# Spectra Processing, Compound Annotation, Functional Insight and Causal Analysis using MetaboAnalyst 6.0

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# Schedule

## Part I: 2:15 p.m. – 4:15 p.m

- **2:15 – 3:00:** Background
  - ✓ General introduction
  - ✓ LC-MS & MS/MS spectral processing
  - ✓ From peaks to functions
- **3:00 – 3:20**: Live demo
- **3:20 – 4:15:** Hands on practice

## Part II: 4:30 p.m. – 6:30 p.m.

- **4:30 – 5:10**: Background
  - ✓ Data processing
  - ✓ Statistical analysis
  - ✓ Causal analysis
- **5:10 – 5:40**: Live demo
- **5:40 – 6:15**: Hands on practice
- **6:15 – 6:30**: Summary & discussion

# Github Repository

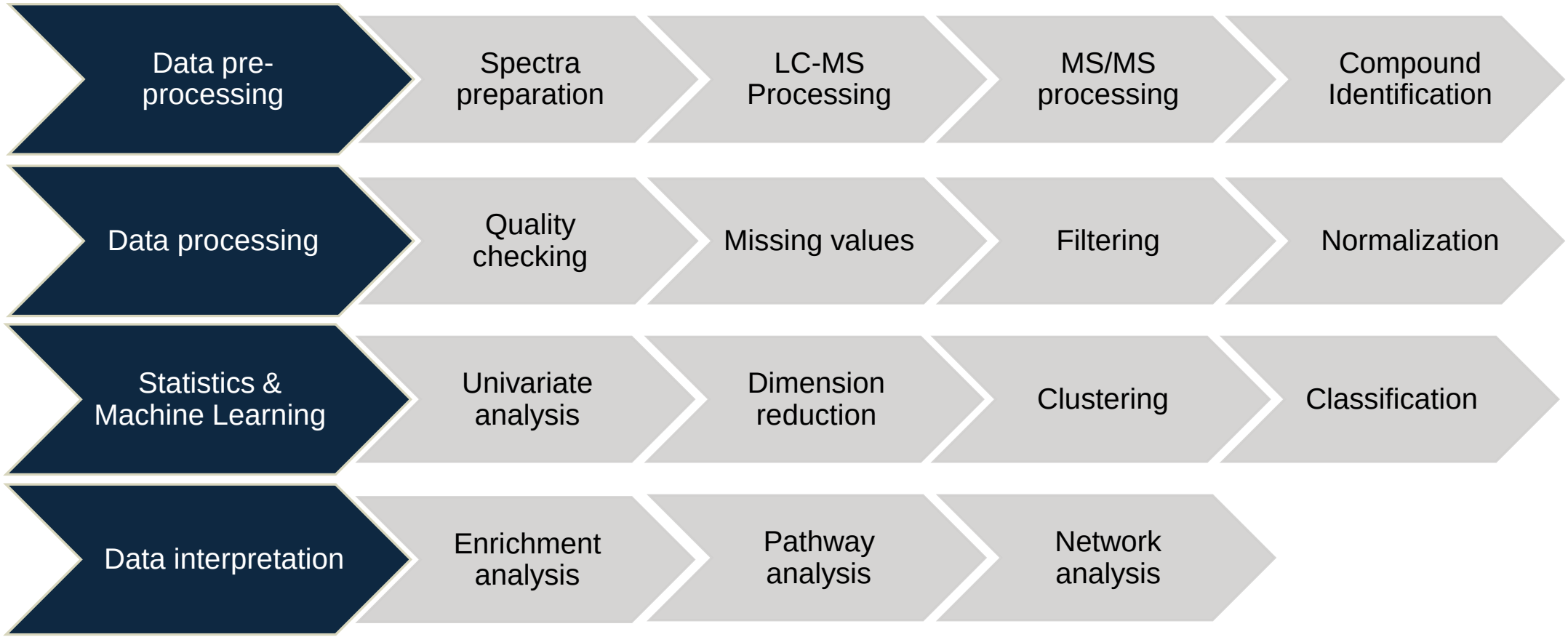
- https://github.com/xia-lab/Metabolomics_2024

- Slides (in PDF format);
- Example data;
- Reference literatures;
- Contact information.

# MetaboAnalyst 6.0 Modules

| **Input Data Type** | **Available Modules** (click on a module to proceed, or scroll down to explore a total of 18 modules including **utilities**) | | | | |
|---|---|---|---|---|---|
| LC-MS Spectra (mzML, mzXML or mzData) | | | Spectra Processing [LC-MS w/wo MS2] | | |
| MS Peaks (peak list or intensity table) | | Peak Annotation [MS2-DDA/DIA] | Functional Analysis [LC-MS] | Functional Meta-analysis [LC-MS] | |
| Generic Format (.csv or .txt table files) | Statistical Analysis [one factor] | Statistical Analysis [metadata table] | Biomarker Analysis | Statistical Meta-analysis | Dose Response Analysis |
| Annotated Features (metabolite list or table) | | Enrichment Analysis | Pathway Analysis | Network Analysis | |
| Link to Genomics & Phenotypes (metabolite list) | | | Causal Analysis [Mendelian randomization] | | |

# Common Tasks for Metabolomics



# Data Processing

# Targeted metabolomics data

| Sample | Label | Acetate | Acetone | Alanine | Betaine | Carnitine | Choline | Citrate | Creatine |
|---|---|---|---|---|---|---|---|---|---|
| Control_01 | 0 | 189.07 | 24.24 | 266.27 | 298.95 | 304.62 | 305.61 | 3969.16 | 366.7 |
| Control_02 | 0 | 386.52 | 26.29 | 612.02 | 313.5 | 122.06 | 78.46 | 2075.6 | 435.99 |
| Control_03 | 0 | 506.28 | 27.84 | 347.32 | 101.49 | 88.82 | 35.88 | 745.5 | 83.39 |
| Control_04 | 0 | 51 | 177.56 | 468.42 | 172.65 | 133.28 | 0 | 10797.57 | 77.69 |
| Control_05 | 0 | | | | | 21 | 187.98 | 1016.97 | 83.39 |
| Disease_01 | 1 | | | | | 04 | 122.4 | 1486.6 | 152.5 |
| Disease_02 | 1 | | | | | 36 | 44.93 | 3327.06 | 263.41 |
| Disease_03 | 1 | 231.59 | 12.91 | 226.63 | 0 | 0 | 35.26 | 758.18 | 50.36 |
| Disease_04 | 1 | 285.55 | 0 | 217.43 | 0 | 77.79 | 0 | 609.23 | 0 |
| Disease_05 | 1 | 353.51 | 15.87 | 699.81 | 98.7 | 458.81 | 112.21 | 3415.49 | 229.79 |

**Targeted metabolomics (samples in rows)**

| Sample | Contr_1 | Contr_2 | Contr_3 | Contr_4 | Contr_5 | Disease_1 | Disease_2 | Disease_3 | Disease_4 | Disease_5 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Label** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **1** |
| Acetate | 189.07 | 386.52 | 506.28 | 51 | 733.45 | 315.12 | 325.39 | 231.59 | 285.55 | 353.51 |
| Acetoacetate | 0 | 0 | 145.96 | 232.69 | 0 | 148.37 | 0 | 56.19 | 0 | 0 |
| Acetone | 24.24 | 26.29 | | | | .29 | | 12.91 | 0 | 15.87 |
| Alanine | 266.27 | 612.02 | | | | .53 | | 226.63 | 217.43 | 699.81 |
| Betaine | 298.95 | 313.5 | | | | 5.1 | | 0 | 0 | 98.7 |
| Carnitine | 304.62 | 122.06 | 88.82 | 133.28 | 89.21 | 32.04 | 89.36 | 0 | 77.79 | 458.81 |
| Choline | 305.61 | 78.46 | 35.88 | 0 | 187.98 | 122.4 | 44.93 | 35.26 | 0 | 112.21 |
| Citrate | 3969.16 | 2075.6 | 745.5 | 10797.57 | 1016.97 | 1486.6 | 3327.06 | 758.18 | 609.23 | 3415.49 |
| Creatine | 366.7 | 435.99 | 83.39 | 77.69 | 83.39 | 152.5 | 263.41 | 50.36 | 0 | 229.79 |

**Targeted metabolomics (samples in columns)**

# Untargeted metabolomics data

| Sample | men_Pt004_RPLCpos | men_Pt007_RPLCpos | men_Pt008_RPLCpos | men_Pt010_RPLCpos | men_Pt013_RPLCpos | men_Pt020_RPLCpos |
|---|---|---|---|---|---|---|
| Label | Covid | Covid | Covid | Covid | Covid | Covid |
| 68.9948__646.68 | 1158657.48719 | 1151894.23428 | 1189086.78073 | 1285945.06468 | 1196004.21814 | 1018581.17851 |
| 69.995__641.77 | 107433.72307 | 126812.99598 | 124835.38621 | 160081.36667 | 132737.27627 | 120183.30701 |
| 70.0663__32.7 | 10385.57069 | 13137.07876 | 15305.24912 | 5714.296 | 1831.73589 | 627.95873 |
| 81.5222__642.31 | 209861.50958 | 194875.48234 | 210983.40708 | 211579.43879 | 209288.2791 | 208780.21243 |
| 84.0461__30.06 | 53988.89851 | 44693.63728 | 54104.51066 | 34887.7869 | 23436.84666 | 46781.95438 |
| 84.9606__643.25 | 186784.7 | 181153.3814 | 285718.9142 | 291334.92371 | 189987.5361 | 134815.70457 |
| 84.9607__664.62 | | | | | 2.48874 | 124820.09035 |
| 84.9607__716.7 | 4 | | | | 6.55494 | 749034.65826 |
| 85.0302__30.53 | | | | | 7.91671 | 2616.3663 |
| 86.0976__89.04 | | | | | 7.66029 | 129637.63897 |
| 87.5108__642.38 | 29017.3417 | 48430.91348 | 29443.40354 | 50376.17085 | 45549.23108 | |
| 88.5106__646.42 | 467439.8206 | 452376.17516 | 486908.8982 | 488320.52256 | 506518.4509 | |
| 89.5084__646.45 | 8408192.9225 | 8132241.81496 | 8477437.65518 | 8543761.98101 | 8679306.12814 | |
| 89.6056__646.48 | 101573.6793 | 89544.45735 | 97535.64361 | 116017.93274 | 127775.03719 | |
| 89.9406__643.85 | 169537.75028 | 169867.84414 | 201913.54163 | 156549.447 | 152568.62769 | |
| 90.0093__646.69 | 839967.52451 | 823526.37057 | 859947.96708 | 900633.07574 | 877001.83348 | |
| 90.5085__641.82 | 1539089.51759 | 1263856.98911 | 1444405.93286 | 1983990.84075 | 1470945.60964 | |
| 90.5086__482.02 | 14153278.42237 | 14091173.41408 | 14308006.37643 | 14770096.07378 | 14987402.61328 | |

