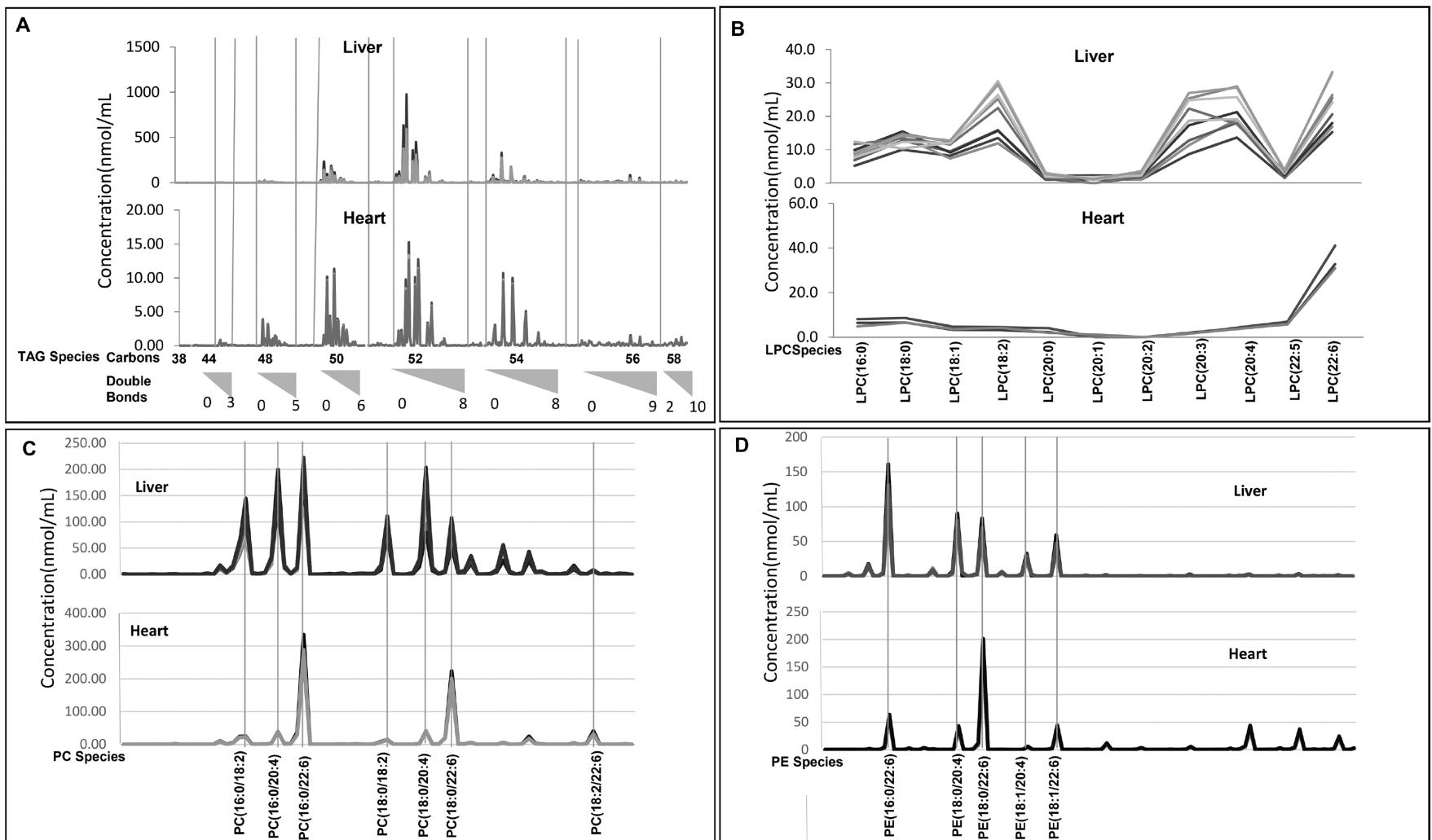


TAG+PC+LPC+FFA+CE>95%

Lipid class composition as a percentage of the total concentration in pooled liver, pooled heart, and human plasma QC sample.



