Statistical Methods for Quantitative MS-based Proteomics: Part II. Differential Abundance Analysis

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Contents

This is part of the online course Proteomics Data Analysis (PDA)

- Playlist PDA Preprocessing

Outline

- Francisella tularensis Example
- Hypothesis testing
- Multiple testing
- Moderated statistics
- Experimental design

Note, that the R-code is included for learners who are aiming to develop R/markdown scripts to automate their quantitative proteomics data analyses. According to the target audience of the course we either work with a graphical user interface (GUI) in a R/shiny App msqrob2gui (e.g. Proteomics Bioinformatics course of the EBI and the Proteomics Data Analysis course at the Gulbenkian institute) or with R/markdowns scripts (e.g. Bioinformatics Summer School at UCLouvain or the Statistical Genomics Course at Ghent University).

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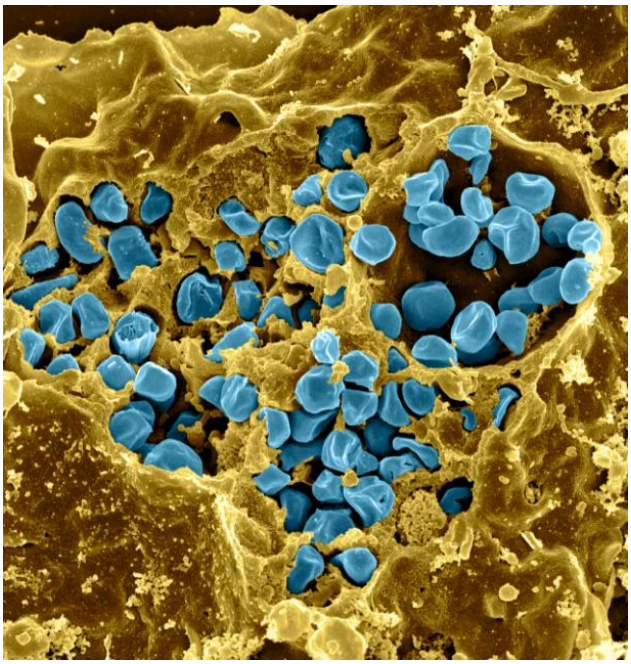
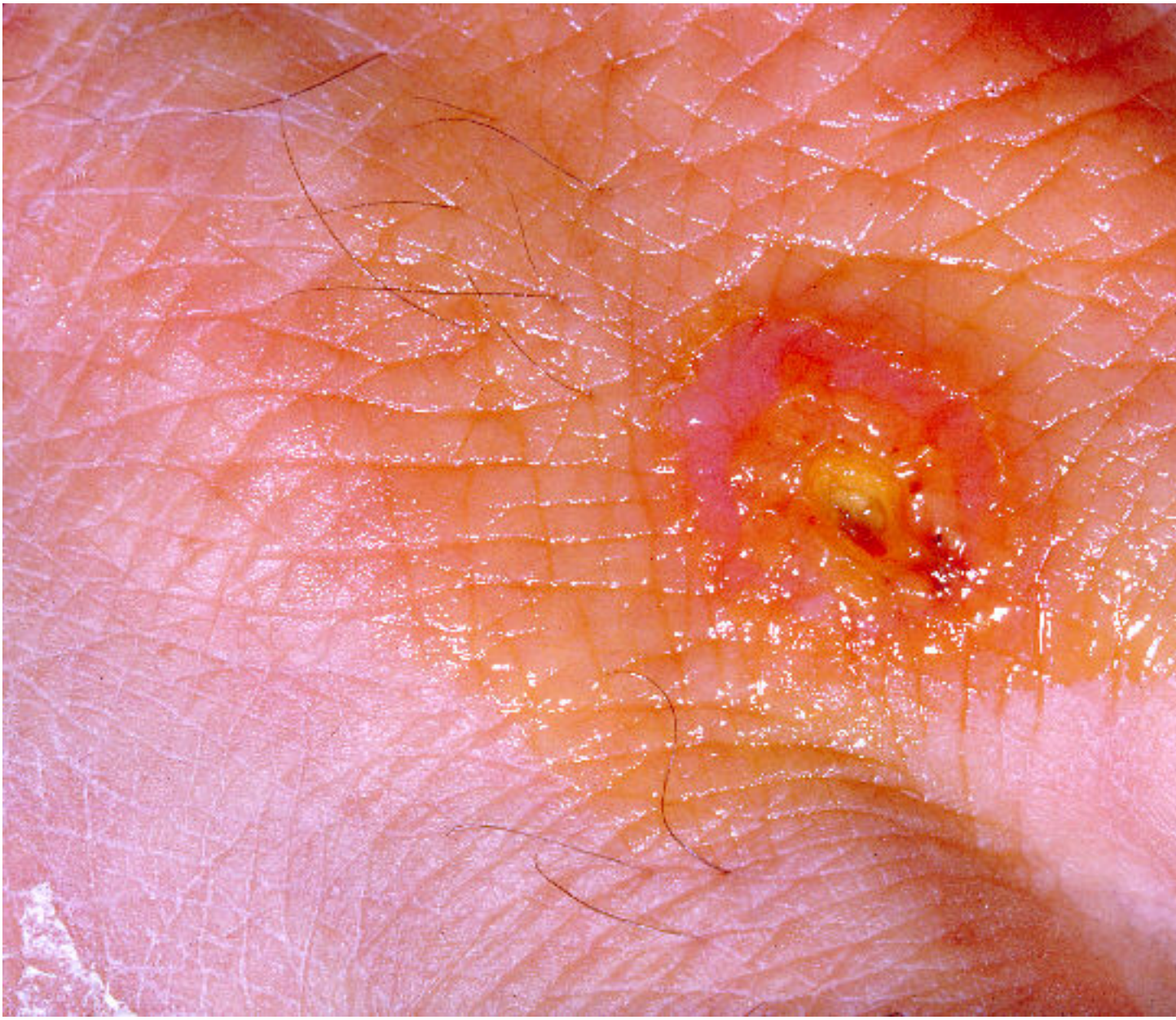
1 Francisella tularensis experiment





- Pathogen: causes tularemia
- Metabolic adaptation key for intracellular life cycle of pathogenic microorganisms.
- Upon entry into host cells quick phasomal escape and active multiplication in cytosolic compartment.
- Franciscella is auxotroph for several amino acids, including arginine.
- Inactivation of arginine transporter delayed bacterial phagosomal escape and intracellular multiplication.
- Experiment to assess difference in proteome using 3 WT vs 3 ArgP KO mutants

1.1 Import the data in R

Click to see code

1. Load libraries

```
library(tidyverse)
library(limma)
```

```
library(QFeatures)
library(msqrob2)
library(plotly)
library(ggplot2)
library(data.table)
```

2. We use a peptides.txt file from MS-data quantified with maxquant that contains MS1 intensities summarized at the peptide level.

```
peptidesTable <- fread("https://raw.githubusercontent.com/statOmics/PDA/data/quantification/francisella
int64 <- which(sapply(peptidesTable,class) == "integer64")
for (j in int64) peptidesTable[[j]] <- as.numeric(peptidesTable[[j]])
```

3. Maxquant stores the intensity data for the different samples in columnns that start with Intensity. We can retreive the column names with the intensity data with the code below:

```
quantCols <- grep("Intensity ", names(peptidesTable))
```

4. Read the data and store it in QFeatures object

```
pe <- readQFeatures(
  assayData = peptidesTable,
  fnames = 1,
  quantCols =  quantCols,
  name = "peptideRaw")
```

```
## Checking arguments.
## Loading data as a 'SummarizedExperiment' object.
## Formatting sample annotations (colData).
## Formatting data as a 'QFeatures' object.
## Setting assay rownames.
```

```
rm(peptidesTable)
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8805615 470.3   15503455 828.0         NA 15503455 828.0
## Vcells 34589285 263.9   81348285 620.7      16384 81348285 620.7
```

```
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8805455 470.3   15503455 828.0         NA 15503455 828.0
## Vcells 34582632 263.9   81348285 620.7      16384 81348285 620.7
```

5. Update data with information on design

```
colData(pe)$genotype <- pe[[1]] |>
  colnames() |>
  substr(12,13) |>
  as.factor() |>
  relevel("WT")

pe |> colData()
```

```
## DataFrame with 6 rows and 1 column
##                          genotype
```

```
##                           <factor>
## Intensity 1WT_20_2h_n3_3       WT
## Intensity 1WT_20_2h_n4_3       WT
## Intensity 1WT_20_2h_n5_3       WT
## Intensity 3D8_20_2h_n3_3       D8
## Intensity 3D8_20_2h_n4_3       D8
## Intensity 3D8_20_2h_n5_3       D8
```

1.2 Preprocessing

Click to see code to log-transfrom the data

1. Log transform

- Peptides with zero intensities are missing peptides and should be represent with a `NA` value rather than `0`.

```
pe <- zeroIsNA(pe, "peptideRaw") # convert 0 to NA
```

- Logtransform data with base 2

```
pe <- logTransform(pe, base = 2, i = "peptideRaw", name = "peptideLog")
```

2. Filtering

- We remove peptides that could not be mapped to a protein or that map to multiple proteins (the protein identifier contains multiple identifiers separated by a ;).

```
pe <- filterFeatures(
    pe, ~ Proteins != "" & ## Remove failed protein inference
        !grepl(";", Proteins)) ## Remove protein groups
```

```
## 'Proteins' found in 2 out of 2 assay(s).
```

- Remove reverse sequences (decoys) and contaminants. Note that this is indicated by the column names Reverse and depending on the version of maxQuant with Potential.contaminants or Contaminants.

```
pe <- filterFeatures(pe,~Reverse != "+")
```

```
## 'Reverse' found in 2 out of 2 assay(s).
```

```
pe <- filterFeatures(pe,~ Contaminant != "+")
```

```
## 'Contaminant' found in 2 out of 2 assay(s).
```

- Drop peptides that were identified in less than three sample. We tolerate the following proportion of NAs: pNA = (n-3)/n.

```
nObs <- 3
n <- ncol(pe[["peptideLog"]])
pNA <- (n-nObs)/n
pe <- filterNA(pe, pNA = pNA, i = "peptideLog")
nrow(pe[["peptideLog"]])
```

```
## [1] 5740
```

We keep 5740 peptides upon filtering.