**Untargeted metabolomics LC-MS peaks**

| Sample | Class | Bin.9.98 | Bin.9.94 | Bin.9.90 | Bin.9.86 | Bin.9.82 | Bin.9.78 | Bin.9.74 | Bin.9.70 | Bin.9.66 |
|---|---|---|---|---|---|---|---|---|---|---|
| P002 | patient | 2.00E-05 | 2.00E-05 | 2.00E-05 | 2.00E-05 | 4.00E-05 | 2.00E-05 | 1.00E-05 | 0 | 2.00E-05 |
| P012 | patient | -3.00E-05 | -3.00E-05 | -3.00E-05 | -1.00E-05 | -1.00E-05 | 0 | 3.00E-05 | 3.00E-05 | 4.00E-05 |
| P014 | patient | 0.00024 | 0.00017 | 0.00016 | 0.00018 | 0.00014 | 0.00015 | 0.00016 | 2.00E-04 | 0.00021 |
| P027 | patient | 9.00E-05 | 0.00013 | 9.00E-05 | 9.00E-05 | 0.00014 | 1.00E-04 | 0.00014 | 0.00016 | 0.00013 |
| P034 | patient | 0.00012 | 0.00015 | 2.00E-05 | 3.00E-05 | 7.00E-05 | 7.00E-05 | 3.00E-05 | 4.00E-05 | 0.00012 |
| P037 | patient | 1.00E-05 | -1.00E-05 | 2.00E-05 | -3.00E-05 | -6.00E-05 | 6.00E-05 | 4.00E-05 | 6.00E-05 | -9.00E-05 |
| P038 | patient | 1.00E-05 | 1.00E-05 | 1.00E-05 | -5.00E-05 | -1.00E-05 | -5.00E-05 | 2.00E-05 | 5.00E-05 | 6.00E-05 |
| P041 | patient | -9.00E-05 | -6.00E-05 | -7.00E-05 | 1.00E-05 | -0.00012 | -6.00E-05 | -1.00E-05 | -1.00E-05 | -3.00E-05 |
| P042 | patient | 0.00015 | 8.00E-05 | 0.00017 | 3.00E-05 | 3.00E-05 | -0.00011 | 2.00E-05 | 6.00E-05 | 0.00015 |
| P049 | patient | | | | | | | | -05 | 2.00E-05 |
| P056 | patient | | | | | | | | -05 | 0 |
| P058 | patient | | | | | | | | -05 | -3.00E-05 |
| P060 | patient | | | | | | | | -05 | 2.00E-05 |
| P064 | patient | | | | | | | | -05 | 7.00E-05 |
| P065 | patient | | | | | | | | -05 | 5.00E-05 |
| P070 | patient | -1.00E-05 | -1.00E-05 | -1.00E-05 | -3.00E-05 | -1.00E-05 | 0 | 0 | -2.00E-05 | 3.00E-05 |
| P080 | patient | 0 | 1.00E-05 | 0 | 0 | 1.00E-05 | 0 | 2.00E-05 | 2.00E-05 | 3.00E-05 |
| P085 | patient | 1.00E-05 | 7.00E-05 | 5.00E-05 | 5.00E-05 | 6.00E-05 | 3.00E-05 | 2.00E-05 | 1.00E-04 | 6.00E-05 |
| P086 | patient | -1.00E-05 | 1.00E-05 | 1.00E-05 | -1.00E-05 | -1.00E-05 | 3.00E-05 | -2.00E-05 | 7.00E-05 | -2.00E-05 |
| P089 | patient | 1.00E-05 | 0 | 1.00E-05 | 2.00E-05 | 3.00E-05 | 3.00E-05 | 4.00E-05 | 5.00E-05 | 6.00E-05 |
| P092 | patient | -3.00E-05 | -3.00E-05 | -3.00E-05 | -1.00E-05 | 0 | 0 | 3.00E-05 | -1.00E-05 | 3.00E-05 |
| P099 | patient | 0 | 0 | 2.00E-05 | -1.00E-05 | -4.00E-05 | 3.00E-05 | 5.00E-05 | 1.00E-05 | 4.00E-05 |
| P113 | patient | -2.00E-05 | 1.00E-05 | -4.00E-05 | -2.00E-05 | -7.00E-05 | -4.00E-05 | -1.00E-05 | 1.00E-05 | -4.00E-05 |
| P013b | patient | -2.00E-05 | -2.00E-05 | -1.00E-05 | -1.00E-05 | -2.00E-05 | 0 | 0 | 1.00E-05 | 0 |

**Untargeted metabolomics NMR bins**

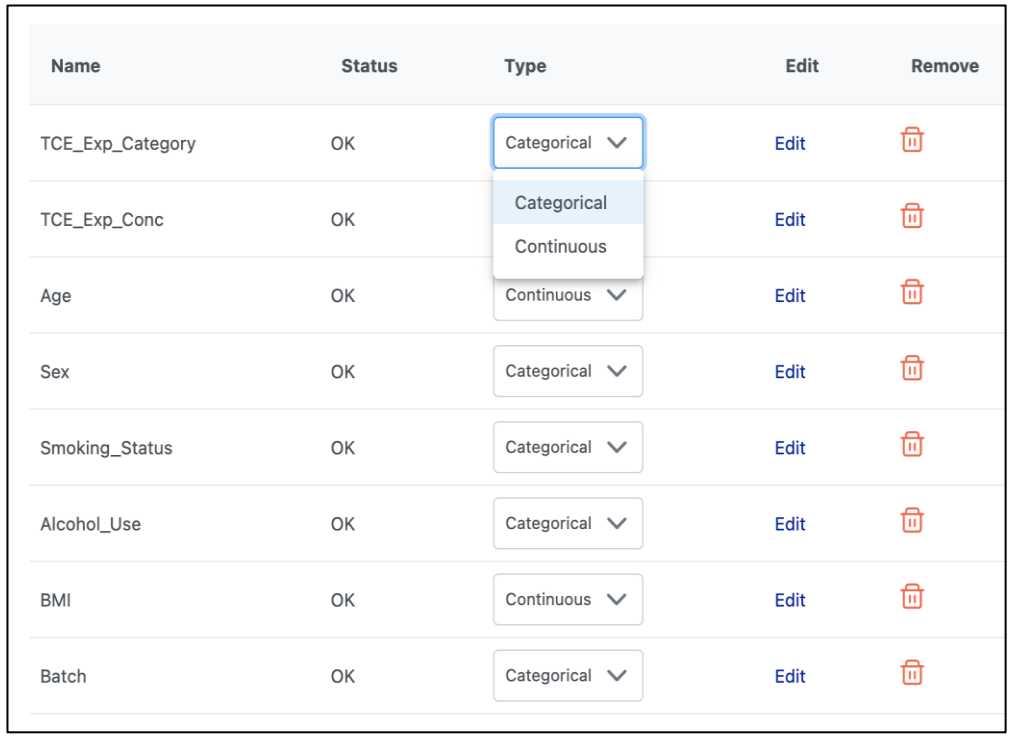
# Metadata table

- Common in observational field studies
  - Clinical
  - Exposomics
  - Epidemiology
- Study design & context
  - Primary experimental factors
  - Covariates

| Sample | Diagnosis | Gender | Treatment | Age |
|---|---|---|---|---|
| S1 | COVID | Male | non_Treated | 62 |
| S2 | COVID | Male | non_Treated | 44 |
| S3 | COVID | Male | Treated | 54 |
| S4 | COVID | Male | non_Treated | 62 |
| S5 | COVID | Male | Treated | 82 |
| S6 | COVID | Male | Treated | 65 |
| S7 | COVID | Female | Treated | 49 |
| S8 | COVID | Female | Treated | 42 |
| S9 | COVID | Female | Treated | 56 |
| S10 | COVID | Female | Treated | 56 |
| S11 | COVID | Female | Treated | 69 |
| S12 | HC | Male | non_Treated | 24 |
| S13 | HC | Female | non_Treated | 38 |
| S14 | HC | Female | non_Treated | 42 |
| S15 | HC | Female | non_Treated | 40 |
| S16 | HC | Female | non_Treated | 56 |
| S17 | HC | Male | non_Treated | 57 |
| S18 | HC | Male | non_Treated | 57 |
| S19 | HC | Male | non_Treated | 60 |
| S20 | HC | Male | non_Treated | 62 |
| S21 | HC | Male | non_Treated | 55 |

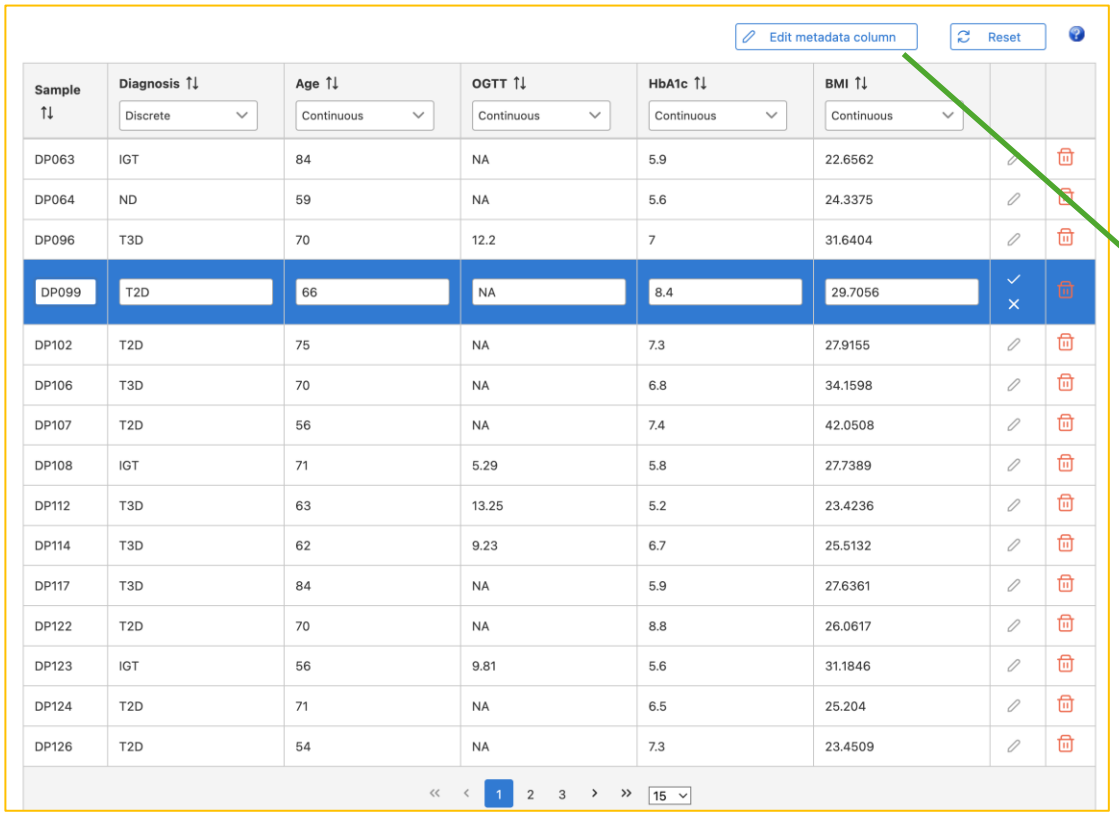
# Understanding & formatting metadata

- Essential for downstream analysis
- Categorize as “Categorical” or “Continuous”
  - For categorical, must have at least 2 groups with at least 3 replicates each
- No missing values
- First meta-data column will be considered primary variable by default

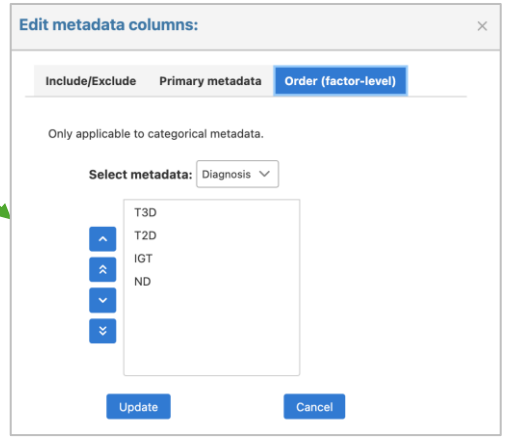


| Name | Status | Type | Edit | Remove |
|---|---|---|---|---|
| TCE_Exp_Category | OK | Categorical | Edit | |
| TCE_Exp_Conc | OK | Categorical / Continuous | Edit | |
| Age | OK | Continuous | Edit | |
| Sex | OK | Categorical | Edit | |
| Smoking_Status | OK | Categorical | Edit | |
| Alcohol_Use | OK | Categorical | Edit | |
| BMI | OK | Continuous | Edit | |
| Batch | OK | Categorical | Edit | |