Heat map of fold changes of 787 individual lipid species across the 13 lipid classes in all individual animals (A). Fold change is $\log_2(\text{concentration}/\text{average for each lipid in all of the animals})$. Selected lipid species distribution patterns in tissues (B). Error bars denote standard deviation (SD) from $n = 3$ replicates.



Lipid profiles of the detected 491 TAG (A), 11 LPC (B), 80 PC (C), and 108 PE (D) species in heart and liver. Solid lines for heart tissue include three individual runs from the pooled heart samples; for liver, it includes three individual runs from the pooled liver samples, individual female ($n = 3$), and male liver samples ($n = 3$). The triangles at y-axis indicate the number of double bonds increasing from left to right within each group.

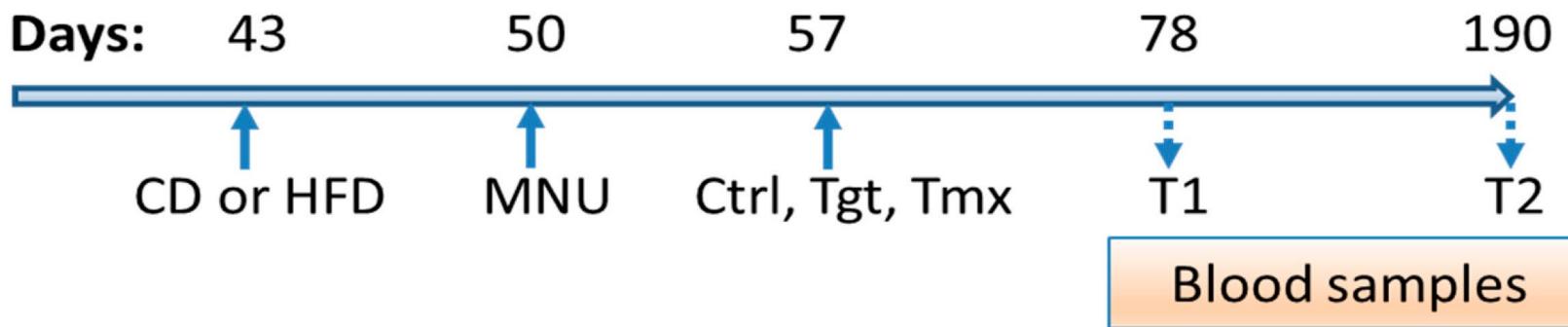
Summary:

- (1) more male and female mice need to be included in the study to better gauge the biological variations;
- (2) the current study was limited to examine only liver and heart tissues. To generate an atlas of the tissue-specific lipid distribution in mice, various tissues/organs across the body need to be quantitatively analyzed.
- (3) The QC and QC spike data showed that the lipidomics data from the Lipidlyzer platform were very reproducible with CV values <10%.
- (4) The PCA scores plots indicated that there were differences in lipid distribution between tissues.
- (5) The total concentration of the CE lipid class, and the specific CE(16:1) and CE(18:1) species, showed sex differences in the liver.
- (6) The total concentration of the TAG class was the highest in the liver. Almost all 491 TAG species were more abundant in the liver than the heart.
- (7) The heart had higher levels of docosahexaenoic acid containing phospholipids including PC(18:0/22:6), PC(18:2/22:6), LPC(22:6), PE(18:0–1/22:6), and PE(16:0/22:6), which could be related to the heart health status. SM(20:0) and SM(20:1) were also more abundant in the heart.
- (8) Our results demonstrate the usefulness of the Lipidlyzer platform in identifying differences in lipid profile at the tissue level and between male and female mice in specific tissues.