3. Normalization by median centering

```
pe <- normalize(pe,
                i = "peptideLog",
```

```
                name = "peptideNorm",
                method = "center.median")
```

4. Summarization. We use the standard sumarisation in aggregateFeatures, which is a robust summarisation method.

```
pe <- aggregateFeatures(pe,
    i = "peptideNorm",
    fcol = "Proteins",
    na.rm = TRUE,
    name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

```
## Aggregated: 1/1
```

5. Filter proteins that contain many missing values

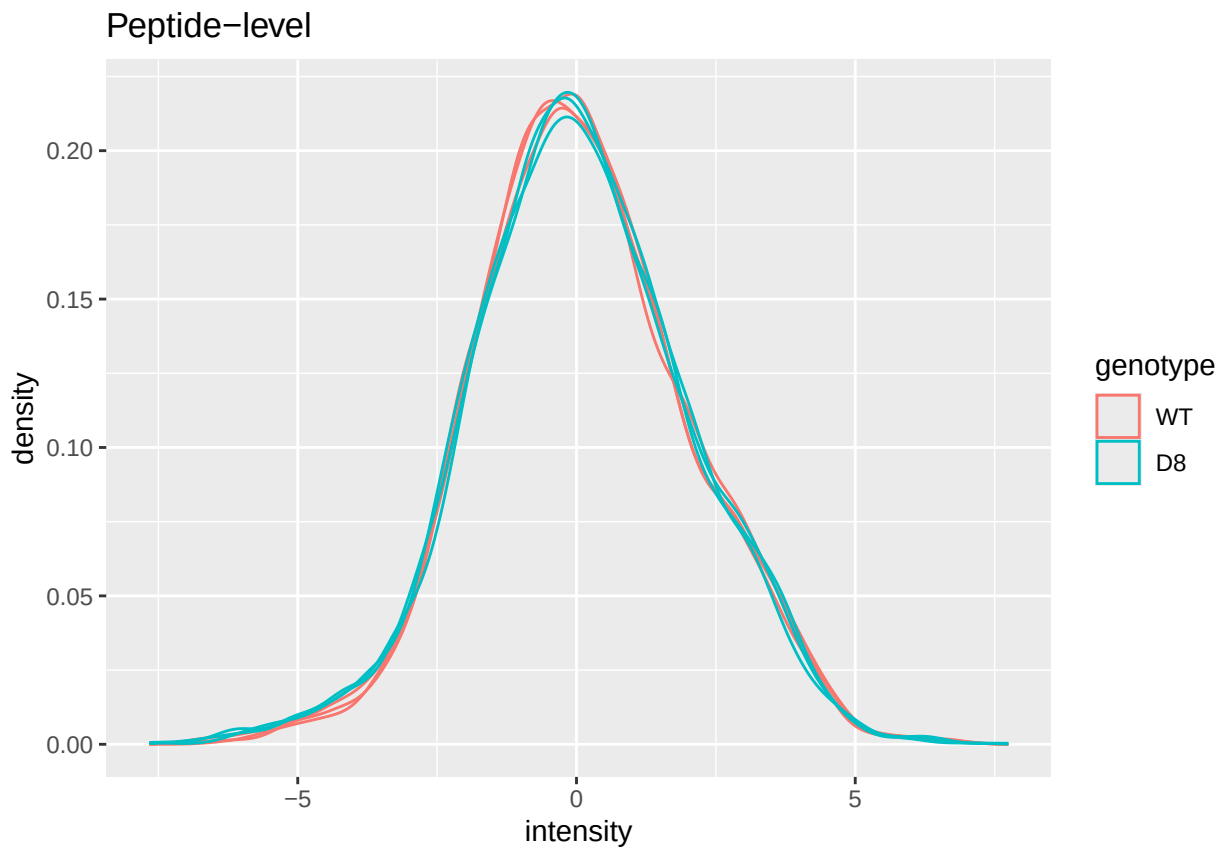
We want to have at least 4 observed proteins so that most proteins have at least 2 observations in each group. So we tolerate a proportion of (n-4)/n NAs.

```
nObs <- 4
n <- ncol(pe[["protein"]])
pNA <- (n-nObs)/n
pe <- filterNA(pe, pNA = pNA, i = "protein")
```

Plot of preprocessed data

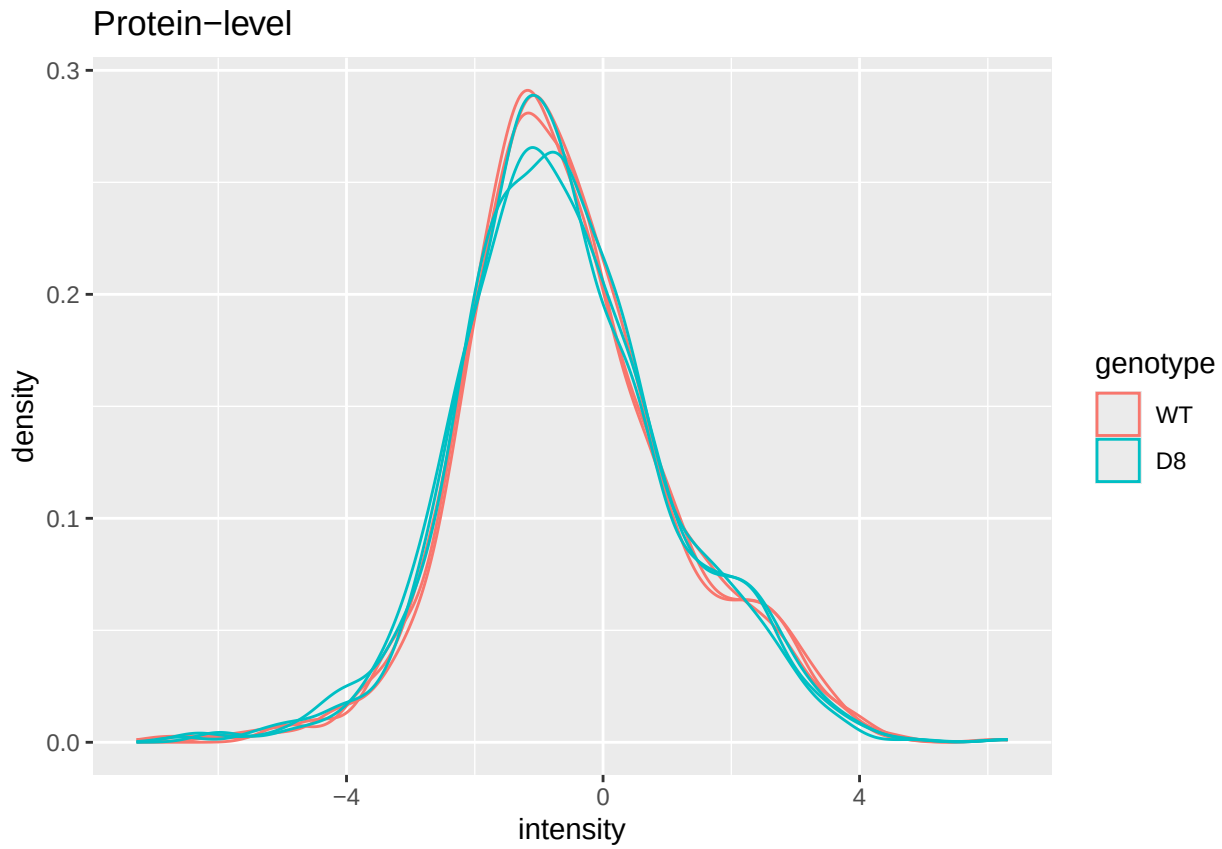
```
pe[["peptideNorm"]] |>
  assay() |>
  as.data.frame() |>
  gather(sample, intensity) |>
  mutate(genotype = colData(pe)[sample,"genotype"]) |>
  ggplot(aes(x = intensity,group = sample,color = genotype)) +
    geom_density() +
    ggtitle("Peptide-level")
```

```
## Warning: Removed 4421 rows containing non-finite outside the scale range
## (`stat_density()`).
```




```
pe[["protein"]] |>
  assay() |>
  as.data.frame() |>
  gather(sample, intensity) |>
  mutate(genotype = colData(pe)[sample,"genotype"]) |>
  ggplot(aes(x = intensity,group = sample,color = genotype)) +
    geom_density() +
    ggtitle("Protein-level")
```

```
## Warning: Removed 149 rows containing non-finite outside the scale range
## (`stat_density()`).
```



1.3 Summarized data structure

1.3.1 Design

```
pe |>
  colData() |>
  knitr::kable()
```

	genotype
Intensity 1WT_20_2h_n3_3	WT
Intensity 1WT_20_2h_n4_3	WT
Intensity 1WT_20_2h_n5_3	WT
Intensity 3D8_20_2h_n3_3	D8
Intensity 3D8_20_2h_n4_3	D8
Intensity 3D8_20_2h_n5_3	D8

- WT vs KO
- 3 vs 3 repeats

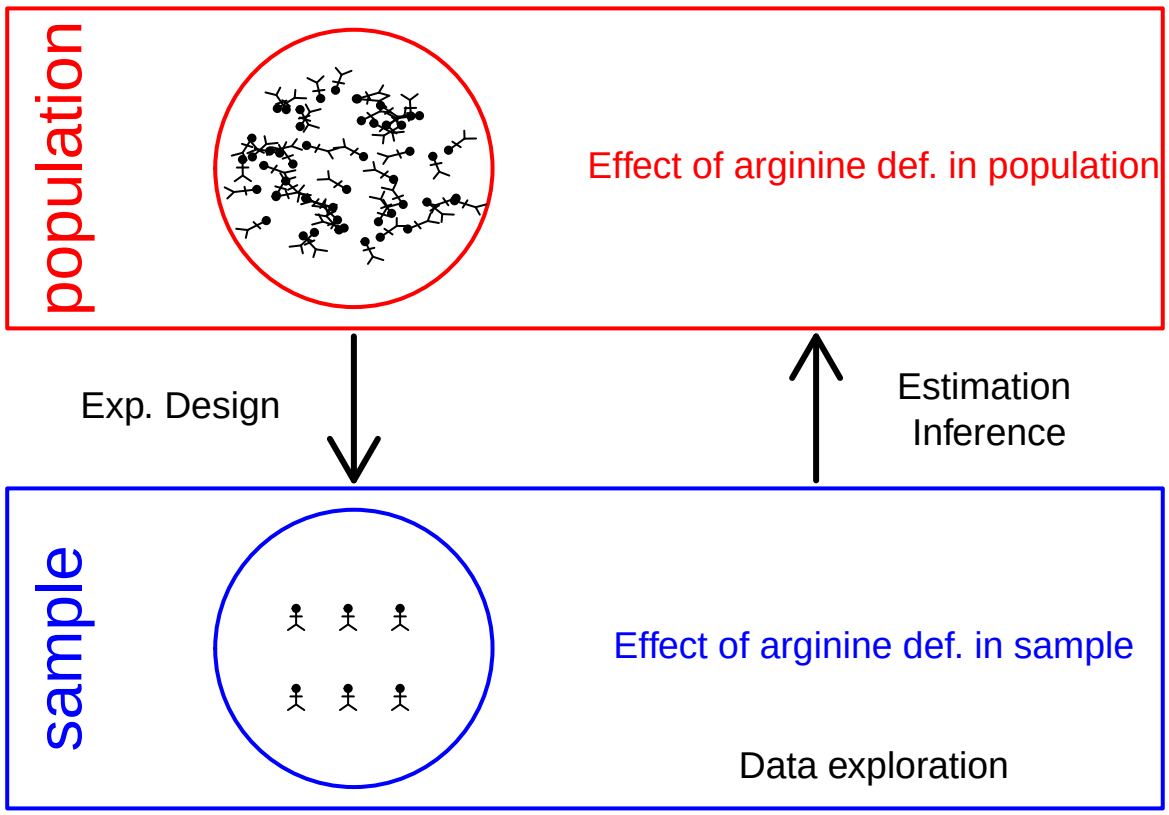
1.3.2 Summarized intensity matrix