# View and edit metadata




Edit



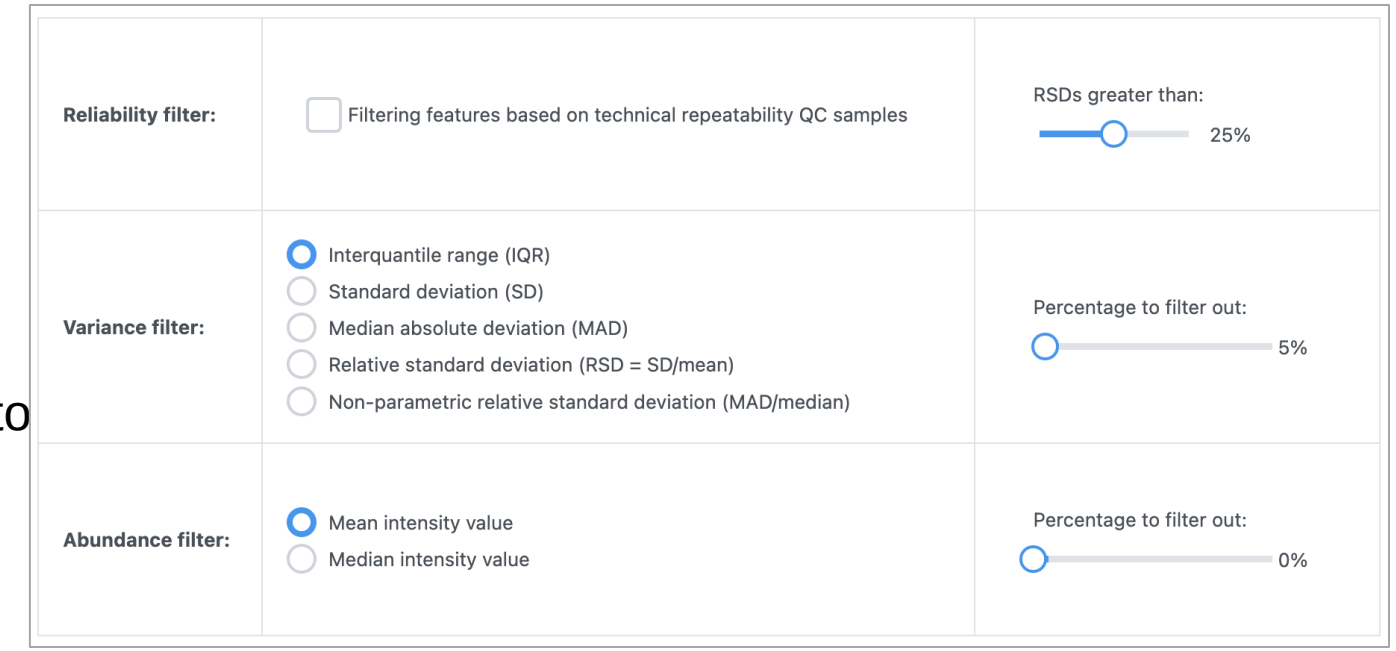
# Feature filtering (I)

- ❖Not all features are informative
- ❖There are redundancies in omics data for most features
- ❖Filtering non-informative features before statistical analysis can often <u>significantly</u> improve the power

# Feature filtering (II)

## Three types of filters

- **Low reliability filter**
  - Too many missing values
  - Hard to measure metabolites: low repeatability based on QC samples
- **Low abundance filter**
  - Variables of very small values (close to baseline or detection limit).
- **Low variance filter**
  - Variables that are near-constant values throughout the experiment conditions (housekeeping or homeostasis)

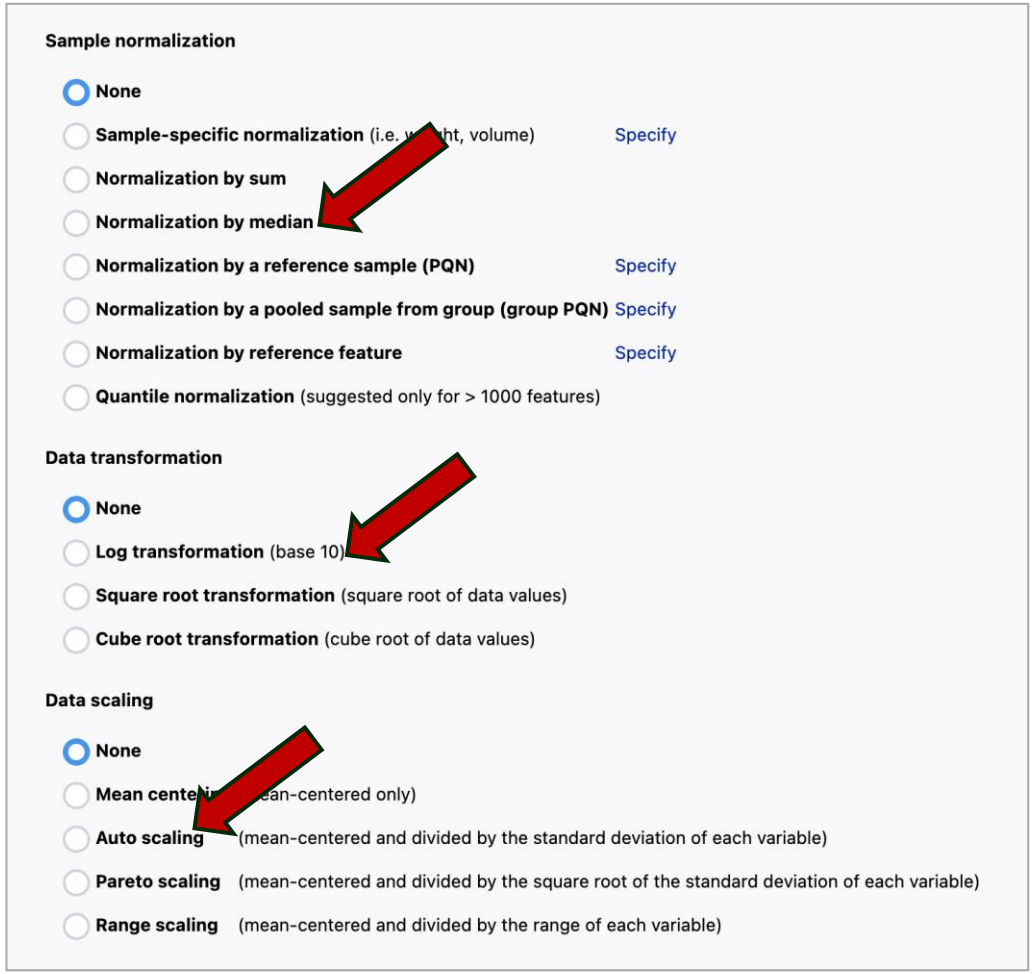



**DO NOT filter features based on their p-values or fold changes at this stage**

# Normalization

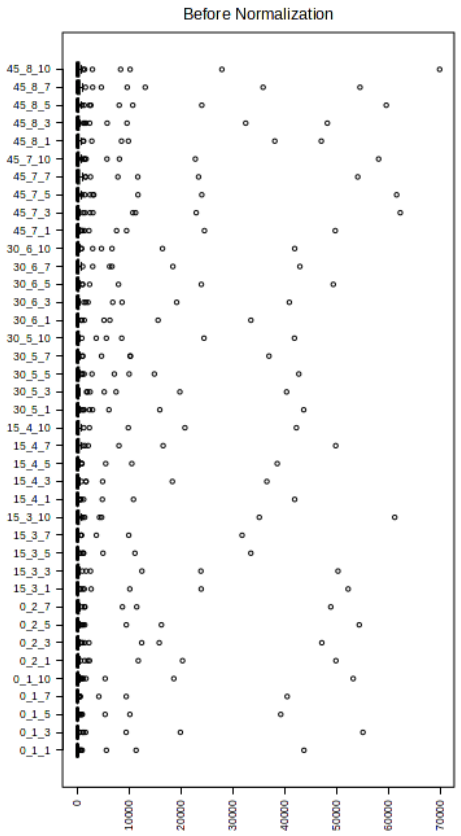
## Three types of normalization

- Sample-wise normalization aims to make each sample (row) comparable to each other (i.e. tissue volume,  urine samples with different dilution effects)
- Data transformation (such as log, cubic root) transform <u>individual values independently</u>
  - Most metabolomics data are log-normal
- Data scaling (auto, pareto) takes into consideration of the distribution (range, SD) of individual features
  - Unique to the current data

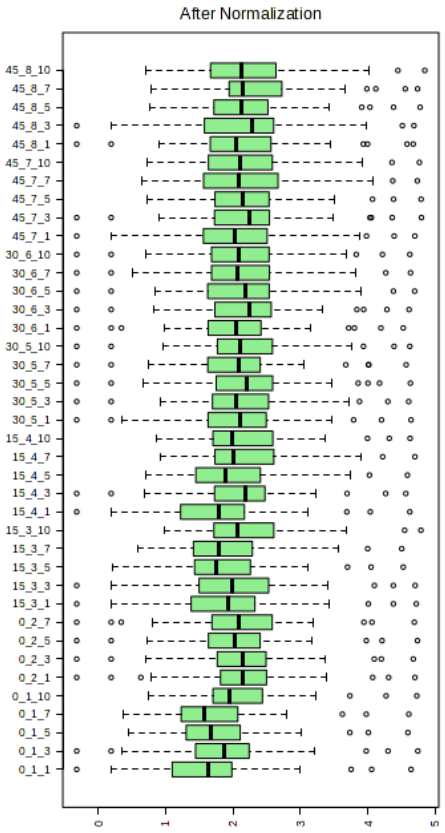


# Sample-level normalization

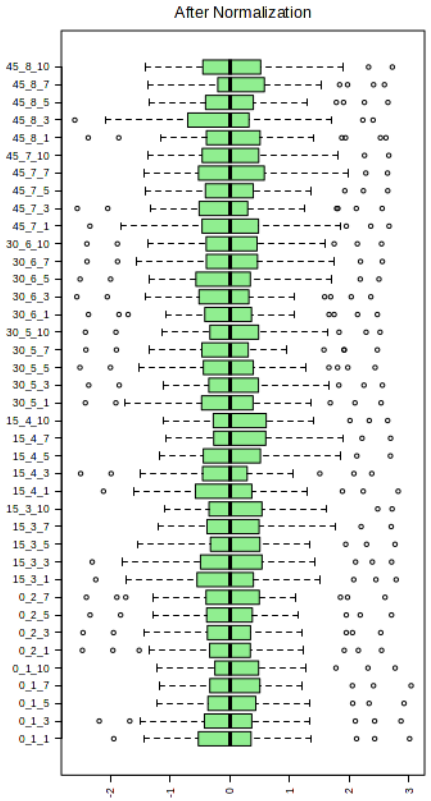
- Adjusting technical or biological bias or inconsistencies