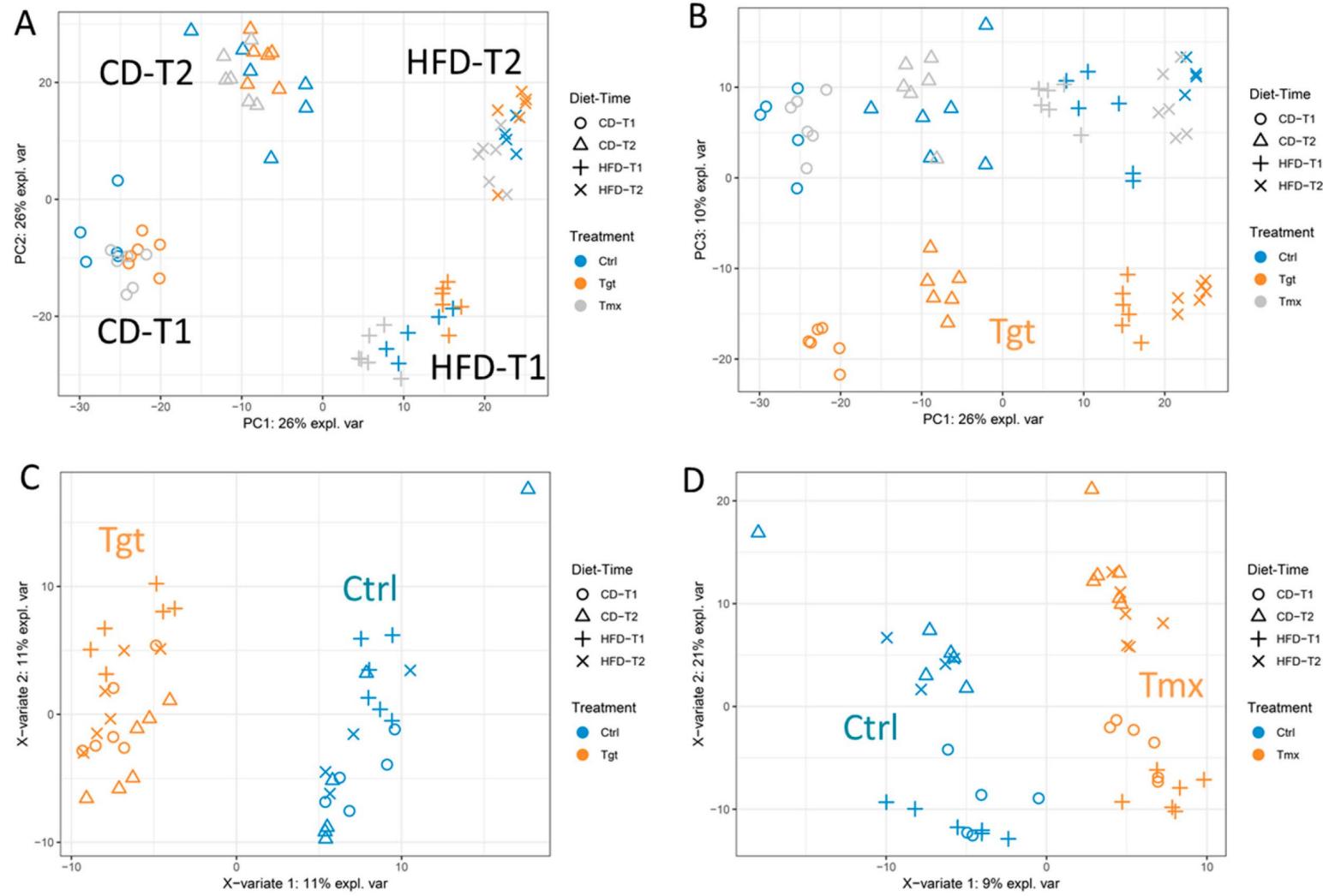
Pharmacometabolomic Pathway Response of Effective Anticancer Agents on Different Diets in Rats with Induced Mammary Tumors

Abstract:

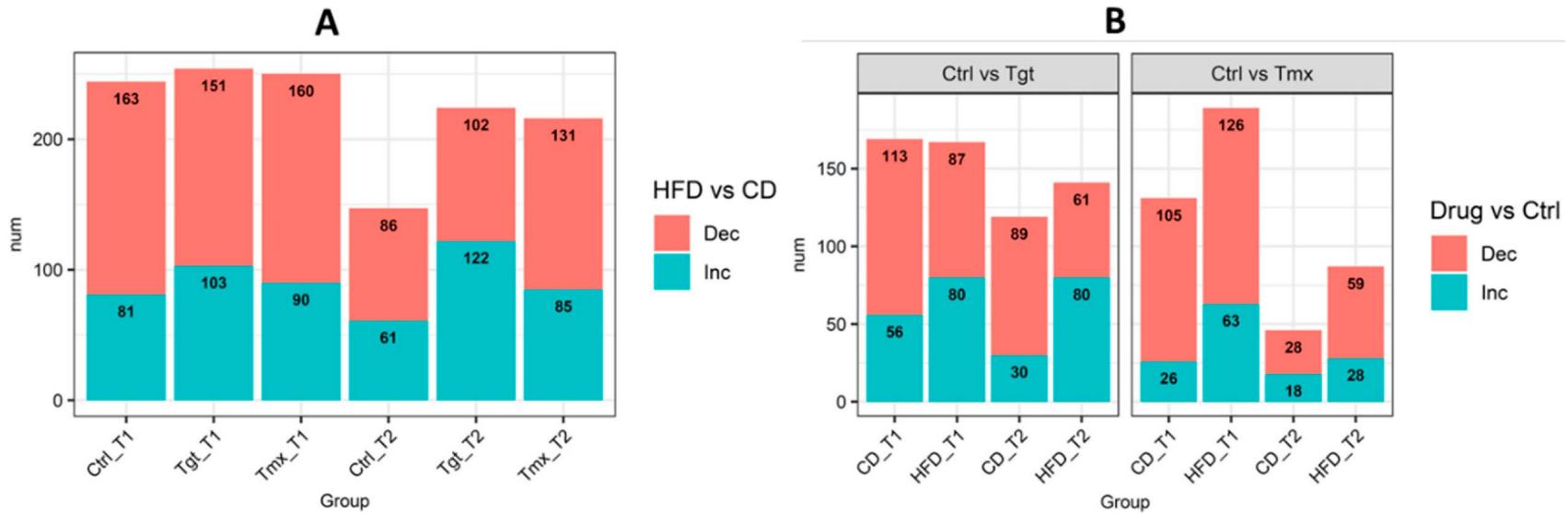
- Metabolomics is an effective approach to characterize the metabotype which can reflect the influence of genetics, physiological status, and environmental factors such as drug intakes, diet.
- Diet may change the chemopreventive efficacy of given agents due to the altered physiological status of the subject.
- Metabolomics response to a chemopreventive agent targretin or tamoxifen, in rats with methylnitrosourea-induced tumors on a standard diet (4% fat, CD) or a high fat diet (21% fat, HFD) was evaluated, and found that
 - ✓ The metabolome was substantially affected by diet and/or drug treatment;
 - ✓ Multiple metabolites were identified as potential pharmacodynamic biomarkers related to targretin or tamoxifen regardless of diet and time;
 - ✓ The primary bile acid pathway was significantly affected by targretin treatment in rats on both diets, and the lysolipid pathway was significantly affected by tamoxifen treatment in rats on the high fat diet.