```
pe[["protein"]] |>
  assay() |>
  head() |>
  knitr::kable()
```

	Intensity 1WT_20_2h_n3_1	Intensity 1WT_20_2h_n4_1	Intensity 1WT_20_2h_n5_1	Intensity D8_20_2h_n3_3	Intensity D8_20_2h_n4_3	Intensity D8_20_2h_n5_3
WP_003013731	1.2277864	-0.0333727	0.1810282	-0.1946484	0.1347441	0.0522102
WP_003013909	9.7603080	-0.8862012	-0.8565867	-0.4951824	-0.6100506	-0.5490239
WP_003014068	8.5743424	0.7821886	1.0336891	0.5098828	0.8536632	0.5446984
WP_003014122	2.8382825	-1.1131095	-0.9099676	-1.2104299	-1.2575807	-1.3093650
WP_003014123	3.2803634	-0.4062057	-0.3159891	-0.4216095	-0.3453093	-0.6329528
WP_003014302	2.5696671	1.7486922	1.5869110	1.6919901	1.8142693	1.5398097

- 1025 proteins

1.3.3 Hypothesis testing: a single protein



1.3.3.1 T-test

$$\log_2 \text{FC} = \bar{y}_{p1} - \bar{y}_{p2}$$

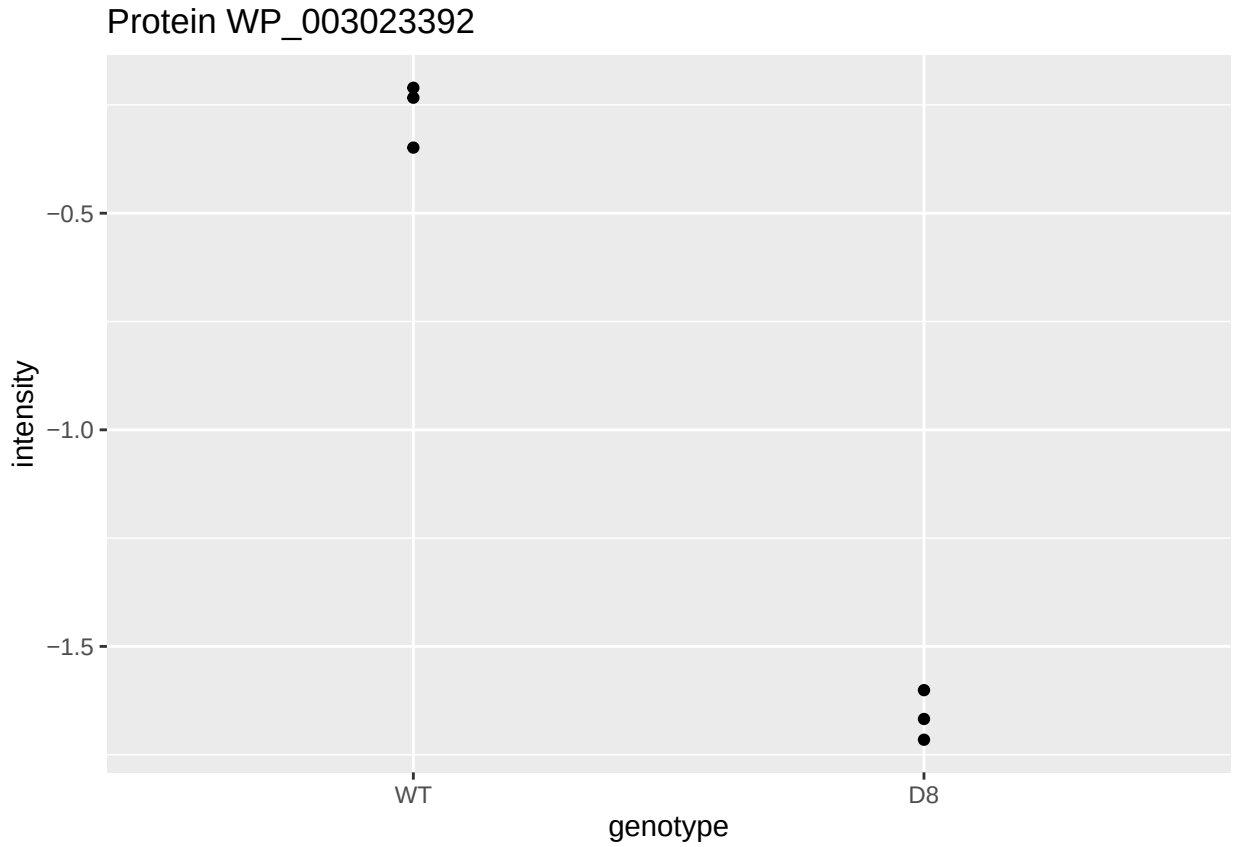
$$T_g = \frac{\log_2 \text{FC}}{\text{se}_{\log_2 \text{FC}}}$$

$$T_g = \frac{\widehat{\text{signal}}}{\widehat{\text{Noise}}}$$

If we can assume equal variance in both treatment groups:

$$\text{se}_{\log_2 \text{FC}} = \text{SD}\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

```
WP_003023392 <- data.frame(
    intensity = assay(pe[["protein"]]["WP_003023392",]) |>
      c(),
    genotype = colData(pe)[,1])

WP_003023392 |>
  ggplot(aes(x=genotype,y=intensity)) +
  geom_point() +
  ggtitle("Protein WP_003023392")
```



$$t = \frac{\log_2 \widehat{\text{FC}}}{\text{se}_{\log_2 \widehat{\text{FC}}}} = \frac{-1.4}{0.0541} = -25.8$$

- Is t = -25.8 indicating that there is an effect?
- How likely is it to observe t = -25.8 when there is no effect of the argP KO on the protein expression?

1.3.3.2 Null hypothesis (H_0) and alternative hypothesis (H_1)

- With data we can never prove a hypothesis (falsification principle of Popper)
- With data we can only reject a hypothesis
- In general we start from *alternative hypothese* H_1: we want to show an effect of the KO on a protein

H_1: On average the protein abundance in WT is different from that in KO

- But, we will assess this by falsifying the opposite:
  H_0: On average the protein abundance in WT is equal to that in KO<-

```
t.test(intensity ~ genotype, data = WP_003023392, var.equal=TRUE)
```

```
##
##  Two Sample t-test
##
## data:  intensity by genotype
## t = 25.828, df = 4, p-value = 1.335e-05
## alternative hypothesis: true difference in means between group WT and group D8 is not equal to 0
## 95 percent confidence interval:
##  1.246966 1.547353
## sample estimates:
## mean in group WT mean in group D8
##       -0.2641239       -1.6612832
```

- How likely is it to observe an equal or more extreme effect than the one observed in the sample when the null hypothesis is true?
- When we make assumptions about the distribution of our test statistic we can quantify this probability: *p-value*. The p-value will only be calculated correctly if the underlying assumptions hold!
- When we repeat the experiment, the probability to observe a fold change for this gene that is more extreme than a 2.63 fold ($\log_2 FC = -1.4$) down or up regulation by random change (if H_0 is true) is 13 out of 1 000 000.

- If the p-value is below a significance threshold α we reject the null hypothesis. *We control the probability on a false positive result at the α-level (type I error)*
- Note, that the p-values are uniform under the null hypothesis, i.e. when H_0 is true all p-values are equally likely.

1.4 Multiple hypothesis testing

- Consider testing DA for all $m = 1066$ proteins simultaneously
- What if we assess each individual test at level α? $\rightarrow$ Probability to have a false positive (FP) among all m simultatenous test $>>> \alpha = 0.05$
- Indeed for each non DA protein we have a probability of 5% to return a FP.
- In a typical experiment the majority of the proteins are non DA.
- So an upperbound of the expected FP is $m \times \alpha$ or $1066 \times 0.05 = 53$.

$\rightarrow$ Hence, we are bound to call many false positive proteins each time we run the experiment.

1.4.1 Multiple testing

1.4.1.1 Family-wise error rate The family-wise error rate (FWER) addresses the multiple testing issue by no longer controlling the individual type I error for each protein, instead it controls:

$$\text{FWER} = \text{P}\left[FP \geq 1\right].$$

The Bonferroni method is widely used to control the type I error:

- assess each test at
$$\alpha_{\text{adj}} = \frac{\alpha}{m}$$
- or use adjusted p-values and compare them to α:
$$p_{\text{adj}} = \min(p \times m, 1)$$

Problem, the method is very conservative!

1.4.1.2 False discovery rate

- FDR: Expected proportion of false positives on the total number of positives you return.
- An FDR of 1% means that on average we expect 1% false positive proteins in the list of proteins that are called significant.
- Defined by Benjamini and Hochberg in their seminal paper Benjamini, Y. and Hochberg, Y. (1995). "Controlling the false discovery rate: a practical and powerful approach to multiple testing". Journal of the Royal Statistical Society Series B, 57 (1): 289–300.

The **False Discovery Proportion (FDP)** is the fraction of false positives that are returned, i.e.

$$FDP = \frac{FP}{R}$$

- However, this quantity cannot be observed because in practice we only know the number of proteins for which we rejected H_0, R.
- But, we do not know the number of false positives, FP.

Therefore, Benjamini and Hochberg, 1995, defined The **False Discovery Rate (FDR)** as

$$\text{FDR} = \text{E}\left[\frac{FP}{R}\right] = \text{E}\left[\text{FDP}\right]$$

the expected FDP.

- Controlling the FDR allows for more discoveries (i.e. longer lists with significant results), while the fraction of false discoveries among the significant results in well controlled on average. As a consequence, more of the true positive hypotheses will be detected.

1.4.1.3 Intuition of BH-FDR procedure

Consider $m = 1000$ tests

- Suppose that a researcher rejects all null hypotheses for which $p < 0.01$.
- If we use $p < 0.01$, we expect $0.01 \times m_0$ tests to return false positives.
- A conservative estimate of the number of false positives that we can expect can be obtained by considering that the null hypotheses are true for all features, $m_0 = m = 1000$.
- We then would expect $0.01 \times 1000 = 10$ false positives ($FP = 10$).
- Suppose that the researcher found 200 genes with $p < 0.01$ ($R = 200$).
- The proportion of false positive results (FDP = false positive proportion) among the list of $R = 200$ genes can then be estimated as
$$\widehat{\text{FDP}} = \frac{FP}{R} = \frac{10}{200} = \frac{0.01 \times 1000}{200} = 0.05.$$

1.4.1.4 Benjamini and Hochberg (1995) procedure for controlling the FDR at α

1. Let $p_{(1)} \leq \ldots \leq p_{(m)}$ denote the ordered p-values.
2. Find the largest integer k so that
$$\frac{p_{(k)} \times m}{k} \leq \alpha$$
or
$$p_{(k)} \leq k \times \alpha/m$$
3. If such a k exists, reject the k null hypotheses associated with $p_{(1)}, \ldots, p_{(k)}$. Otherwise none of the null hypotheses is rejected.

The adjusted p-value (also known as the q-value in FDR literature):

$$q_{(i)} = \tilde{p}_{(i)} = \min \left[\min_{j=i,\ldots,m} \left(m p_{(j)}/j \right), 1 \right].$$

In the hypothetical example above: $k = 200$, $p_{(k)} = 0.01$, $m = 1000$ and $\alpha = 0.05$.

1.4.1.5 Francisella Example Click to see code

```
ttestMx <- function(y,group) {
    test <- try(t.test(y[group],y[!group],var.equal=TRUE),silent=TRUE)
    if(is(test,"try-error")) {
      return(c(log2FC=NA,se=NA,tstat=NA,p=NA))
      } else {
      return(c(log2FC= (test$estimate%*%c(1,-1)),se=test$stderr,tstat=test$statistic,pval=test$p.value))
      }
 }

 res <- apply(
    assay(pe[["protein"]]),
    1,
    ttestMx,
    group = colData(pe)$genotype=="D8") |>
  t()

 colnames(res) <- c("logFC","se","tstat","pval")

 res <- res |>
   as.data.frame() |>
   na.exclude() |>
   arrange(pval)

 res$adjPval <- p.adjust(res$pval, "fdr")

 alpha <- 0.05

 res$adjAlphaForm <- paste0(1:nrow(res)," x ",alpha,"/",nrow(res))

 res$adjAlpha <- alpha * (1:nrow(res))/nrow(res)