**Raw**



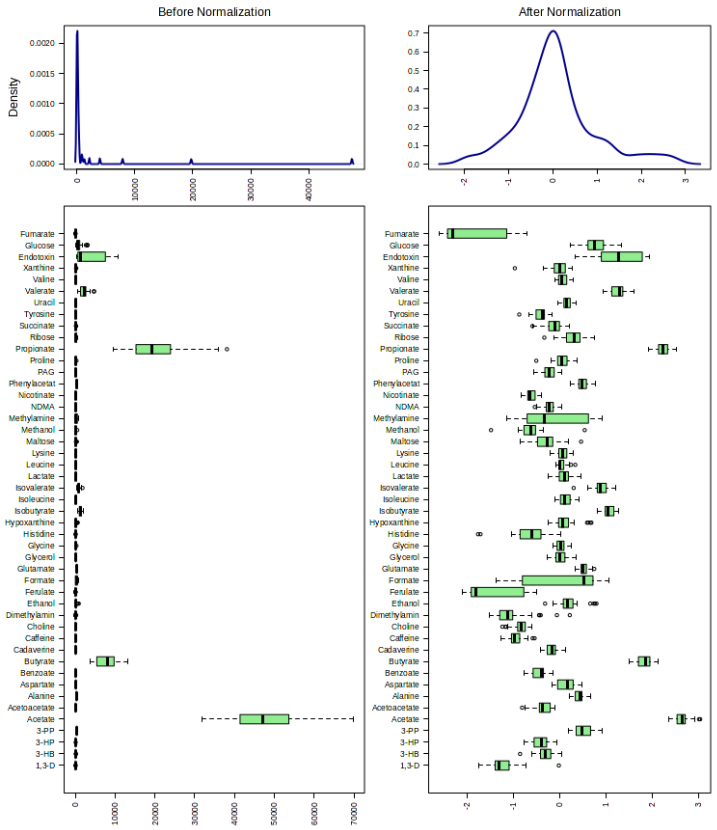

**Log**



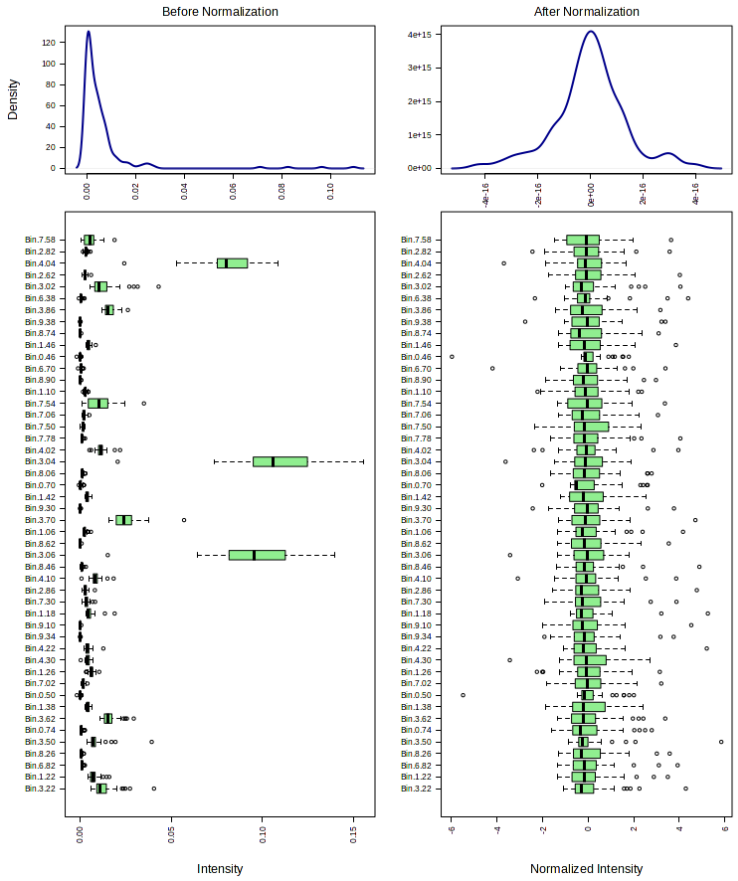

**Median norm + Log**

# Feature-level normalization

- Making features more “normal” and comparable




**Log transformation**




**Auto-scaling**

# Statistical Analysis

-- identify significant features & patterns

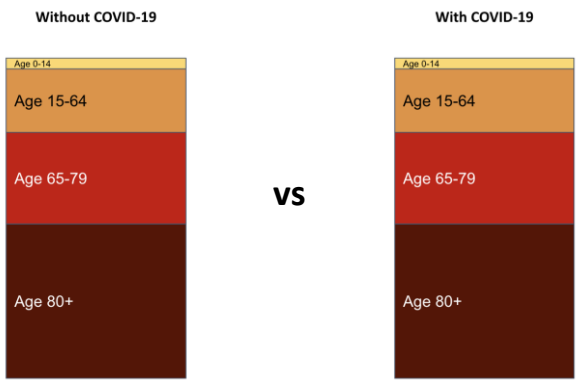
# Univariate Analysis

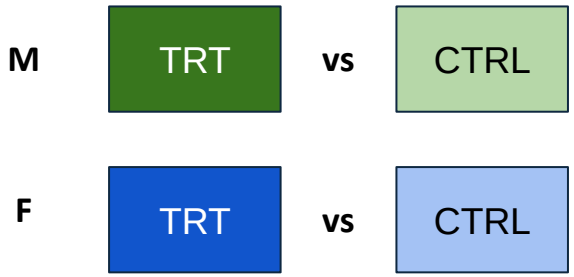
Test each feature individually (ignore their correlations)

1. T-tests & Fold Change Analysis
   - Compare the means between 2 conditions
2. ANOVA & post-hoc analysis
   - One factor with more than 2 levels (One-way ANOVA)
   - Two factors (Two-way ANOVA)
3. Linear modeling (i.e., limma): more flexible analysis
   - Multiple factors (metadata table)
     - Time series
     - Covariates analysis

# Dealing with covariates

- Experimental design:
  - Control group that ‘matches’ your group of interest
- Data analysis:
  1. Reduce the number of factors
     - use PCA + other tools to choose only factors with most influence on the data
  2. Stratification - for factors with few classes, split up the data and analyze separately
     - Analyze male / female separately
  3. Taking into account
     - Covariates

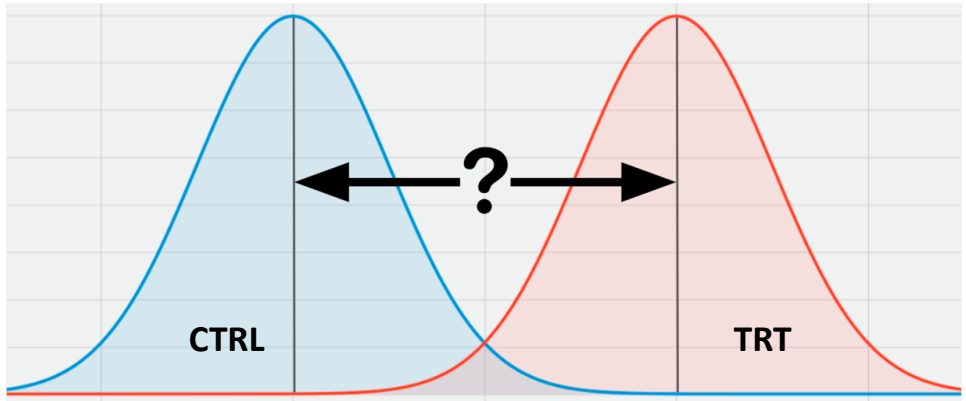


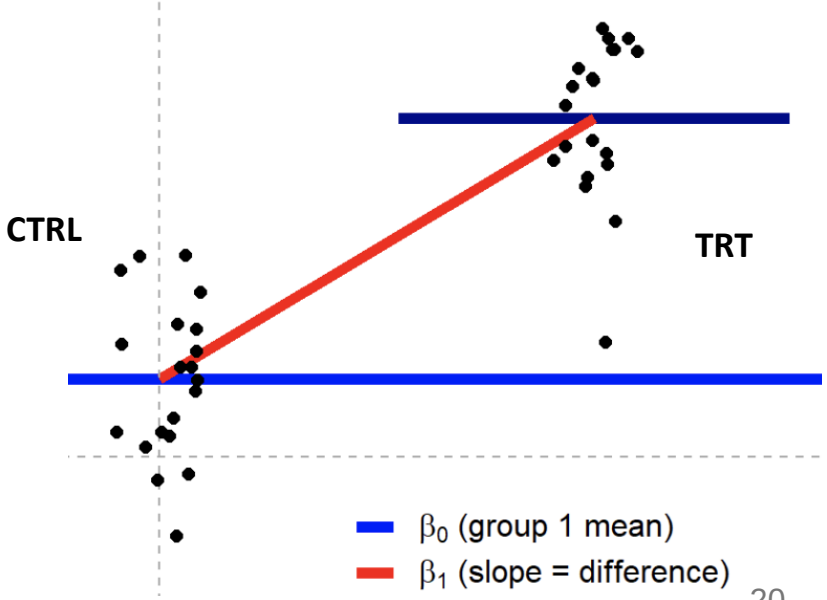




$$y = b_0 + b_1 * x_1 + b_2 * x_2 + \ldots + b_n * x_n$$

# T-test vs. linear regression

- You can do t-test with linear regression:
- $y = B_0 + B_1 * x$
  - y: level of metabolite A
  - x: variable of interest
  - Categorical variables expressed using 'dummy variables'
  - Null hypothesis: $B_1 = 0$
  - Alternative hypothesis: $B_1 > 0$



# Linear regression is flexible

- Linear regression is more flexible than the classical t-test:
  - Predictor variables (x) can be continuous or categorical
  - You can have **multiple predictor variables**
- Multiple linear regression:
  - $y = B_0 + B_1 * x_{diagnosis} + B_2 * x_{age}$
  - Coefficient estimates: relationship between $x_i$ and y *with all other variables held constant*
  - We can generate a t-stat for any coefficient using the same formula from before: $t_i$ = ($B_i$ coefficient estimate) / (standard error of $B_i$ coefficient estimate)
- Coefficients are unstable when the predictors are highly correlated
  - Use PCA/heatmap to detect redundancies and consolidate factors

# Method overview

## Single-factor study design

**Univariate Analysis**

Fold Change Analysis  T-tests  Volcano plot

One-way Analysis of Variance (ANOVA)

Correlation Heatmaps  Pattern Search  Correlation Networks (DSPC)

**Advanced Significance Analysis**

Significance Analysis of Microarray (and Metabolites) (SAM)

Empirical Bayesian Analysis of Microarray (and Metabolites) (EBAM)

**Chemometrics Analysis**

Principal Component Analysis (PCA)

Partial Least Squares - Discriminant Analysis (PLS-DA)

Sparse Partial Least Squares - Discriminant Analysis (sPLS-DA)

Orthogonal Partial Least Squares - Discriminant Analysis (orthoPLS-DA)

**Cluster Analysis**

Hierarchical Clustering: Dendrogram  Heatmaps

Partitional Clustering: K-means  Self Organizing Map (SOM)

**Classification & Feature Selection**

Random Forest

Support Vector Machine (SVM)

## Multi-factor study design

**Data and Metadata Overview**

Metadata Visualization

Users can explore the metadata patterns and correlations through intuitive graphics. It is very useful for users to identify highly dependent metadata and quickly assess the overall patterns of the metadata.

Interactive PCA Visualization

Users can visualize data using different colors or shapes based on selected metadata in an 2D and 3D (interactive) PCA plots. It is very useful to detect overall patterns of data with regard to different metadata.

Hierarchical Clustering and Heatmap Visualization

This method displays data and metadata in the form of colored cells. It provides direct visualization of feature abundances across different samples and metadata.

**Univariate Analysis**

Linear Models with Covariate Adjustment

This approach uses linear models (limma or lm) to perform significance testing with covariate adjustments. Users can choose different metadata to be included in the analysis.

Correlation and Partial Correlation Analysis

This approach allows users to explore the correlations or partial correlations (with covariate adjustments) between metabolomics features and different metadata of interest.

Two-way ANOVA (ANOVA2)

This approach provides classical two-way ANOVA based on the two factors selected by users. For time-series data, users should choose within-subjects ANOVA.

**Multivariate Analysis**

ANOVA Simultaneous Component Analysis (ASCA)

This approach is designed to identify major patterns with regard to the two given factors and their interaction. The implementation was based on the algorithm described by AK Smildle, et al. with additional improvements on feature selection and model validation.

Multivariate Empirical Bayes Analysis of Variance (MEBA) for Time Series

This approach is designed to compare temporal profiles across different biological conditions. It is based on the timecourse method described by YC Tai. et al.

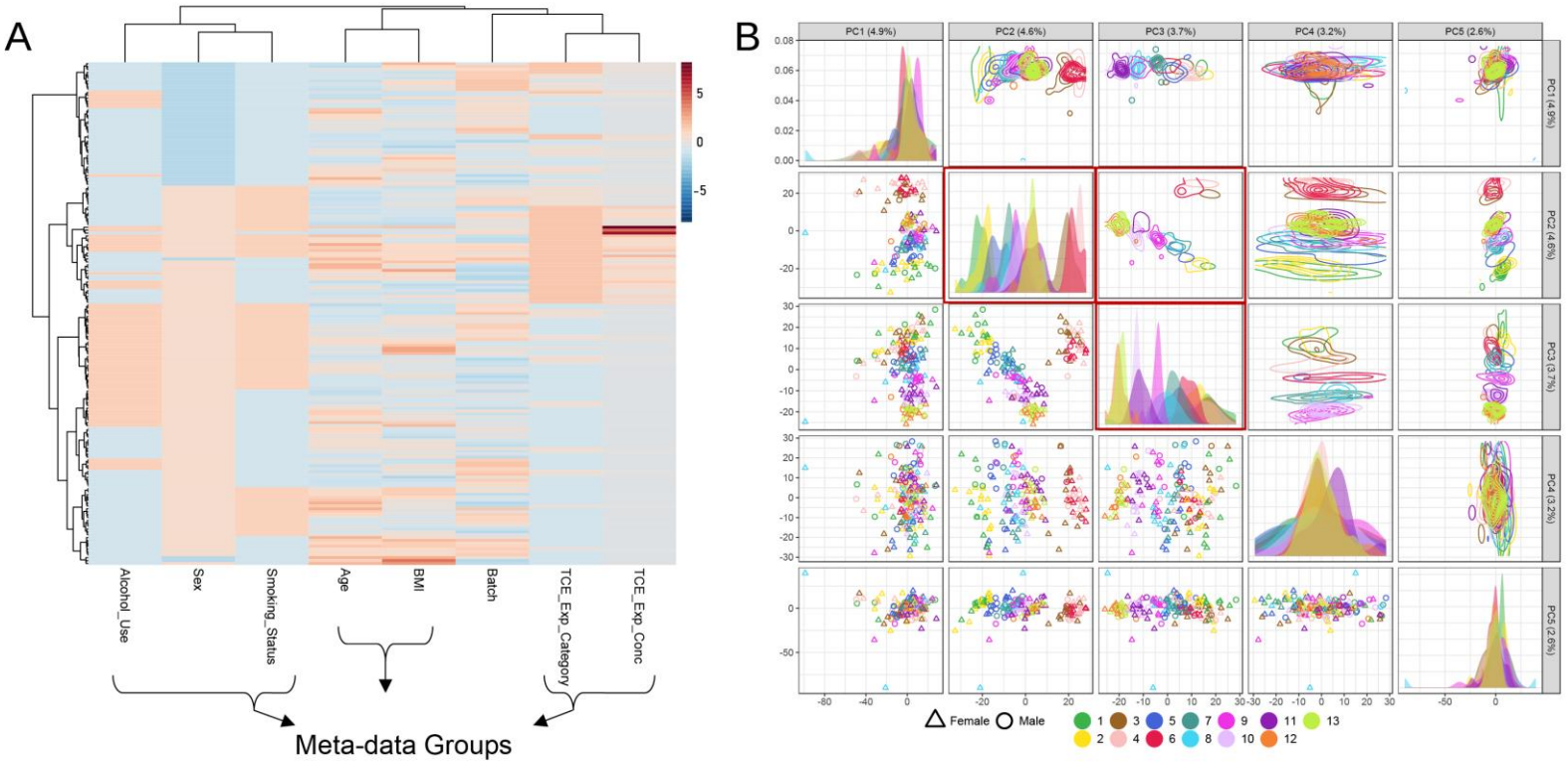
**Supervised Classification**

Random Forest

This machine learning approach is designed to perform classification and feature selection analysis. Users can also test contribution of meta-data to class prediction.