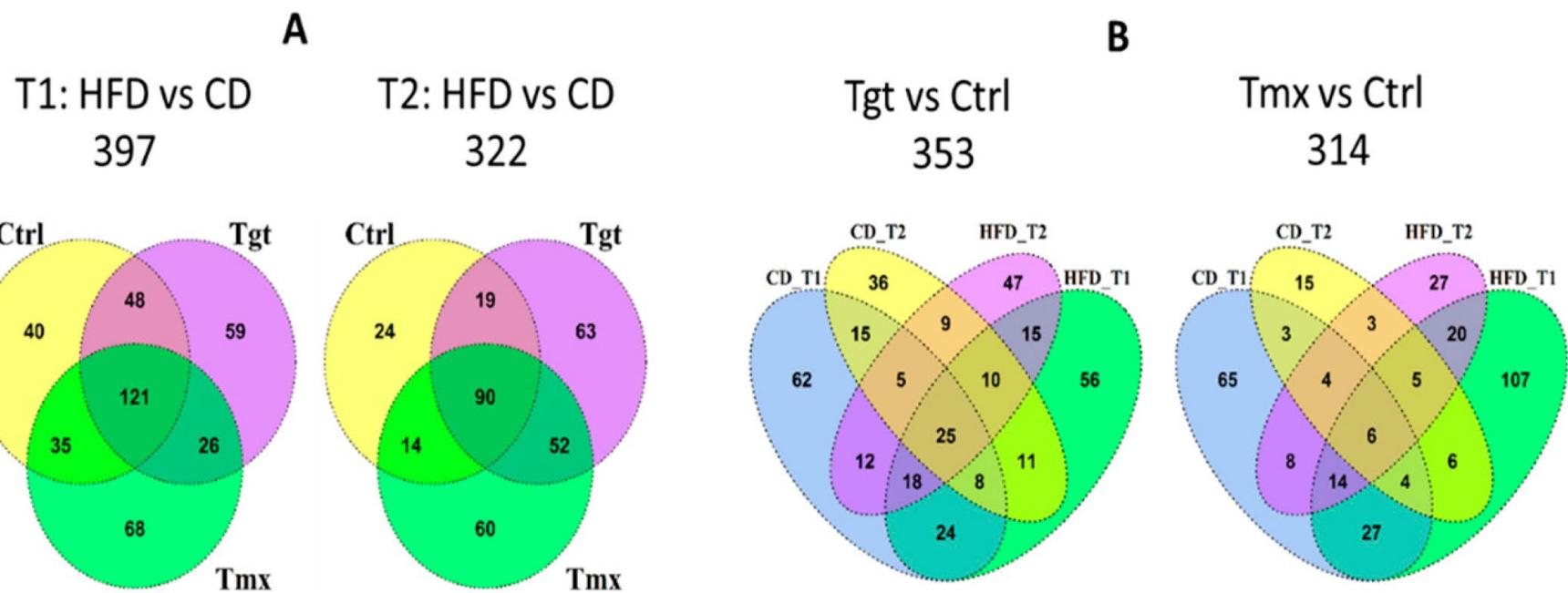
Diet and Treatment process. Diets: standard diet (CD, 4% fat, high calcium and high soy protein); high fat diet (HFD, 21% fat, low in calcium, and no soy proteins); Times: T1, 78 days of age; T2, 190 days of age; Treatments: Ctrl, control; Tgt, targretin; Tmx, tamoxifen