 res$"pval < adjAlpha" <- res$pval < res$adjAlpha

 res$"adjPval < alpha" <- res$adjPval < alpha
```

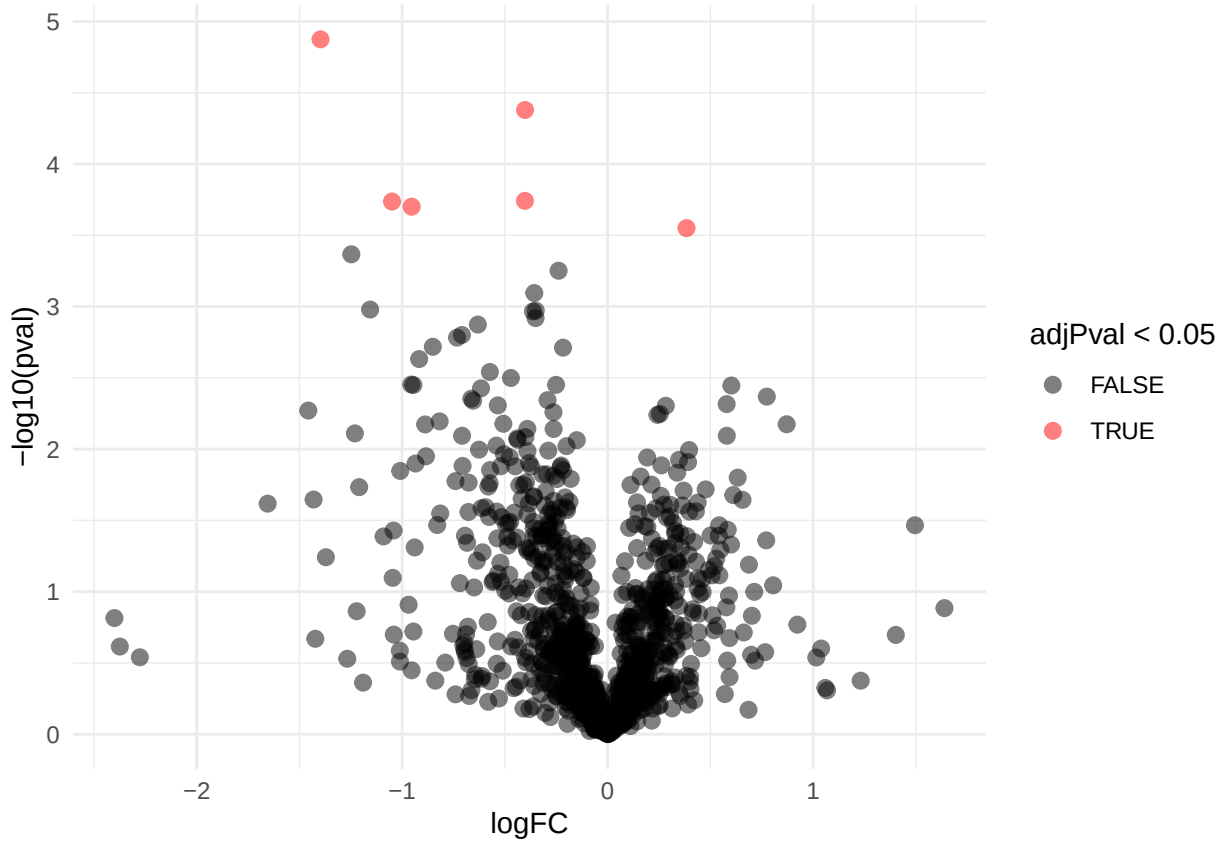
FWER: Bonferroni method:$\alpha_{\text{adj}} = \alpha/m = 0.05/1025 = 5 \times 10^{-5}$

	logFC	pval	adjPval	adjAlphaForm	adjAlpha	pval < adjAlpha	adjPval < alpha
WP_003023392	-1.3971592	0.0000134	0.0136840	1 x 0.05/1025	0.0000488	TRUE	TRUE
WP_011733588	-0.4018518	0.0000418	0.0214166	2 x 0.05/1025	0.0000976	TRUE	TRUE
WP_003038430	-0.4024957	0.0001814	0.0408656	3 x 0.05/1025	0.0001463	FALSE	TRUE
WP_003026016	-1.0493276	0.0001834	0.0408656	4 x 0.05/1025	0.0001951	TRUE	TRUE
WP_003014552	-0.9536835	0.0001993	0.0408656	5 x 0.05/1025	0.0002439	TRUE	TRUE
WP_011733645	0.3838435	0.0002817	0.0481190	6 x 0.05/1025	0.0002927	TRUE	TRUE
WP_003040849	-1.2473865	0.0004301	0.0629754	7 x 0.05/1025	0.0003415	FALSE	FALSE
WP_003038940	-0.2386737	0.0005609	0.0718619	8 x 0.05/1025	0.0003902	FALSE	FALSE
...	...	...	...	...	...	...	...
WP_003040562	0.0039480	0.9976429	0.9985797	1065 x 0.05/1066	0.0499531	FALSE	FALSE
WP_003041130	0.0002941	0.9992812	0.9992812	1066 x 0.05/1066	0.05	FALSE	FALSE

1.4.1.6 Results Click to see code

```
volcanoT <- res |>
  ggplot(aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)) +
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal()
```

```
volcanoT
```



1.5 Moderated Statistics

Problems with ordinary t-test

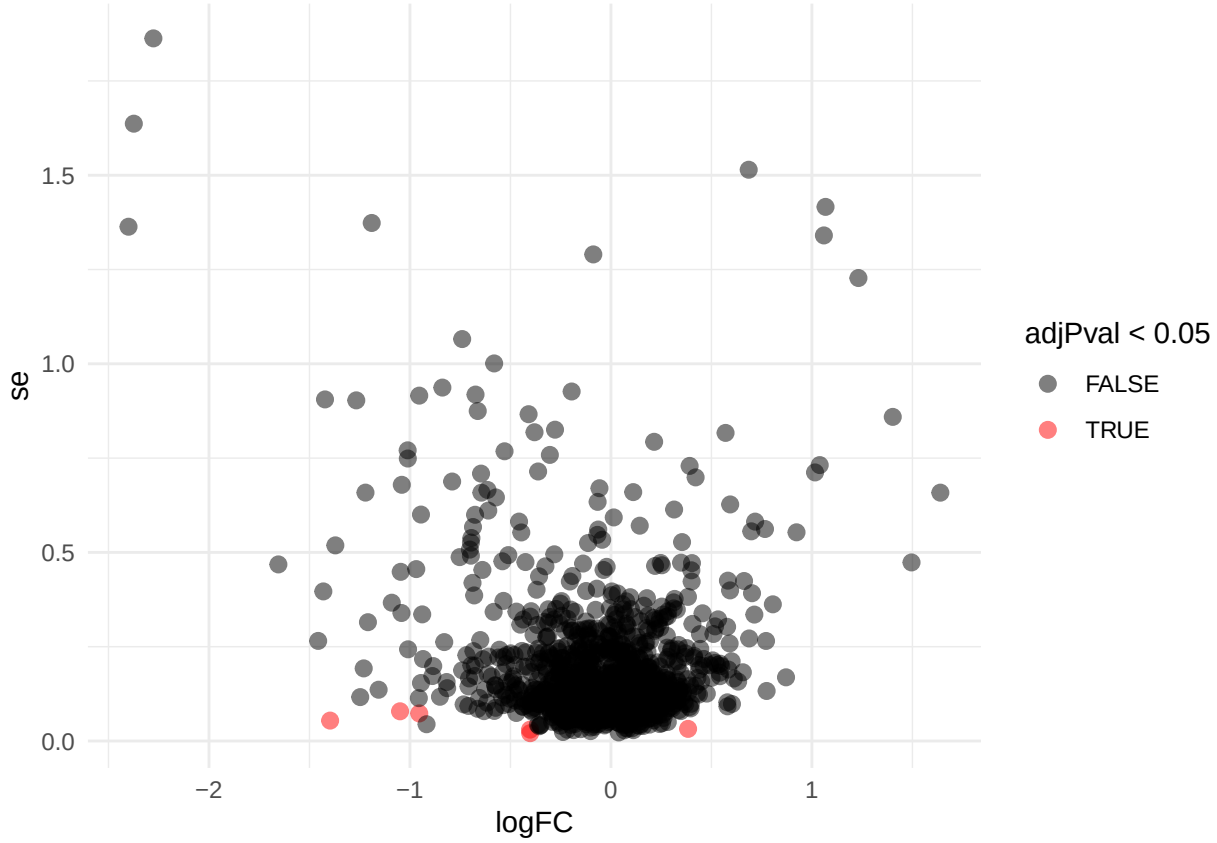
Click to see code

```
problemPlots <- list()
problemPlots[[1]] <- res |>
  ggplot(aes(x = logFC, y = se, color = adjPval < 0.05)) +
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal()

i <- 1
problemPlots[[i+1]] <- colData(pe) |>
    as.data.frame() |>
    mutate(intensity = pe[["protein"]][rownames(res)[i],] |>
             assay() |>
             c()
           ) |>
    ggplot(aes(x=genotype,y=intensity)) +
    geom_point() +
    ylim(-3,0) +
    ggtitle(rownames(res)[i])


i <- 2
```

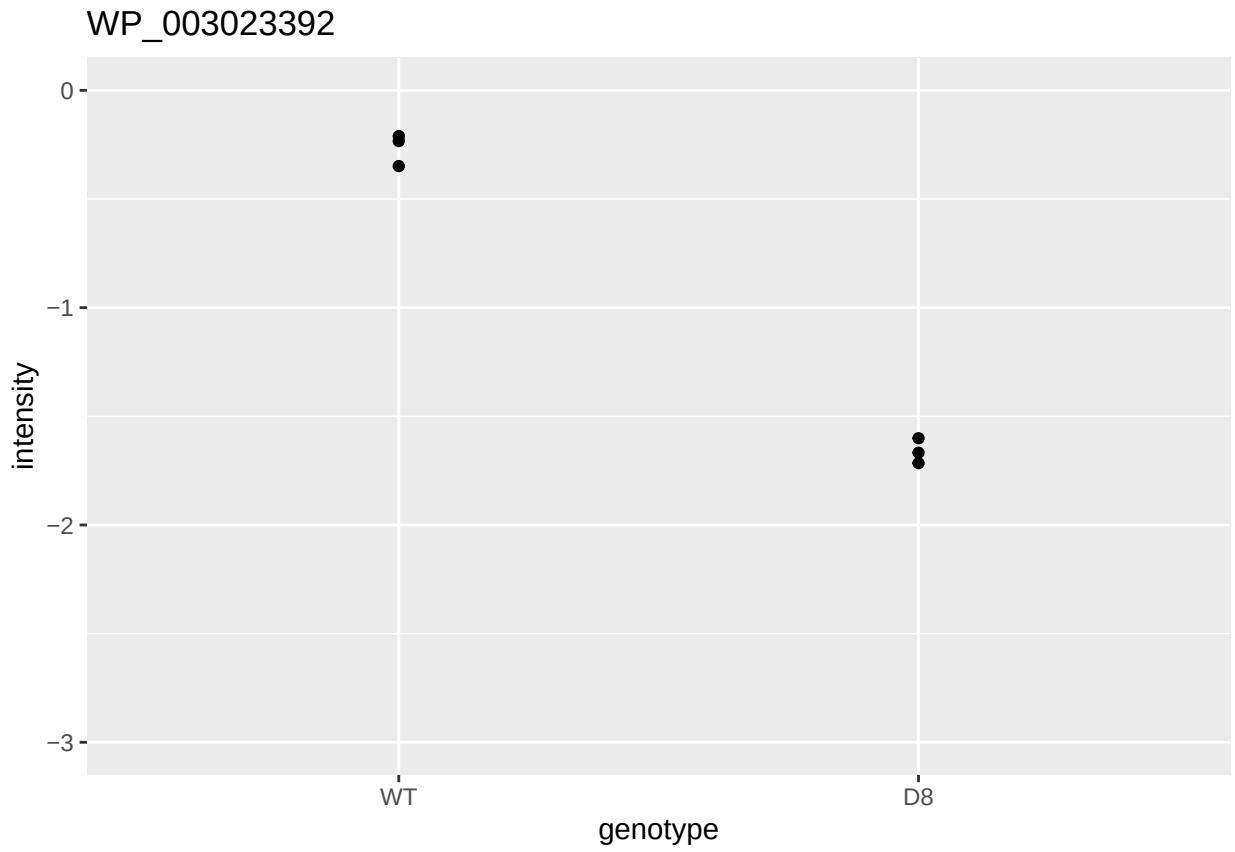
```
problemPlots[[i+1]] <- colData(pe) |>
    as.data.frame() |>
    mutate(intensity = pe[["protein"]][rownames(res)[i],] |>
             assay() |>
             c()
           ) |>
    ggplot(aes(x=genotype,y=intensity)) +
    geom_point() +
    ylim(0,3) +
    ggtitle(rownames(res)[i])
```