# Reducing metadata factors in analysis

- Factors maybe redundant (colinear)
  - ➔ Keep only one
- Overlay factors on omics data using PCA
  - ➔ Keep those with effects



# Linear models for complex design

Simple Metadata | Complex Metadata

**Primary metadata:** Diagnosis

**Reference group:** T3D

**Contrast:** All contrasts (ANOVA-style)

**Covariates (control for):**

**Blocking factor:**

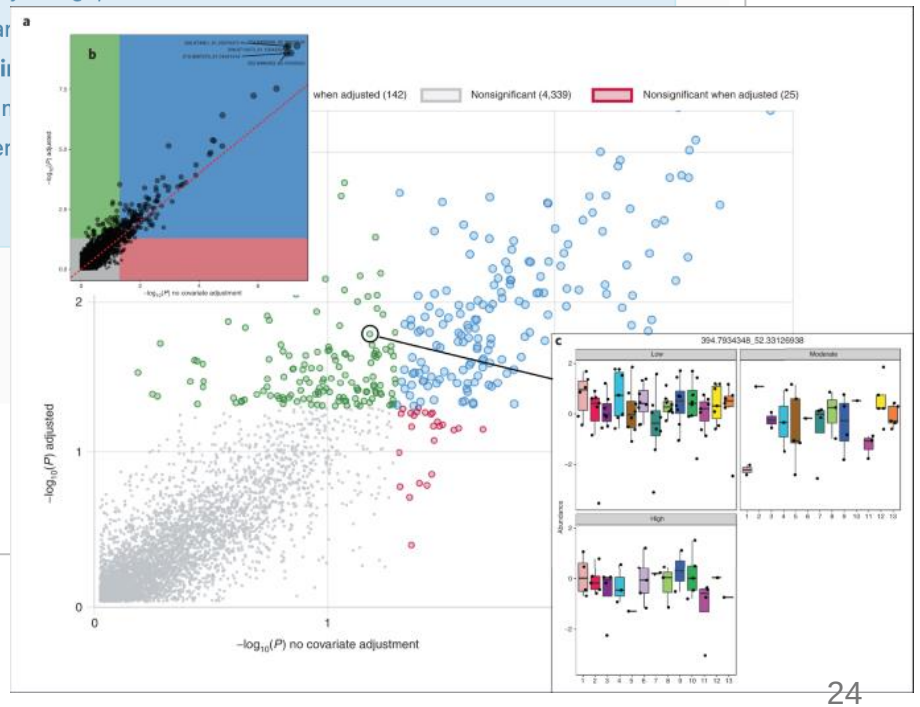
**Adjust using robust trend**

- --- Not Available ---
- Diagnosis
- Age
- OGTT
- HbA1c
- BMI

Submit

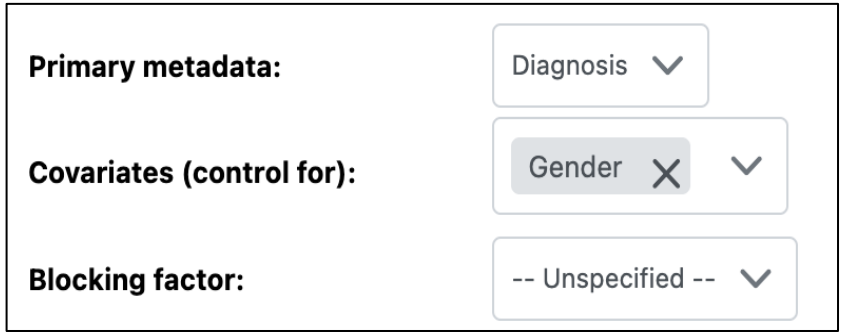
**Did you know?**

To perform multi-factor comparison analysis for complex metadata, we leverage the linear models with covariate adjustments of limma for its high-performance implementation. Some data may include some form of blocking in the study design, which can be modeled as either fixed or random effects. Please note that although you car
effects), we in general recommend **keepi**
effect model not only is computationally r
consistent with the interpretation of differ
(2009) for more technical discussions.



# Fixed vs. random effects

- Fixed effects = covariates
  - Simply variables included in the regression model
  - Age, sex, tissue, etc
- Random effects = blocking factor
  - Accounted for with multi-level modeling
  - Batch, subjects
- In general, we recommend using fixed effects for simplicity & computational efficiency in most cases. Treating the blocks as a fixed effect has the huge advantage of being able to quantify block-by-factor interactions which is perhaps the best method to quantify the robustness of your design structure.

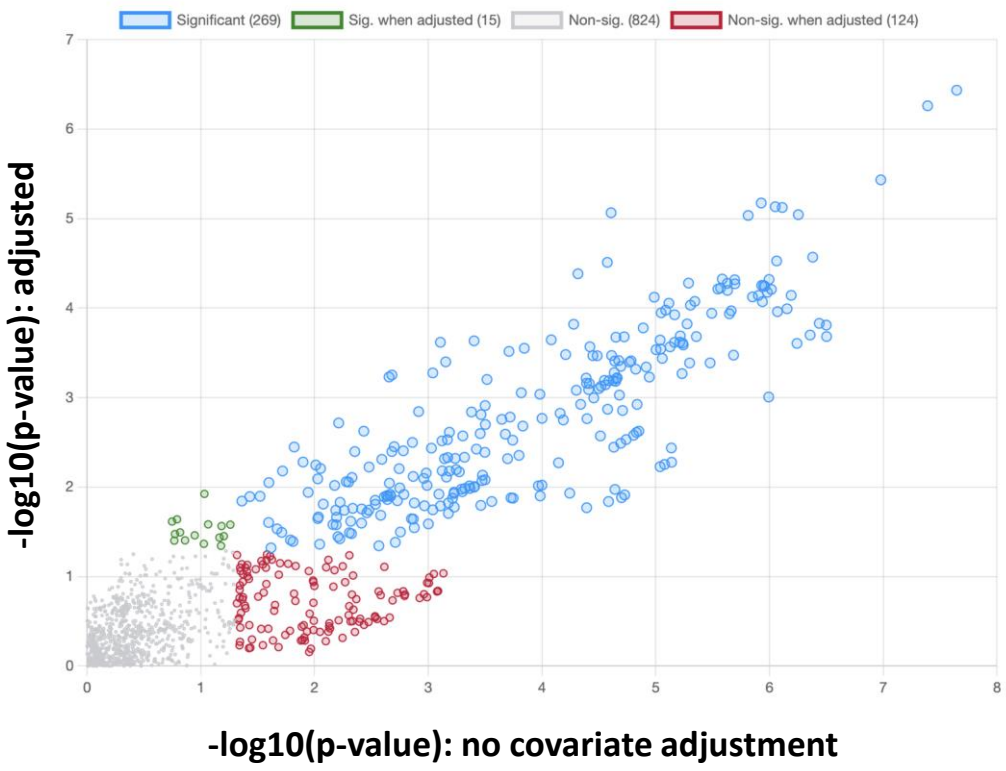


# Interpretation of coefficient results

Multiple linear regression example:

- $y = B_0 + B_1 * x_{diagnosis} + B_2 * x_{age}$

- By including $B_2 * x_{age}$ in the model, we account for effects of age
- Extract $B_1$ from the model:
  - $B_1$ value = magnitude & direction of relationship between metabolite 'y' and $x_{diagnosis}$
  - $B_1$ p-value = statistical significance of relationship




**Linear model with covariate adjustment**

# Multivariate Analysis

# Principal Component Analysis (PCA)

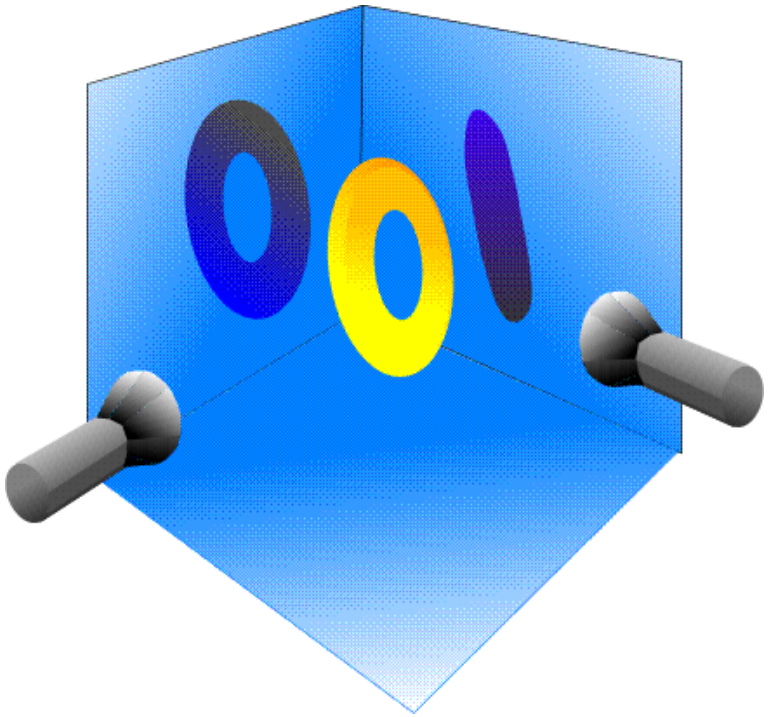
- Project high-dimensional omics data into low dimensions (two or three PCs)
- Works best when there are a large number of variables are correlated
  - This is particularly relevant for untargeted metabolomics where single metabolites can generate several peaks
- PCA is very useful for:
  - Data overview
  - Outlier detection
  - Look at relationships between variables

# Principal Component Analysis (PCA)

- Project high-dimensional data into lower dimensions that capture the most variance of the data
- Assumption:

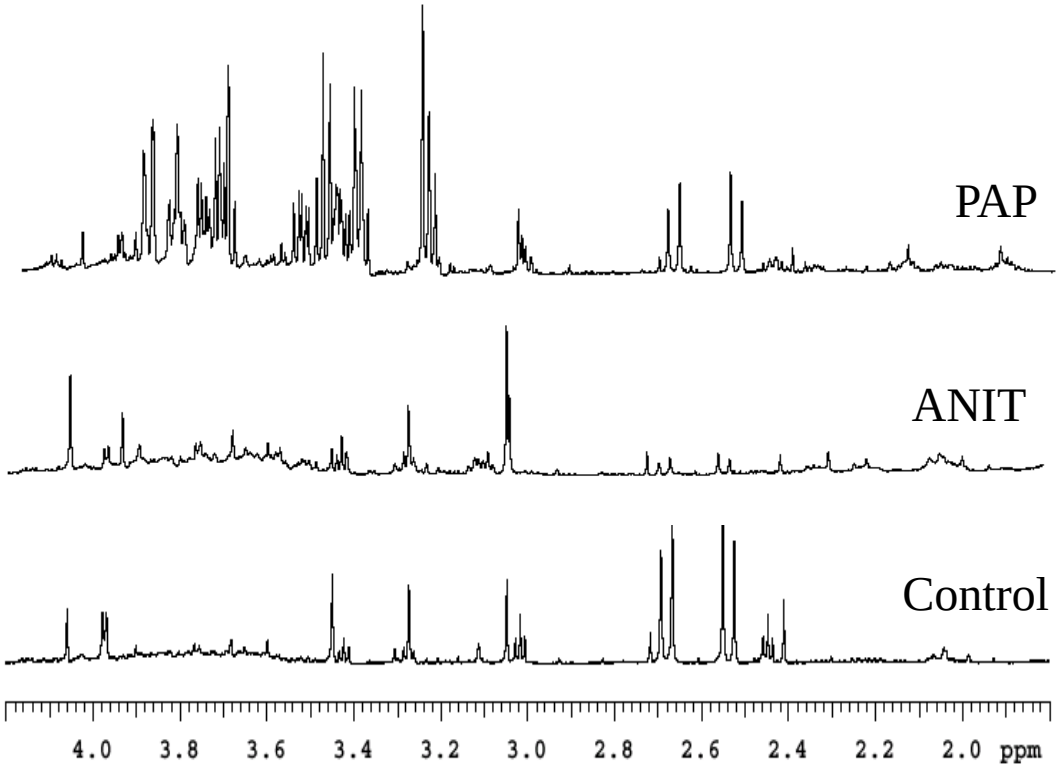
  **Main directions of variance**

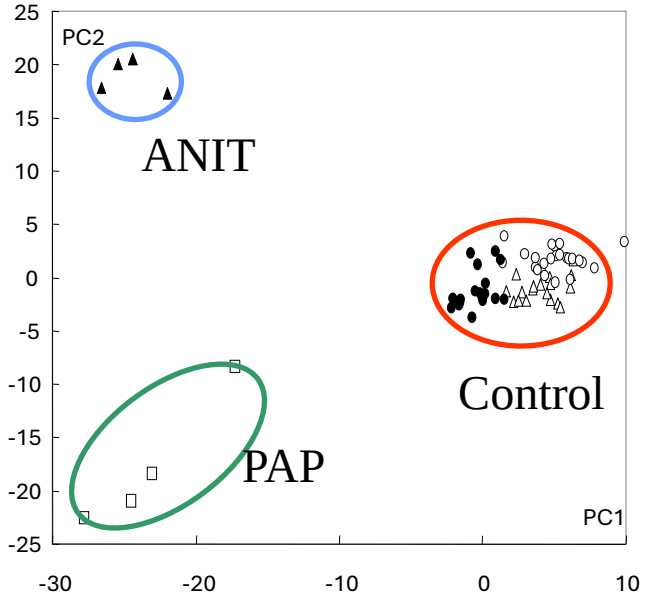
  **≈ major data characteristics**



# PCs capture the main variance in omics data

**Hundreds of variables** → **2 components**




**Scores plot**

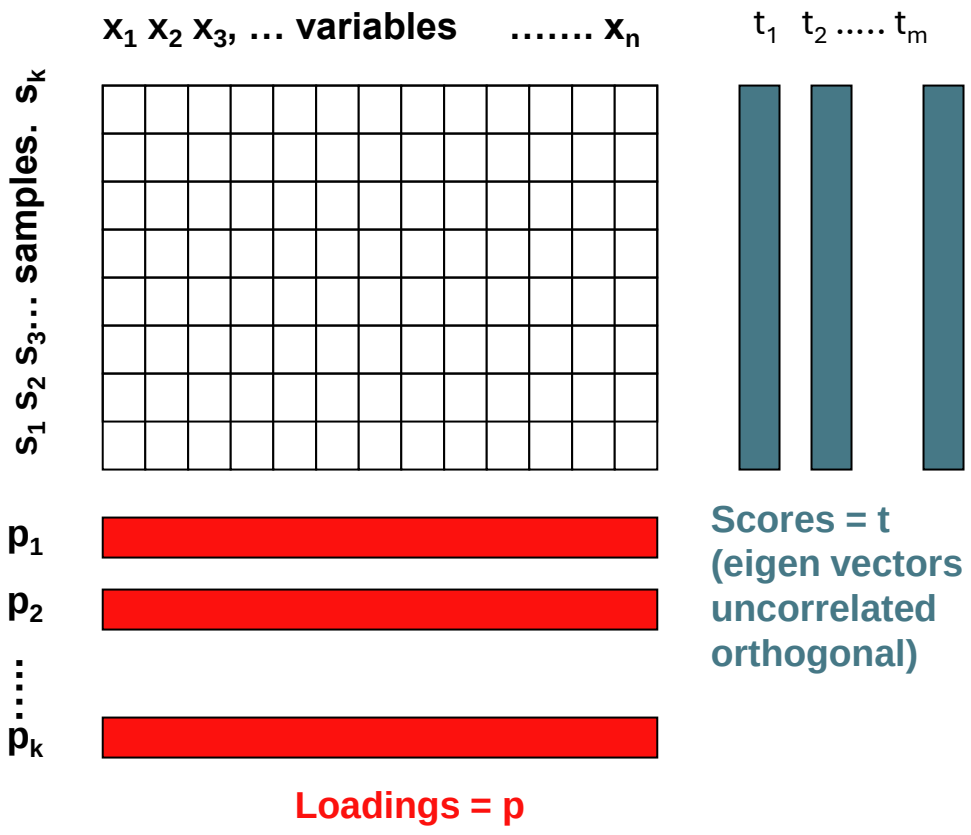
# PCA – under the hood

- Orthogonal linear transformation
- PCA transforms data to a new coordinate system so that the greatest variance of the data comes to lie on the first coordinate (1st PC), the second greatest variance on the 2nd PC etc.

**Scores = Loadings x Data**

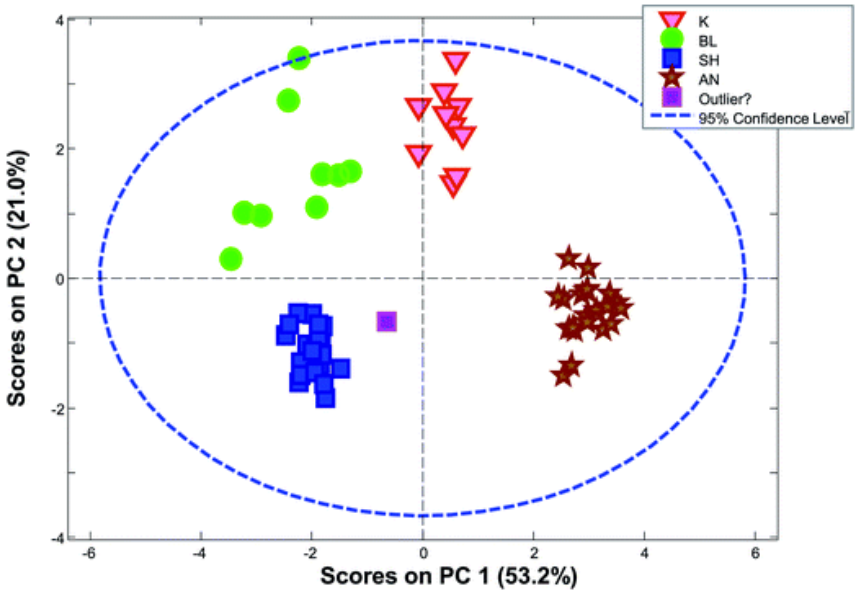
$t_1 = p_1x_1 + p_2x_2 + p_3x_3 + \ldots + p_nx_n$

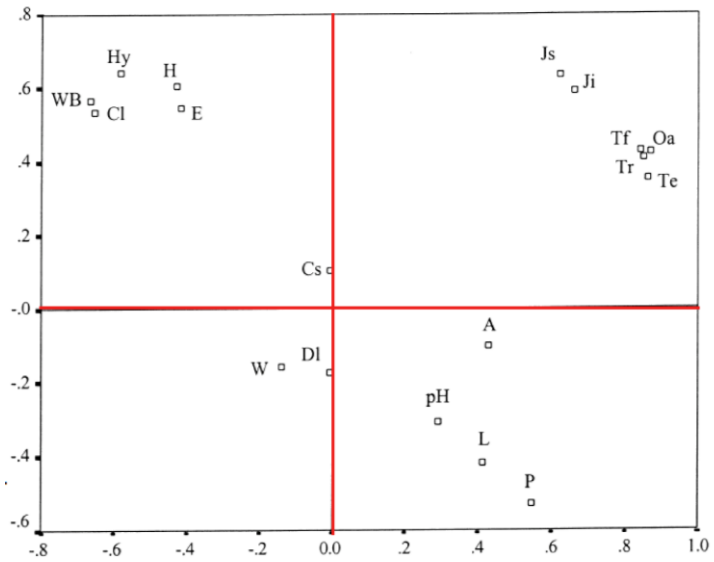
.....



# Scores & loadings plots

➢Sample patterns (scores) are directly related to feature patterns (loadings)




Loadings

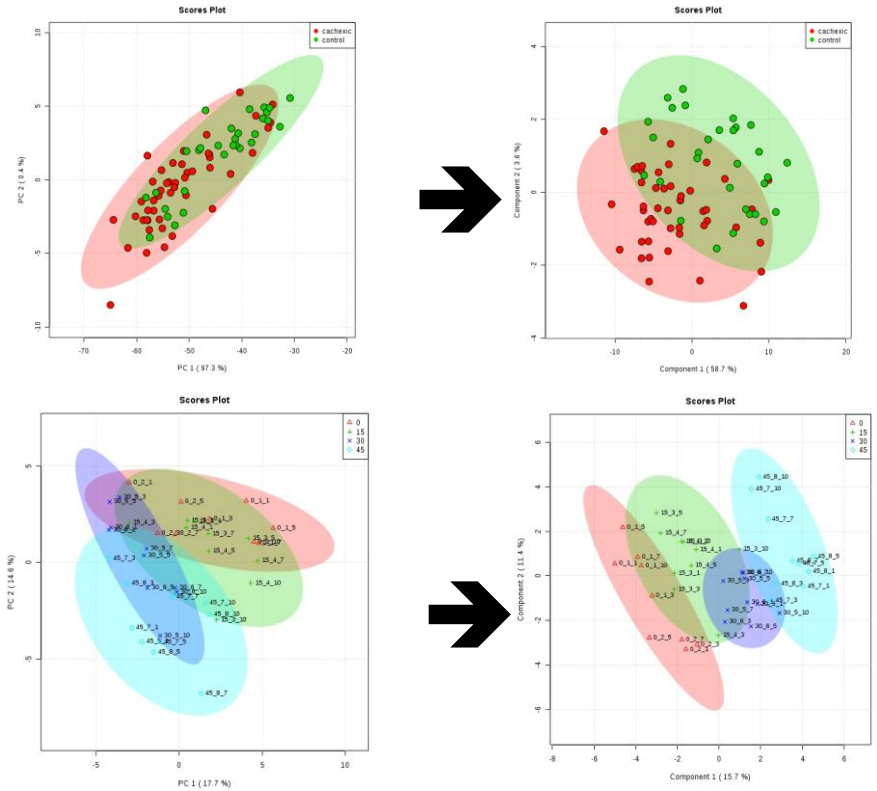
**Scores = Loadings x data**

$$t_1 = p_1x_1 + p_2x_2 + p_3x_3 + \ldots + p_nx_n$$

.....