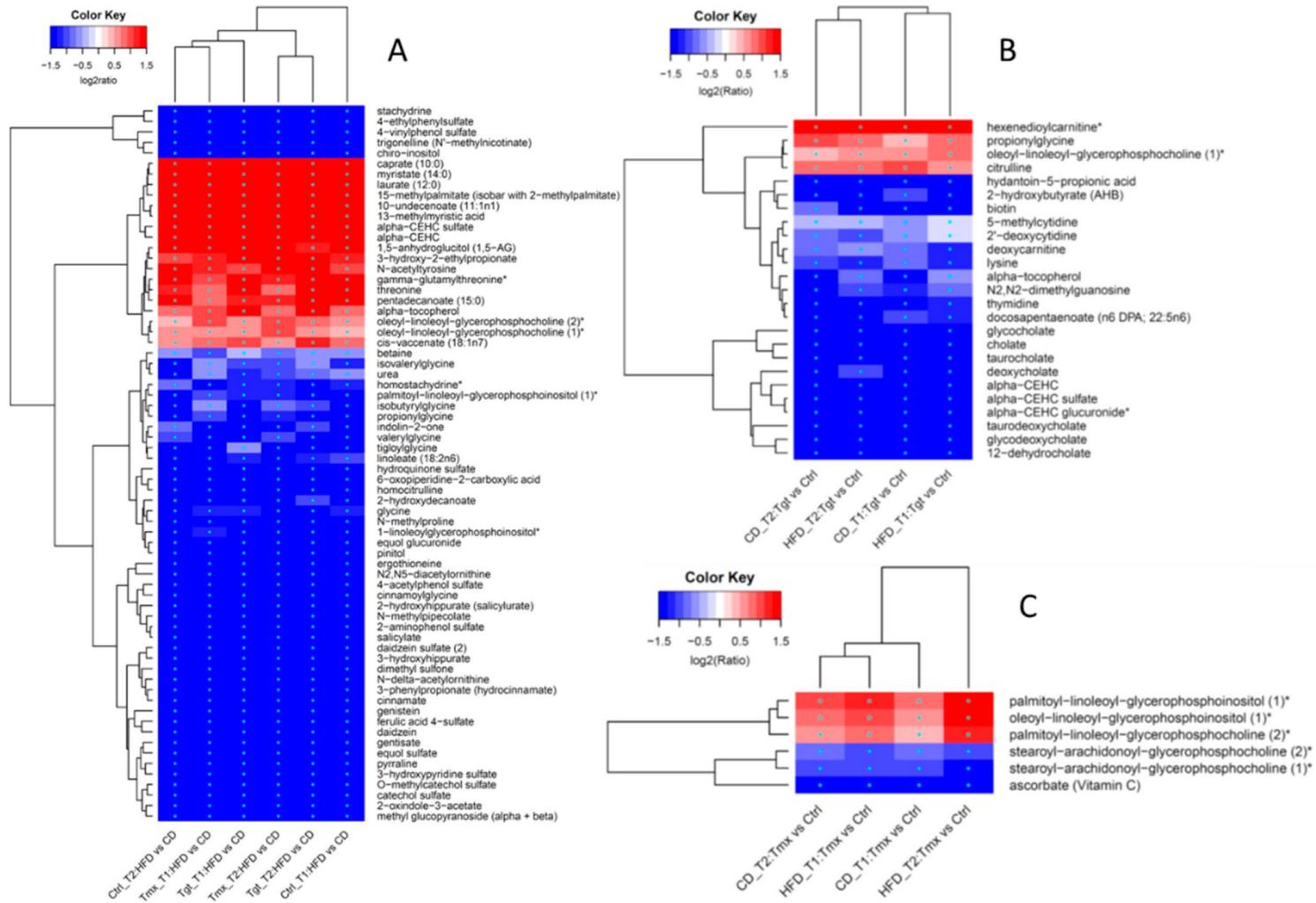
Multivariate analysis of all quantified metabolites. **(A,B)** the scores plots of the PCA analysis, **(C,D)** the scores plots of the PLS-DA analysis. Diets: standard diet (CD); high fat diet (HFD); Times: T1, 78 days of age; T2, 190 days of age; Treatments: Ctrl, control; Tgt, targretin; Tmx, tamoxifen.