```
problemPlots
```

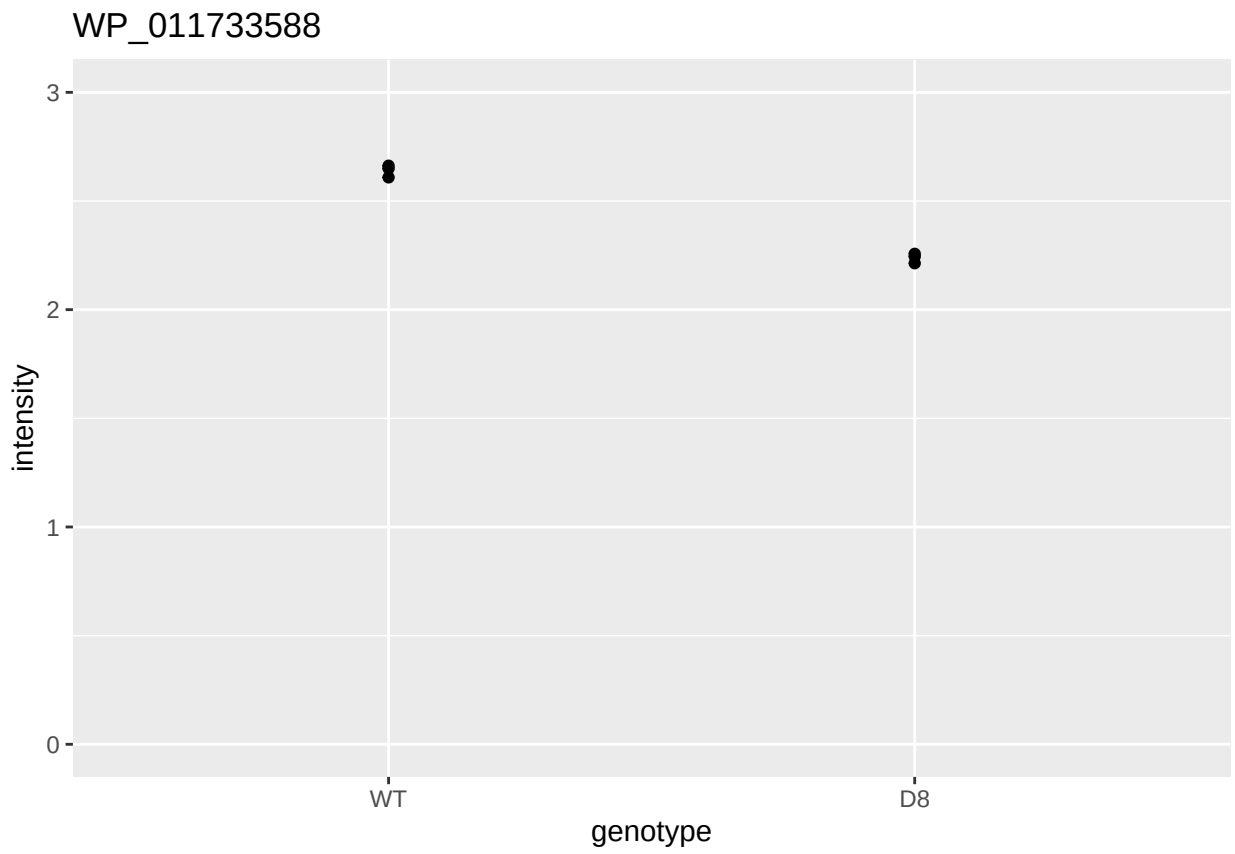
```
## [[1]]
```




```
##
## [[2]]
```




```
##
## [[3]]
```



A general class of moderated test statistics is given by

$$T_g^{mod} = \frac{\bar{Y}_{g1} - \bar{Y}_{g2}}{C \quad \tilde{S}_g},$$

where $\tilde{S}_g$ is a moderated standard deviation estimate.

- C is a constant depending on the design e.g. $\sqrt{1/n_1 + 1/n_2}$ for a t-test and of another form for linear models.
- $\tilde{S}_g = S_g + S_0$: add small positive constant to denominator of t-statistic.
- This can be adopted in Perseus.

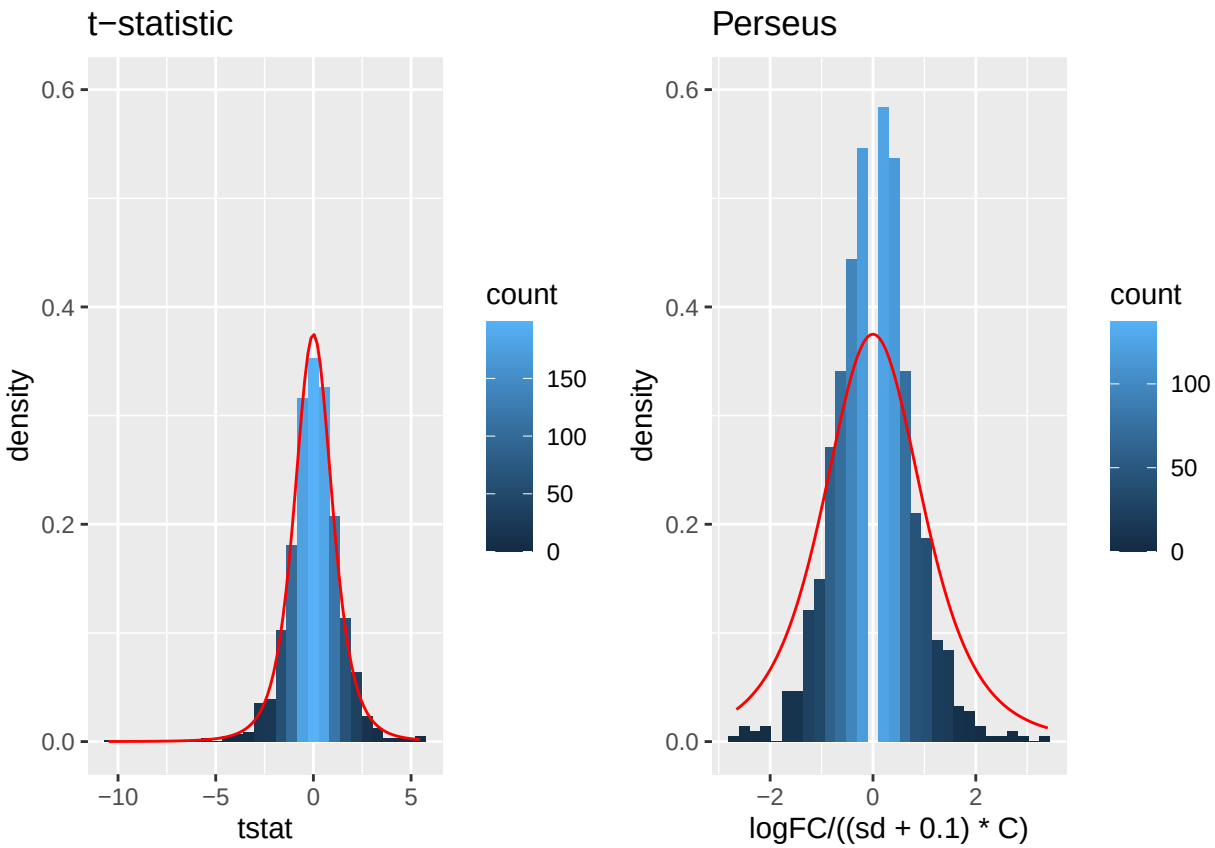
Click to see code

```
simI<-sapply(res$se/sqrt(1/3+1/3),function(n,mean,sd) rnorm(n,mean,sd),n=6,mean=0) |>
  t()

resSim <- apply(
    simI,
    1,
    ttestMx,
    group = colData(pe)$genotype=="D8") |>
  t()
 colnames(resSim) <- c("logFC","se","tstat","pval")
 resSim <- as.data.frame(resSim)
 tstatSimPlot <- resSim |>
   ggplot(aes(x=tstat)) +
     geom_histogram(aes(y=..density.., fill=..count..),bins=30) +
```

```
    stat_function(fun=dt,
   color="red",
   args=list(df=4)) +
  ylim(0,.6) +
  ggtitle("t-statistic")


resSim$C <- sqrt(1/3+1/3)
resSim$sd <- resSim$se/resSim$C
tstatSimPerseus <- resSim |>
  ggplot(aes(x=logFC/((sd+.1)*C))) +
    geom_histogram(aes(y=..density.., fill=..count..),bins=30) +
    stat_function(fun=dt,
                  color="red",
                 args=list(df=4)) +
    ylim(0,.6) +
   ggtitle("Perseus")
```

```
gridExtra::grid.arrange(tstatSimPlot,tstatSimPerseus,nrow=1)
```

```
## Warning: Removed 1 row containing missing values or values outside the scale range
## (`geom_bar()`).
```



- The choice of S_0 in Perseus is ad hoc and the t-statistic is no-longer t-distributed.
- Permutation test, but is difficult for more complex designs.
- Allows for Data Dredging because user can choose S_0

1.5.1 Empirical Bayes

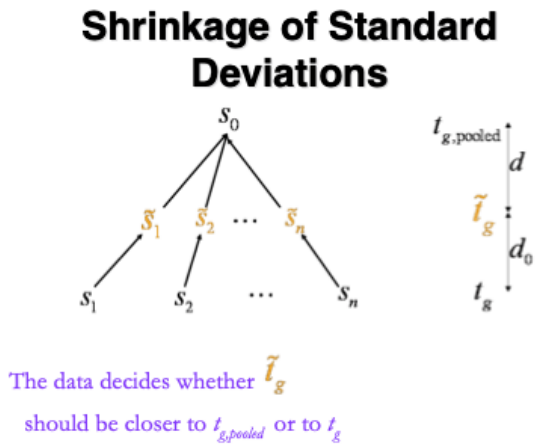


Figure courtesy to Rafael Irizarry

$$T_g^{mod} = \frac{\bar{Y}_{g1} - \bar{Y}_{g2}}{C \quad \tilde{S}_g},$$

- **empirical Bayes** theory provides formal framework for borrowing strength across proteins,
- Implemented in popular bioconductor package **limma** and **msqrob2**

$$\tilde{S}_g = \sqrt{\frac{d_g S_g^2 + d_0 S_0^2}{d_g + d_0}},$$

- S_0^2: common variance (over all proteins)
- Moderated t-statistic is t-distributed with $d_0 + d_g$ degrees of freedom.
- Note that the degrees of freedom increase by borrowing strength across proteins!

Click to see the code

1. We model the protein level expression values using the `msqrob` function. By default `msqrob2` estimates the model parameters using robust regression.

We will model the data with a different group mean for every genotype. The group is incoded in the variable `genotype` of the colData. We can specify this model by using a formula with the factor `genotype` as its predictor: `formula = ~genotype`.

Note, that a formula always starts with a symbol '~'.

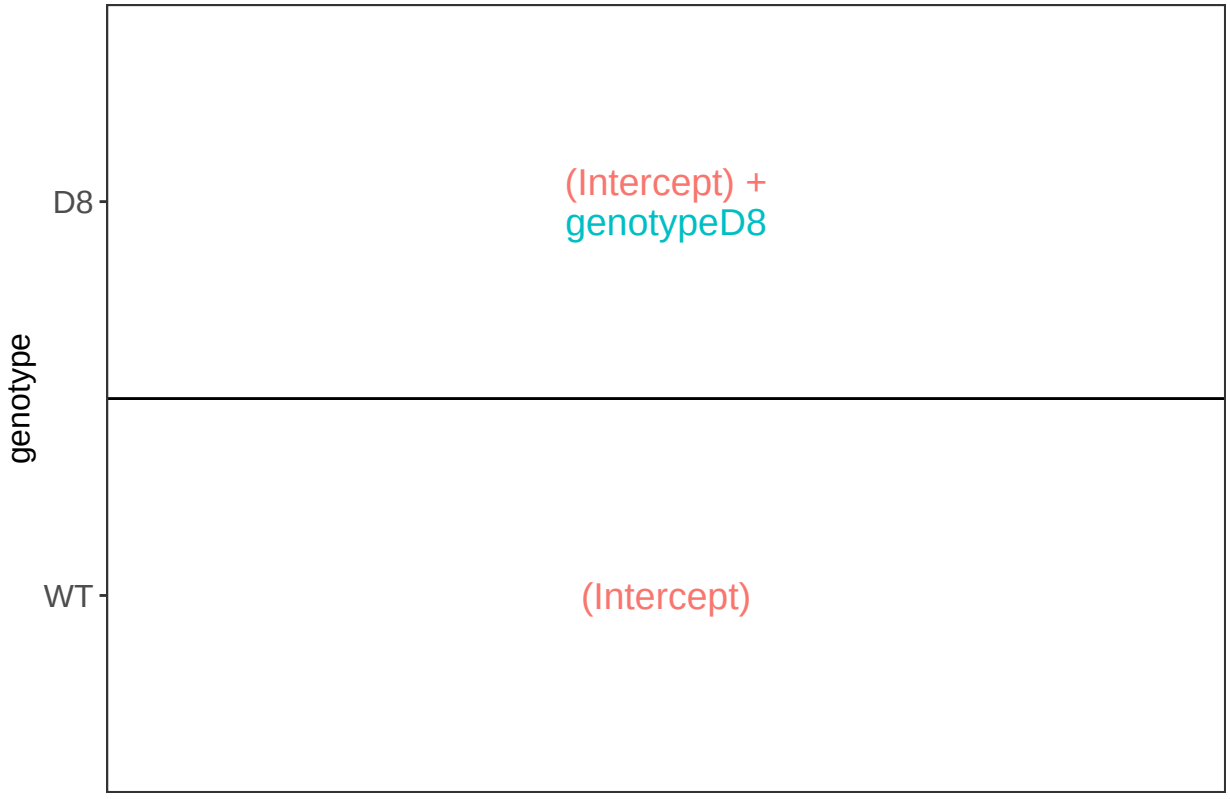
```
pe <- msqrob(object = pe, i = "protein", formula = ~genotype)
```

2. Inference

We first explore the design of the model that we specified using the the package `ExploreModelMatrix`