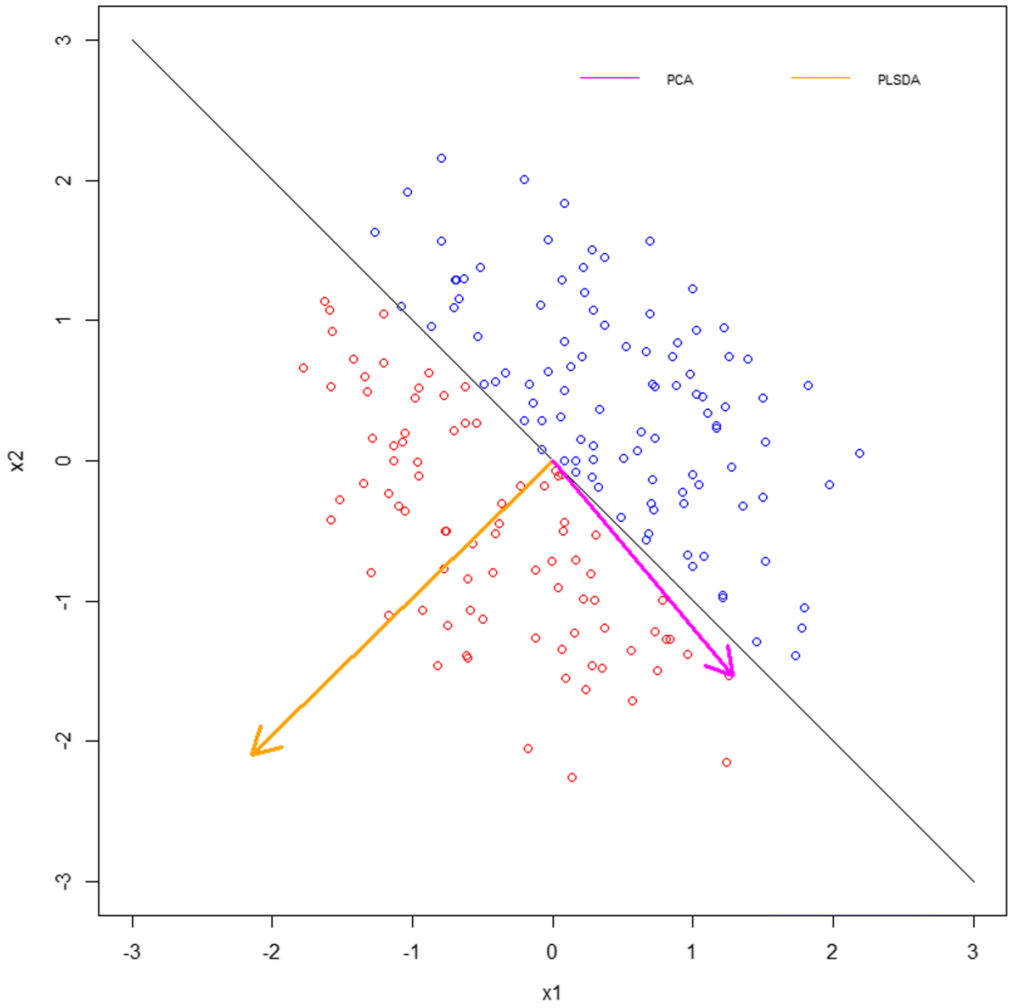
# Partial least squares - discriminant analysis (PLS-DA)


- When the experimental effects are subtle or moderate, PCA will not show good separation patterns
- PLS-DA is a supervised method that uses multiple linear regression technique to find the direction of maximum covariance between a data set (X) and the class membership (Y)



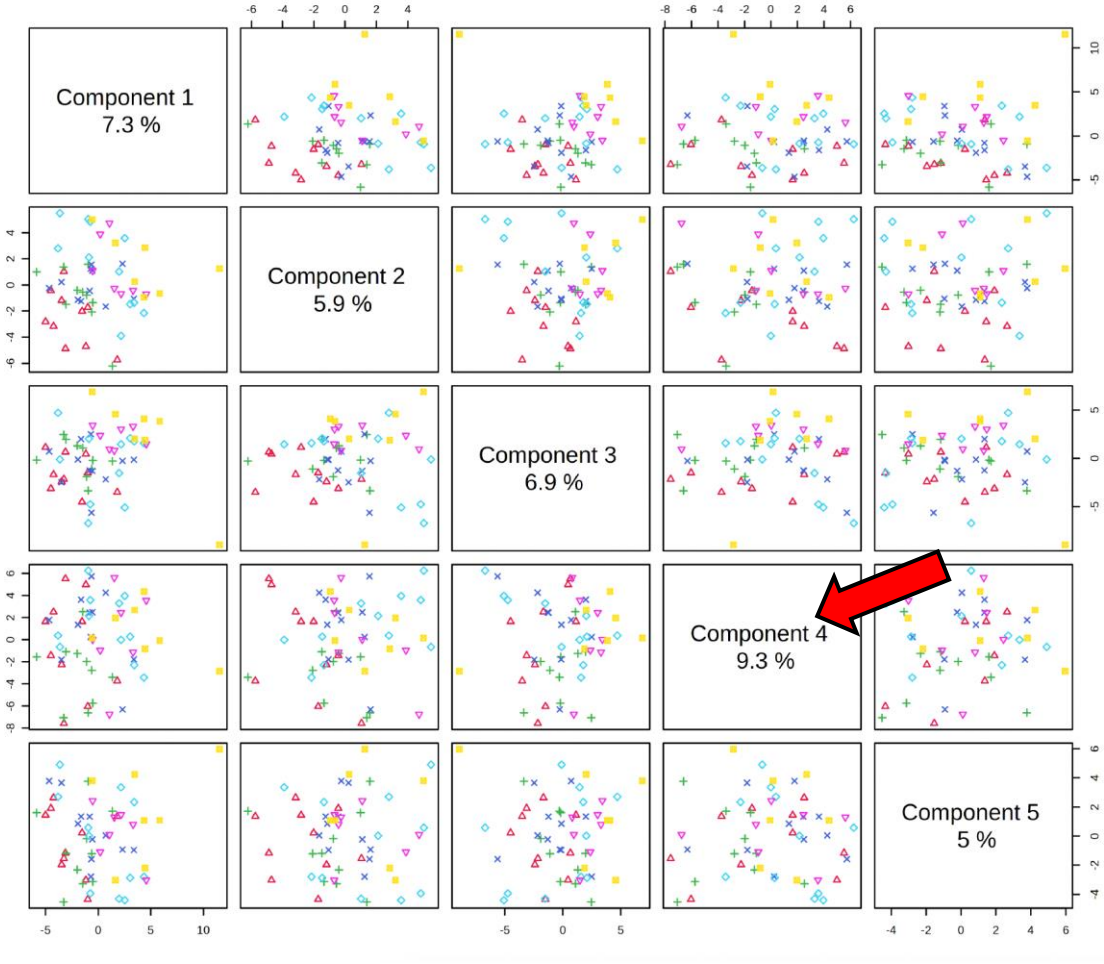

PCA ➔ PLS-DA

# Variance vs co-variance



# Max covariance may not explain max variance

- Variance explained by top components from PLS-DA
- In some cases, the 2nd component may explain more data variance (not covariance!) than the 1st one

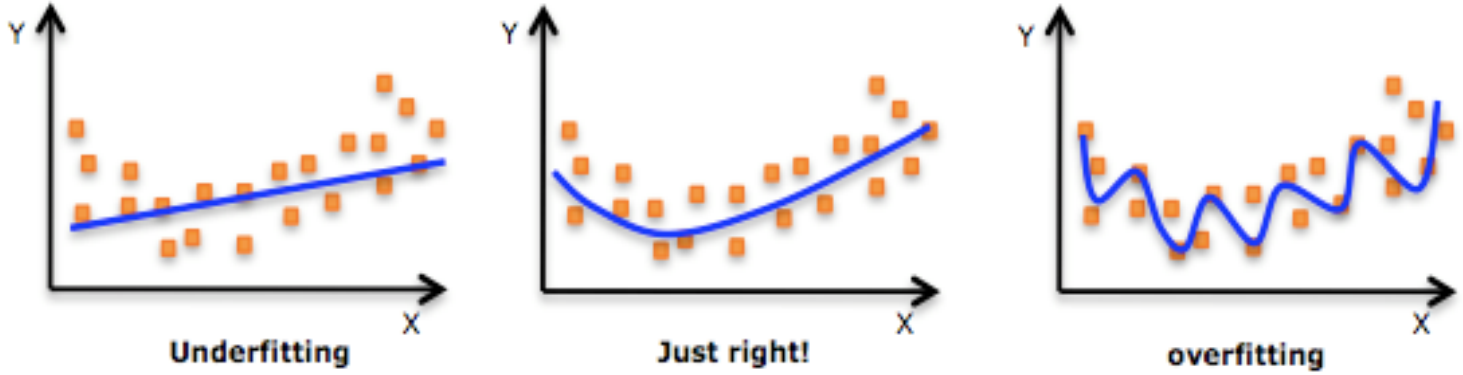



Explained variance by top 5 PLS-DA components

# Working with supervised approaches - overfitting

**Caution!** PLS-DA always produces certain separation patterns with regard the conditions <u>even there is no real difference between them!</u>

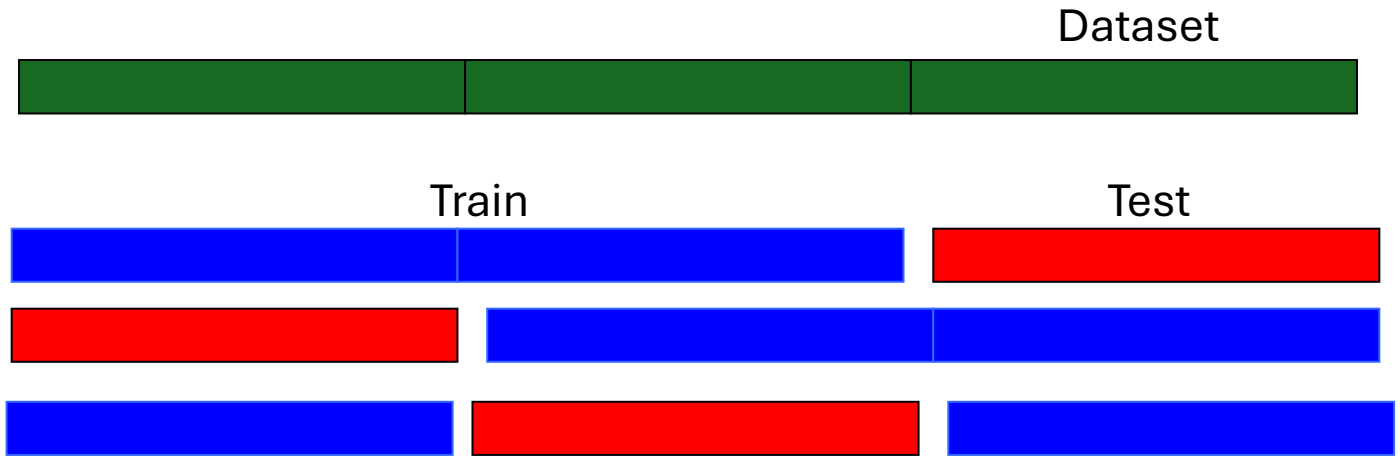
- Fitted model performs well for the current data
- Fitted model is not good for prediction of new data – prediction error is underestimated
- Model is too elaborate, models “noise” that will not be the same for new data



# Performance evaluation for PLS-DA

**Cross validation** – whether the model can predict on new events

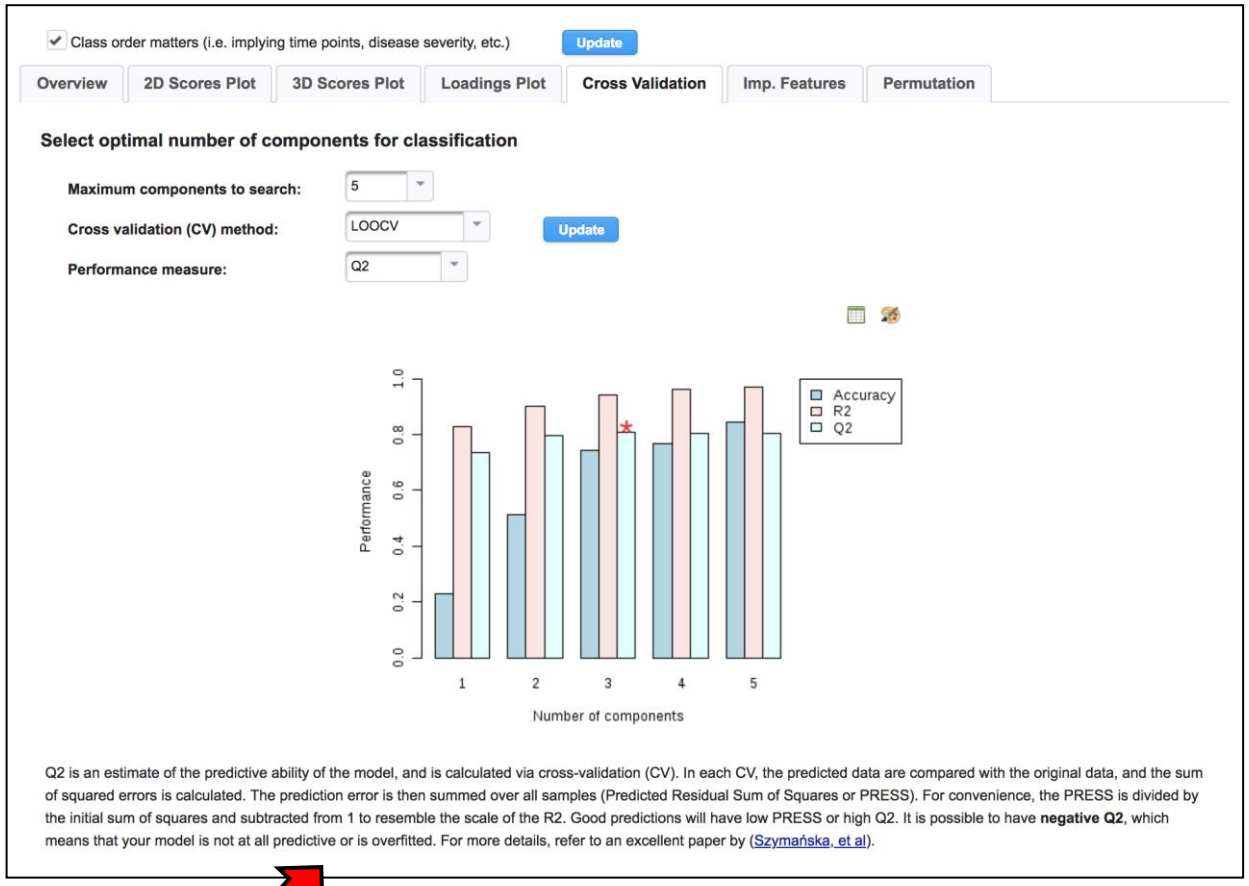
- Prediction accuracy
- Sum of squares captured by the model ($R^2$)
- Cross-validated $R^2$ (also known as $Q^2$)