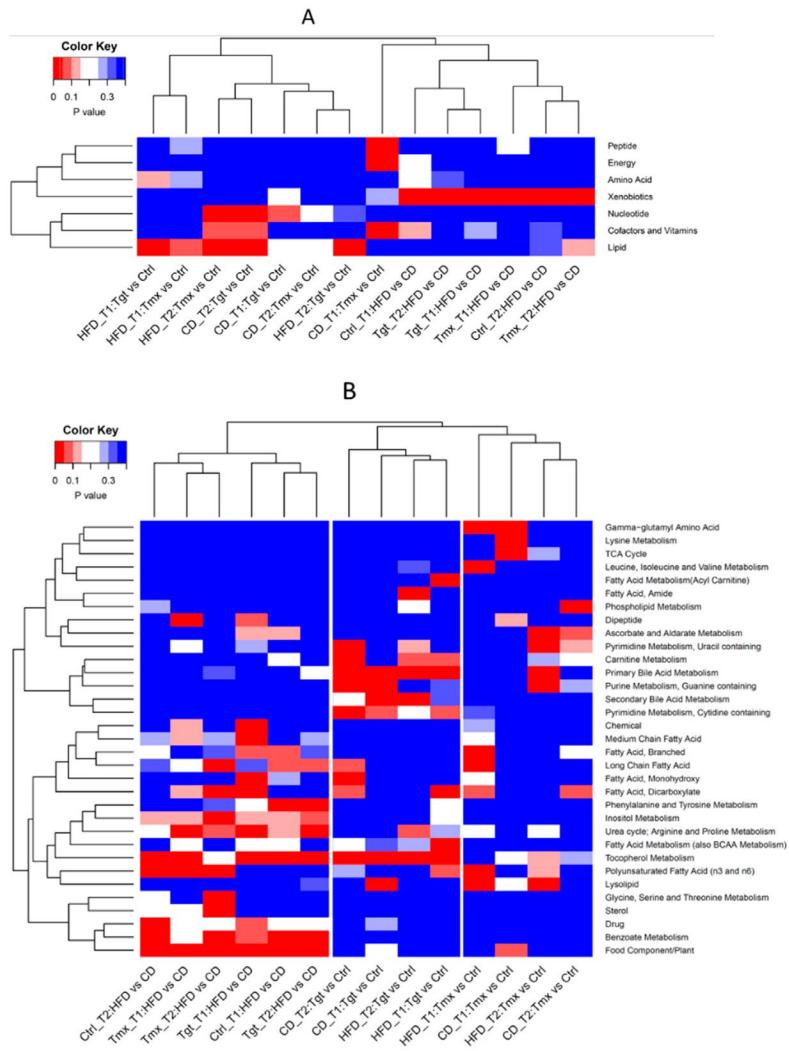
(A) Number of metabolites significantly changed in abundance in HFD vs. CD for each group; **(B)** Number of metabolites significantly changed in abundance in Drug vs. Ctrl for each group. Pink shows metabolites decreased and blue shows metabolites increased. Diets: standard diet (CD); high fat diet (HFD) Times: T1, 78 days of age; T2, 190 days of age; Treatments: Ctrl, control; Tgt, targretin; Tmx, tamoxifen.