```
library(ExploreModelMatrix)
VisualizeDesign(colData(pe),~genotype)$plotlist
```

```
## [[1]]
```



We have two model parameters, the (Intercept) and genotypeD8. This results in a model with two group means:

1. For the wild type (WT) the expected value (mean) of the log2 transformed intensity y for a protein will be modelled using

$$\mathrm{E}[Y|\text{genotype} = \text{WT}] = (\text{Intercept})$$

2. For the knockout genotype D8 the expected value (mean) of the log2 transformed intensity y for a protein will be modelled using

$$\mathrm{E}[Y|\text{genotype} = \text{D8}] = (\text{Intercept}) + \text{genotypeD8}$$

The average log2FC between D8 and WT is thus

$$\log_2 \mathrm{FC}_{D8-WT} = \mathrm{E}[Y|\text{genotype} = \text{D8}] - \mathrm{E}[Y|\text{genotype} = \text{WT}] = \text{genotypeD8}$$

Hence, assessing the null hypothesis that there is no differential abundance between D8 and WT can be reformulated as

$$H_0 : \text{genotypeD8} = 0$$

We can implement a hypothesis test for each protein in msqrob2 using the code below:

```
L <- makeContrast("genotypeD8 = 0", parameterNames = c("genotypeD8"))
pe <- hypothesisTest(object = pe, i = "protein", contrast = L)
```

We can show the list with all significant DE proteins at the 5% FDR using

```
rowData(pe[["protein"]])$genotypeD8 |>
  arrange(pval) |>
  filter(adjPval<0.05)
```

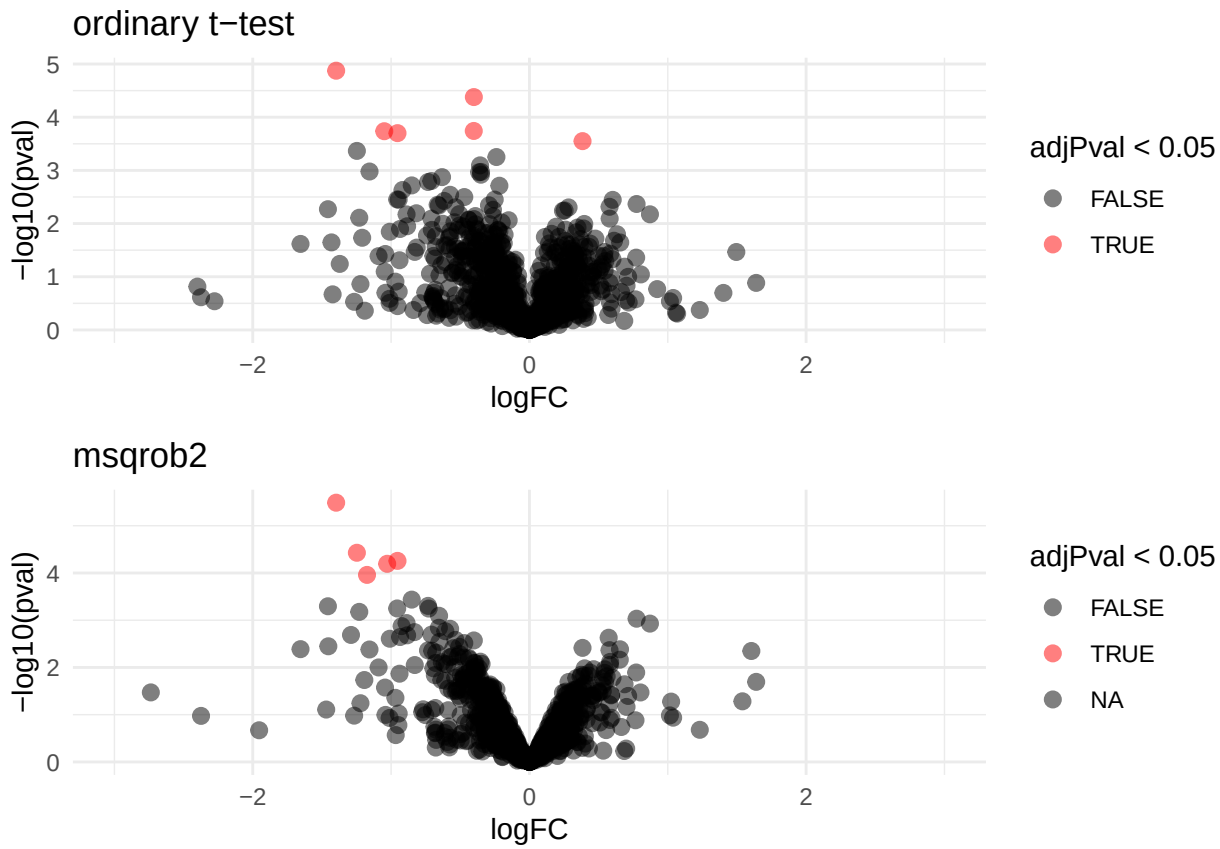
```
##                   logFC         se       df          t         pval     adjPval
## WP_003023392 -1.3971592 0.09349966 6.397042 -14.942933 3.254588e-06 0.003244824
## WP_003040849 -1.2473865 0.12404946 6.397042 -10.055557 3.736229e-05 0.015936958
## WP_003014552 -0.9536835 0.10126476 6.397042  -9.417724 5.550408e-05 0.015936958
## WP_003026016 -1.0283106 0.10528137 6.034774  -9.767261 6.393965e-05 0.015936958
## WP_003033719 -1.1740374 0.13543520 6.186857  -8.668628 1.098307e-04 0.021900233
```

We can also visualise the results using a volcanoplot

```
volcano <- ggplot(
    rowData(pe[["protein"]])$genotypeD8,
    aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)
) +
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal() +
    ggtitle("msqrob2")
```

```
gridExtra::grid.arrange(
  volcanoT +
    xlim(-3,3) +
  ggtitle("ordinary t-test"),
  volcano +
    xlim(-3,3)
,nrow=2)
```

```
## Warning: Removed 28 rows containing missing values or values outside the scale range
## (`geom_point()`).
```

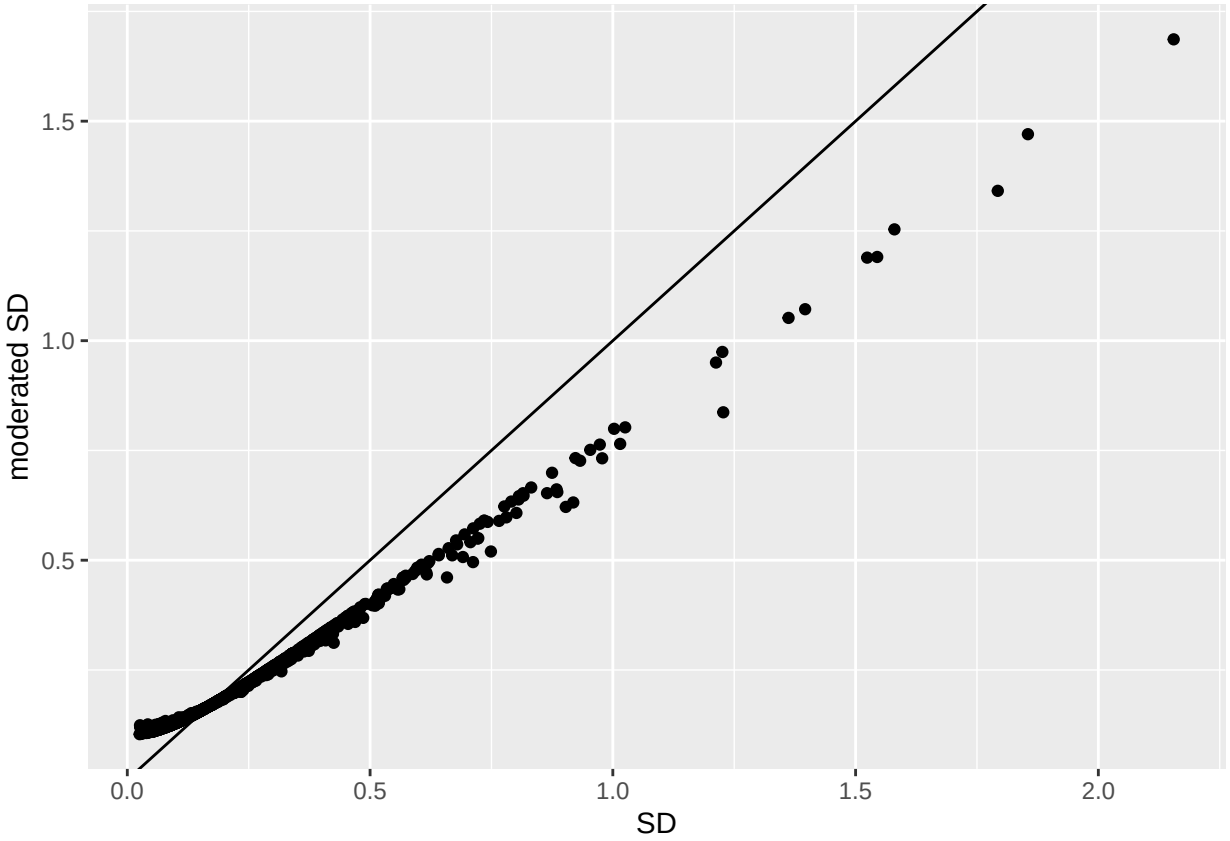


- The volcano plot opens up when using the EB variance estimator
- Borrowing strength to estimate the variance using empirical Bayes solves the issue of returning proteins with a low fold change as significant due to a low variance.

1.5.2 Shrinkage of the variance and moderated t-statistics