**Permutation tests** – whether the model captures real signals compared to the null models (those with group labels randomly assigned)

# Evaluation of PLS-DA Model

- PLS-DA model can be evaluated by cross validation, $R^2$ and $Q^2$
- Using too many components can over-fit
- 3 component model seems to be a good compromise here
- Good $R^2/Q^2$ (>0.7)

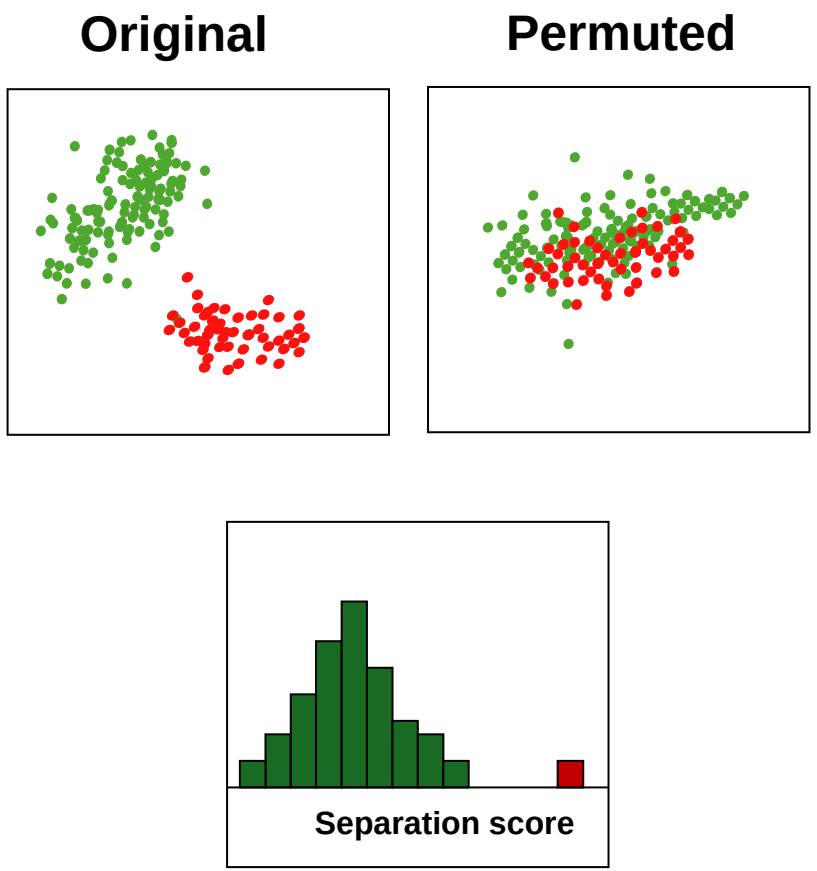



**Can Q2 be negative?!**

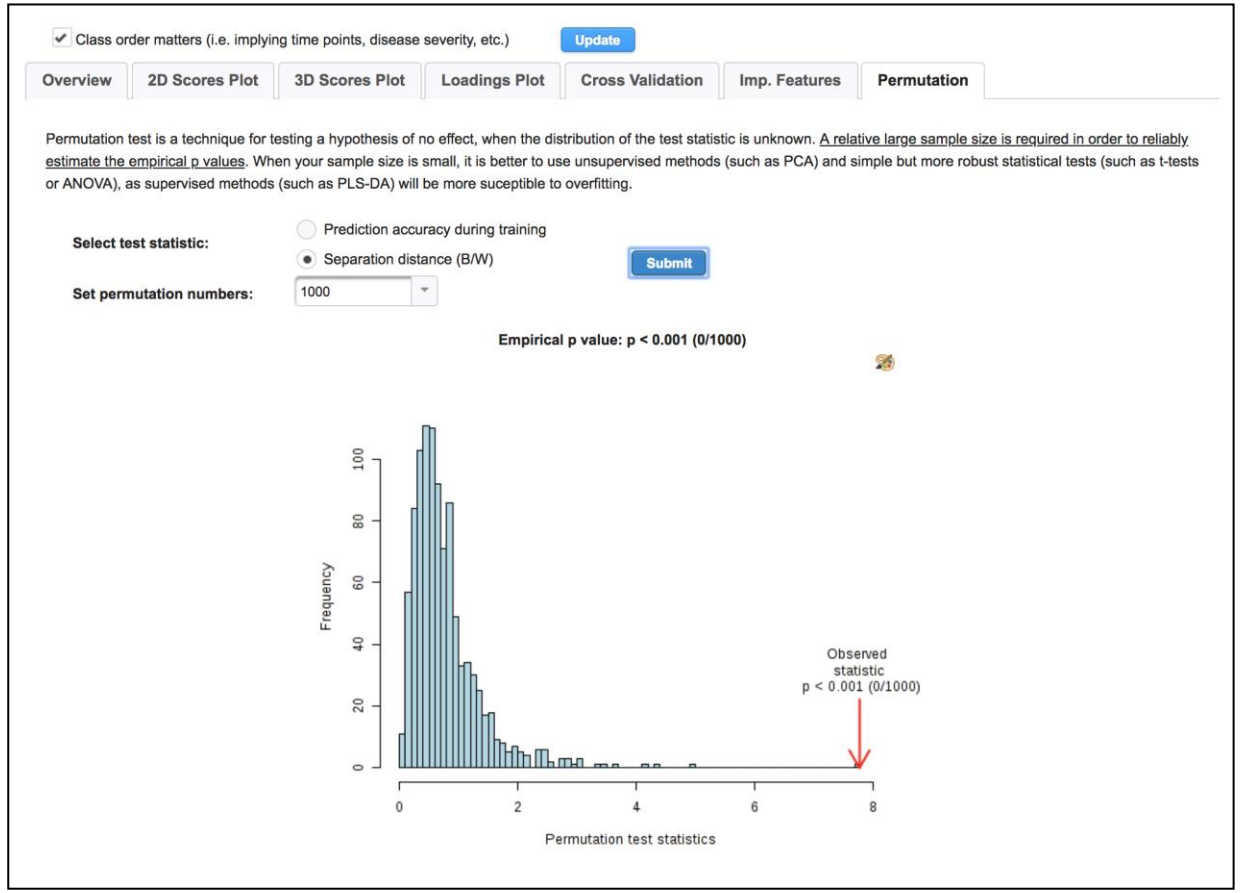
# Permutation Tests

To test whether the model is significantly different from the null models

1. Randomly shuffle the class labels (y) and build the (null) model between new y and x;
2. Test whether there is still the similar performance (i.e. distances in separation patterns);
3. We can compute empirical p values
   - If the result is similar as the permuted results (i.e. null model), then we can not say y and x is significantly correlated



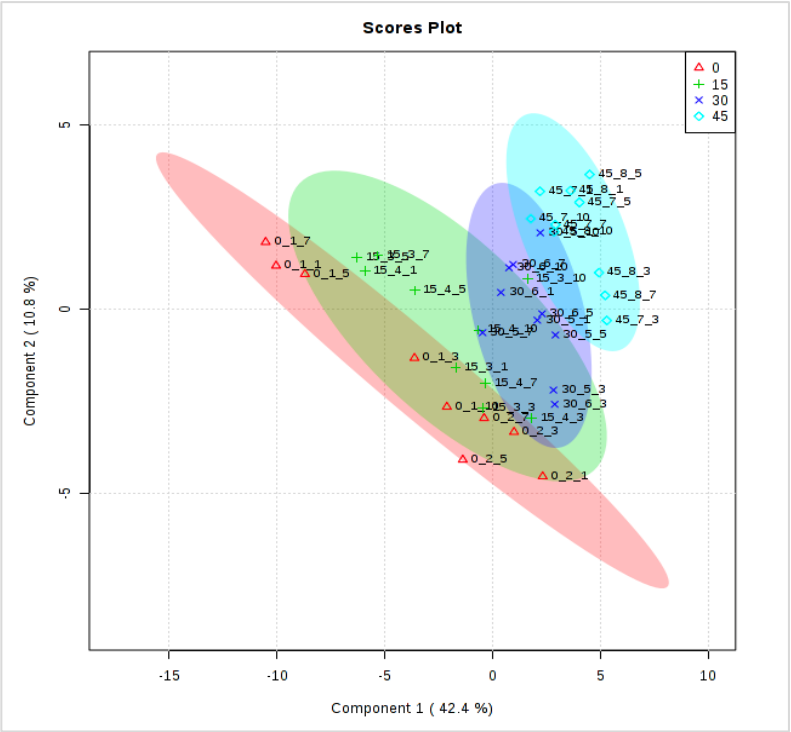
# Model validation by permutations




Permutation is computationally intensive. It is not performed by default. Users need to set the permutation number and press the submit button

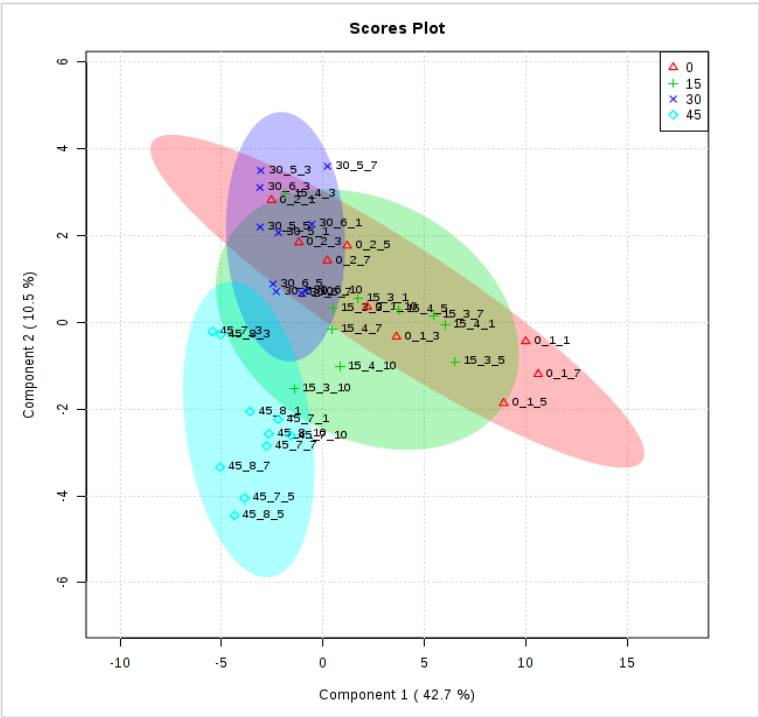
# PLS-DA for multi-group: order matters

Class order matters




Different group names can potentially change separation patterns!

Class order matters




Different group names will not matter