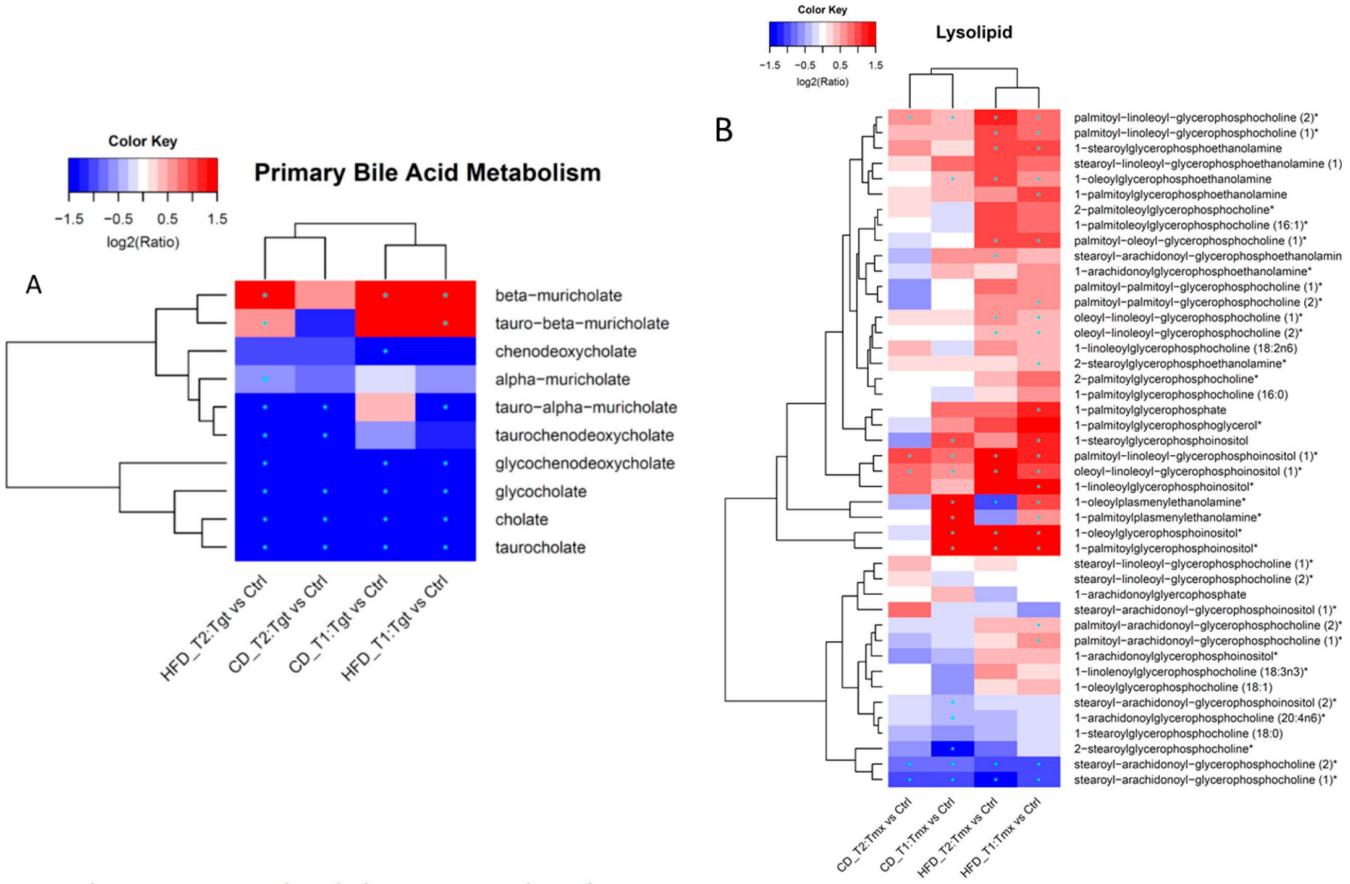
Venn diagrams of metabolites significantly changed in abundance among groups. **(A)** overlap of metabolites significantly changed in abundance at T1 and T2 for HFD vs. CD; **(B)** overlap of metabolites significantly changed in abundance for Tgt vs. control and Tmx vs. Ctrl. Diets: standard diet (CD); high fat diet (HFD); Times: T1, 78 days of age; T2, 190 days of age; Treatments: Ctrl, control; Tgt, targretin; Tmx, tamoxifen.



Hierarchical clustering analysis heatmaps of fold changes on the log2 scales. **(A)** Diet associated (HFD vs. CD); **(B)** Tgt associated (Tgt vs. Ctrl); **(C)** Tmx associated (Tmx vs. Ctrl). * indicates p value < 0.05 , FDR < 0.2 , and fold change ≥ 1.2 . Diets: standard diet (CD); high fat diet (HFD); Times: T1, 78 days of age; T2, 190 days of age; Treatments: Ctrl, control; Tgt, targretin; Tmx, tamoxifen.



Hierarchical clustering analysis heatmaps of *p* values of pathway enrichment using Fisher's exact test. **(A)** super pathways; **(B)** sub-pathways. Diets: standard diet (CD); high fat diet (HFD); Times: T1, 78 days of age; T2, 190 days of age; Treatments: Ctrl, control; Tgt, targretin; Tmx, tamoxifen.



Hierarchical clustering analysis heatmaps of fold changes on the log₂ scales. **(A)** metabolites in sub pathway primary bile acid metabolism related to Tgt treatment; **(B)** metabolites in sub pathway lysolipid related to Tmx treatment. * indicates *p* value < 0.05, FDR < 0.2, and fold change ≥1.2. Diets: standard diet (CD); high fat diet (HFD); Times: T1, 78 days of age; T2, 190 days of age; Treatments: Ctrl, control; Tgt, targretin; Tmx, tamoxifen.