```
qplot(
  sapply(rowData(pe[["protein"]])$msqrobModels,getSigma),
  sapply(rowData(pe[["protein"]])$msqrobModels,getSigmaPosterior)) +
  xlab("SD") +
  ylab("moderated SD") +
  geom_abline(intercept = 0,slope = 1) +
  geom_hline(yintercept = )
```

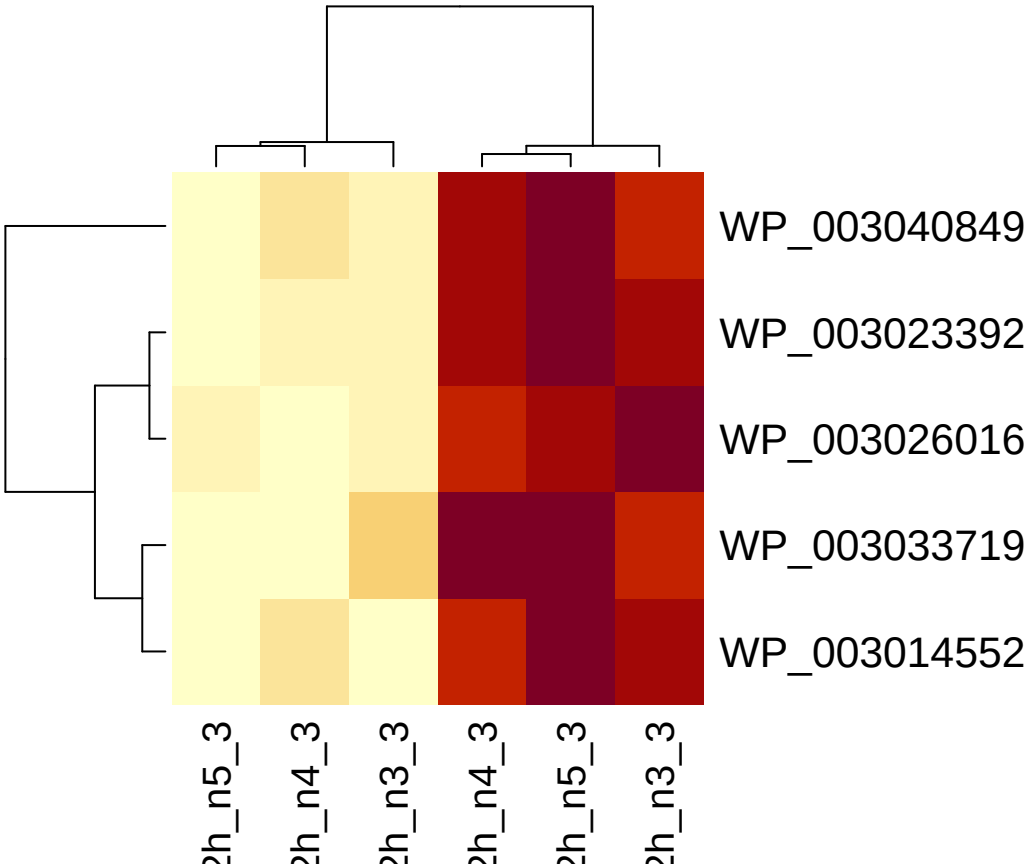
```
## Warning: Removed 28 rows containing missing values or values outside the scale range
## (`geom_point()`).
```



- Small variances are shrunken towards the common variance resulting in large EB variance estimates
- Large variances are shrunken towards the common variance resulting in smaller EB variance estimates
- Pooled degrees of freedom of the EB variance estimator are larger because information is borrowed across proteins to estimate the variance

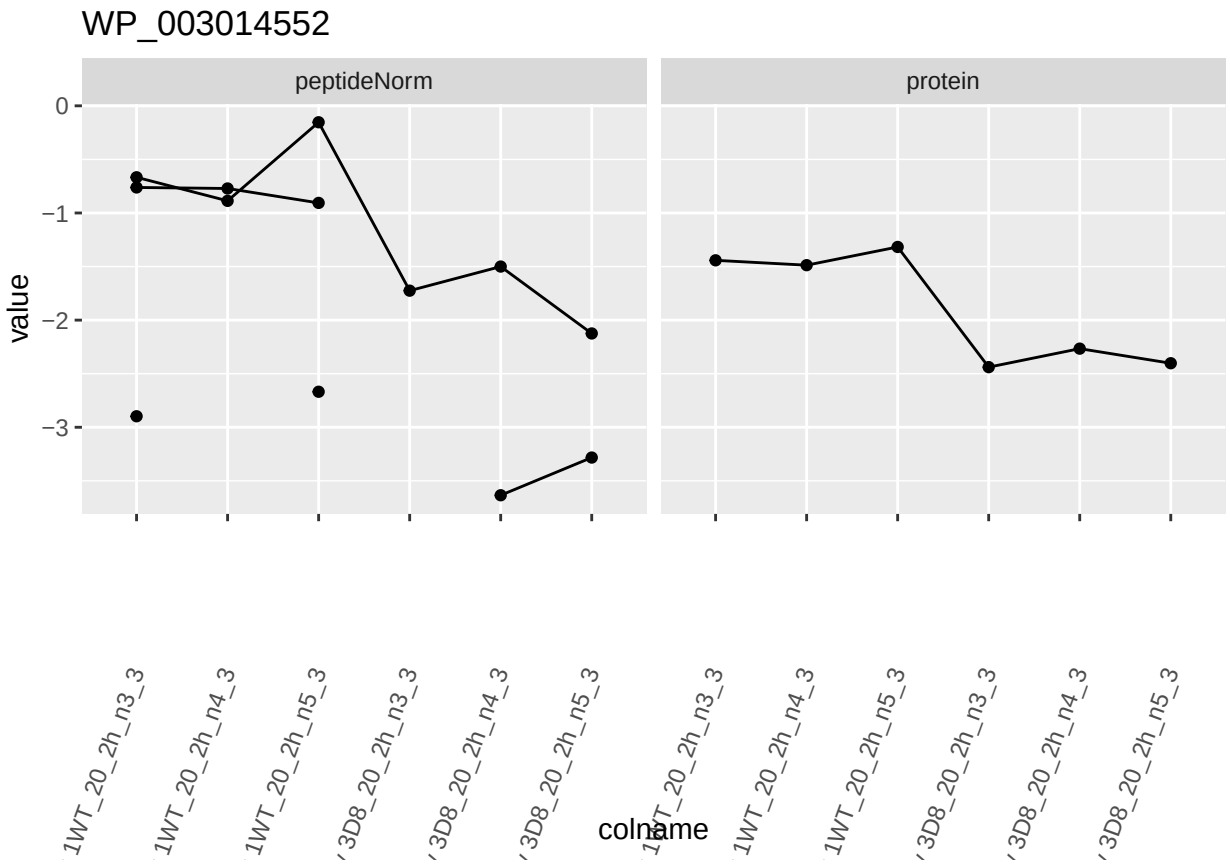
1.6 Plots

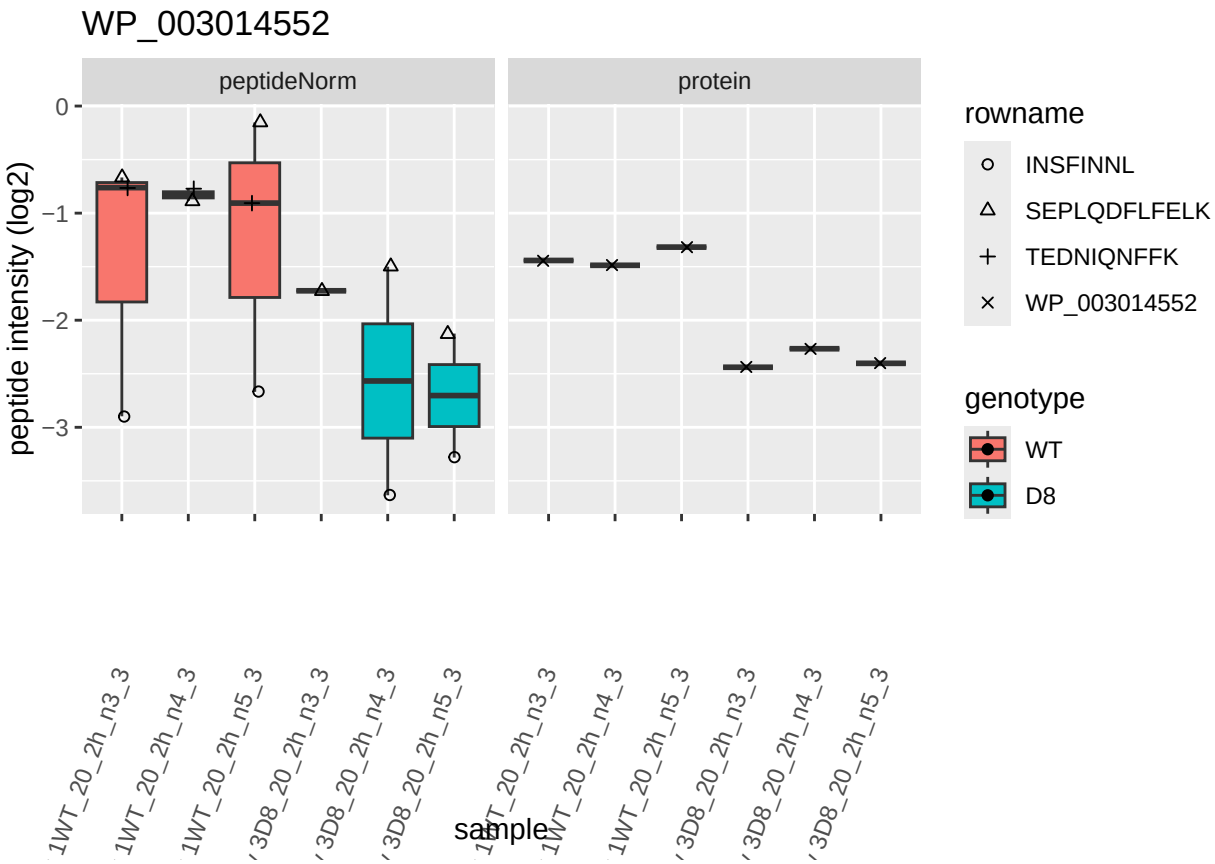
```
sigNames <- rowData(pe[["protein"]])$genotypeD8 |>
    rownames_to_column("protein") |>
    filter(adjPval < 0.05) |>
    pull(protein)
heatmap(assay(pe[["protein"]])[sigNames, ])
```

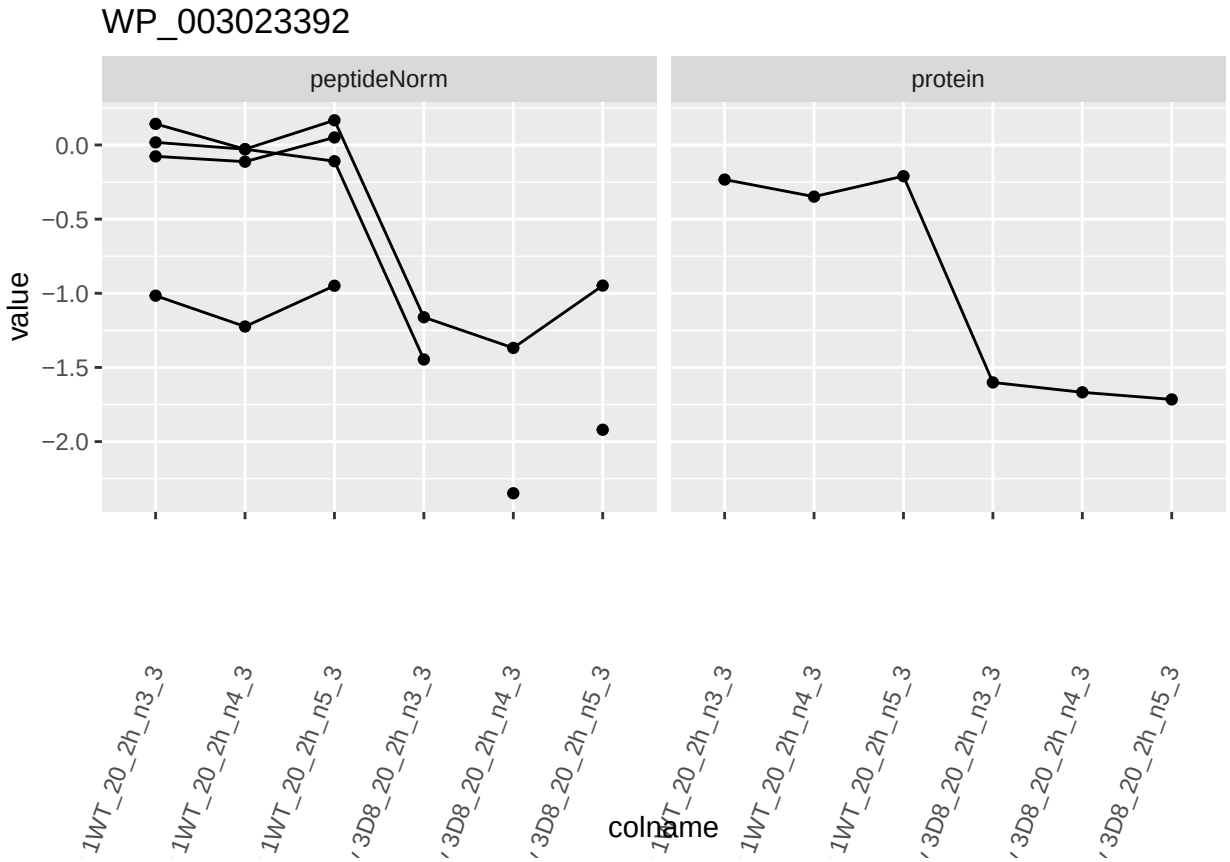


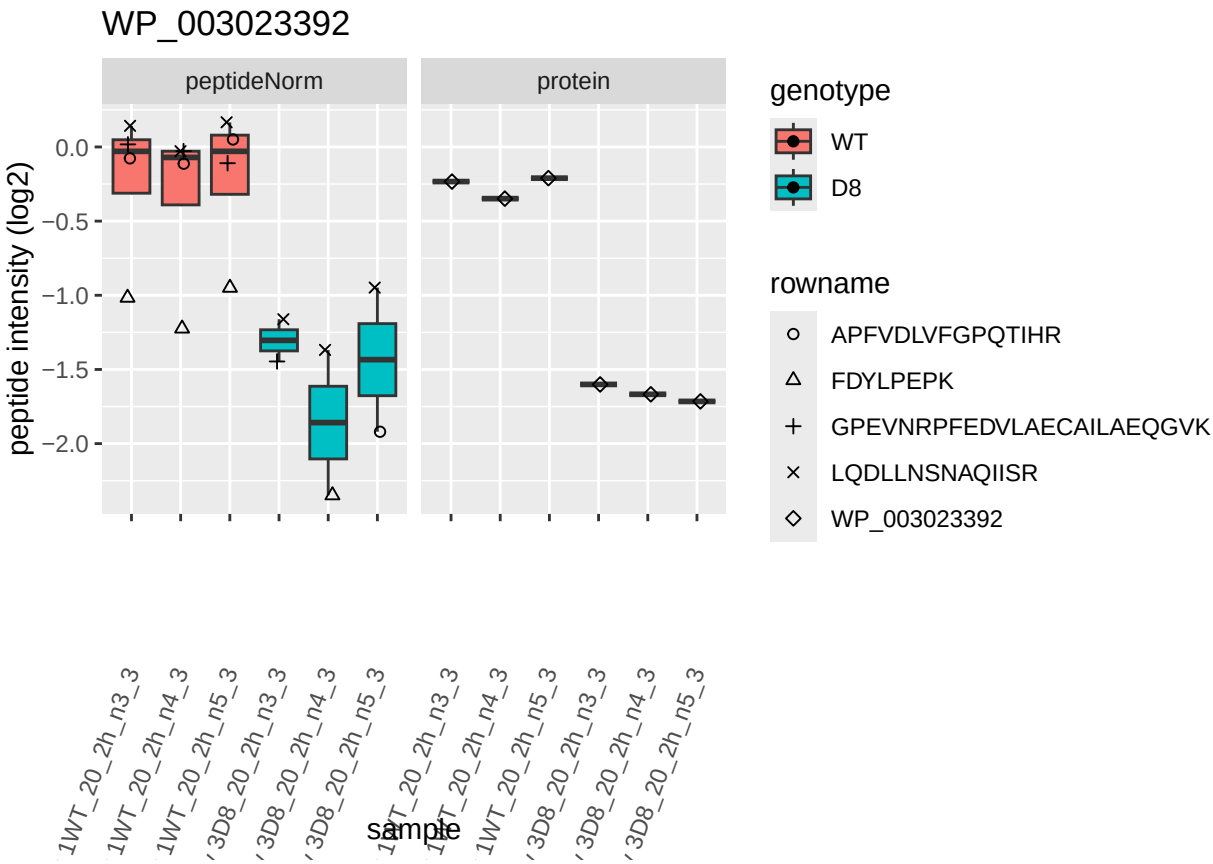

```
for (protName in sigNames)
    {
        pePlot <- pe[protName, , c("peptideNorm", "protein")]
        pePlotDf <- data.frame(longForm(pePlot))
        pePlotDf$assay <- factor(pePlotDf$assay,
            levels = c("peptideNorm", "protein")
        )
        pePlotDf$genotype <- as.factor(colData(pePlot)[pePlotDf$colname, "genotype"])

        # plotting
        p1 <- ggplot(
            data = pePlotDf,
            aes(x = colname, y = value, group = rowname)
        ) +
            geom_line() +
            geom_point() +
            facet_grid(~assay) +
            theme(axis.text.x = element_text(angle = 70, hjust = 1, vjust = 0.5)) +
            ggtitle(protName)
        print(p1)

        # plotting 2
        p2 <- ggplot(pePlotDf, aes(x = colname, y = value, fill = genotype)) +
            geom_boxplot(outlier.shape = NA) +
            geom_point(
                position = position_jitter(width = .1),
```

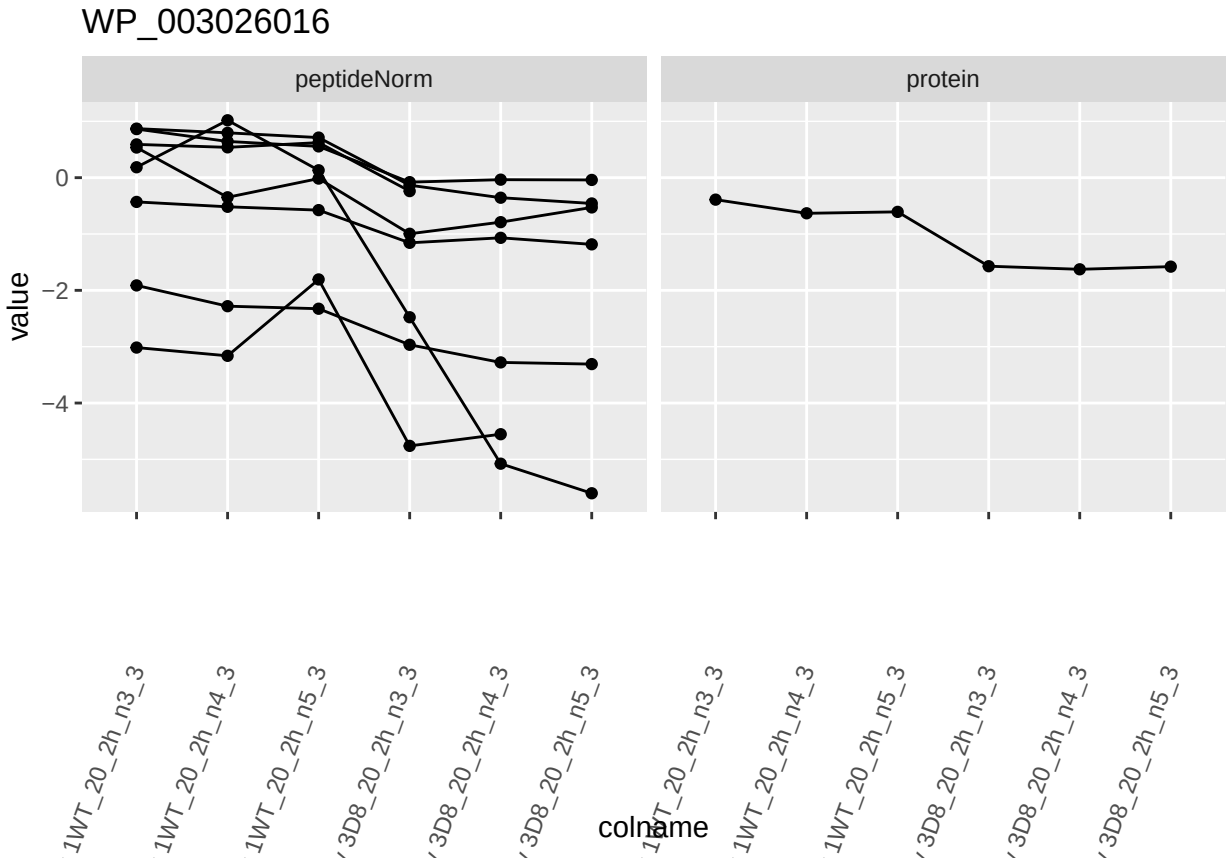
```
                aes(shape = rowname)
            ) +
            scale_shape_manual(values = 1:nrow(pePlotDf)) +
            labs(title = protName, x = "sample", y = "peptide intensity (log2)") +
            theme(axis.text.x = element_text(angle = 70, hjust = 1, vjust = 0.5)) +
            facet_grid(~assay)
        print(p2)
}
```

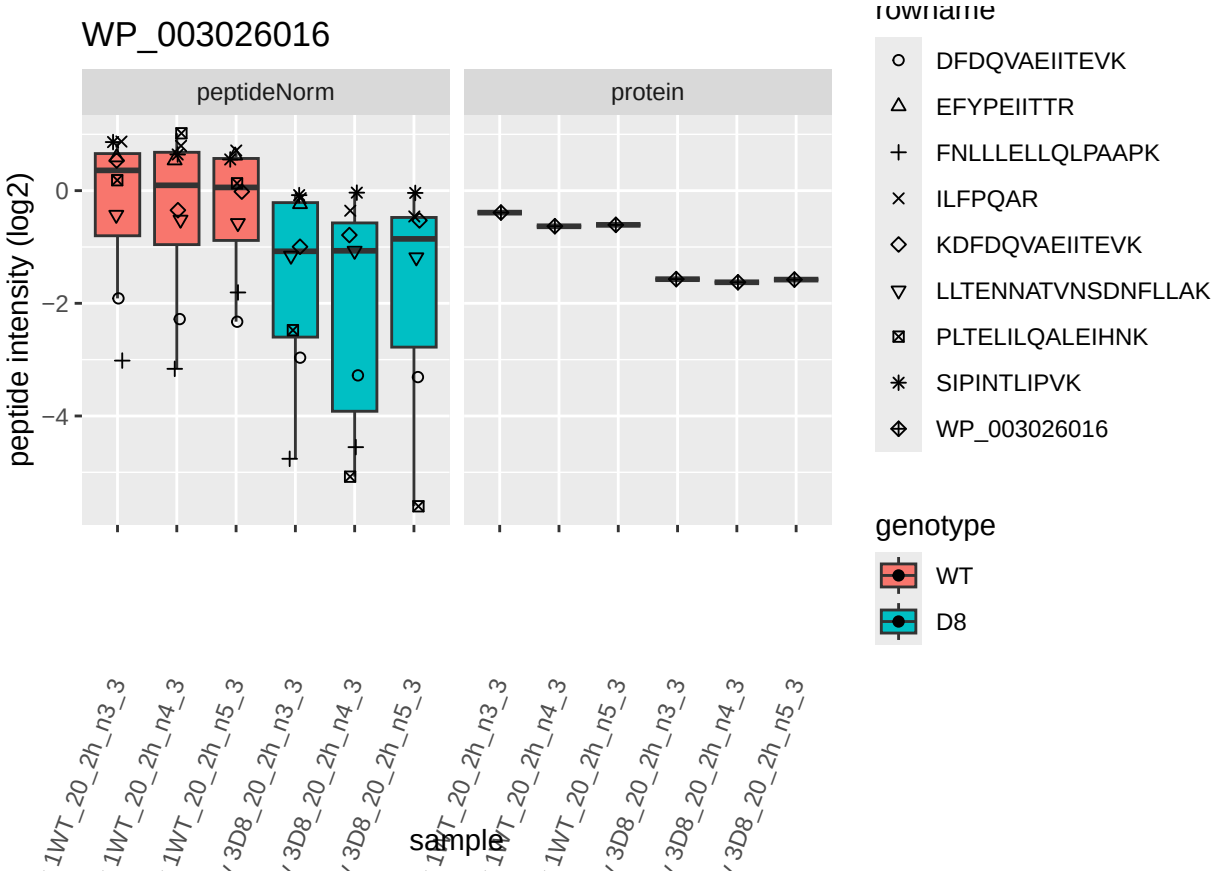


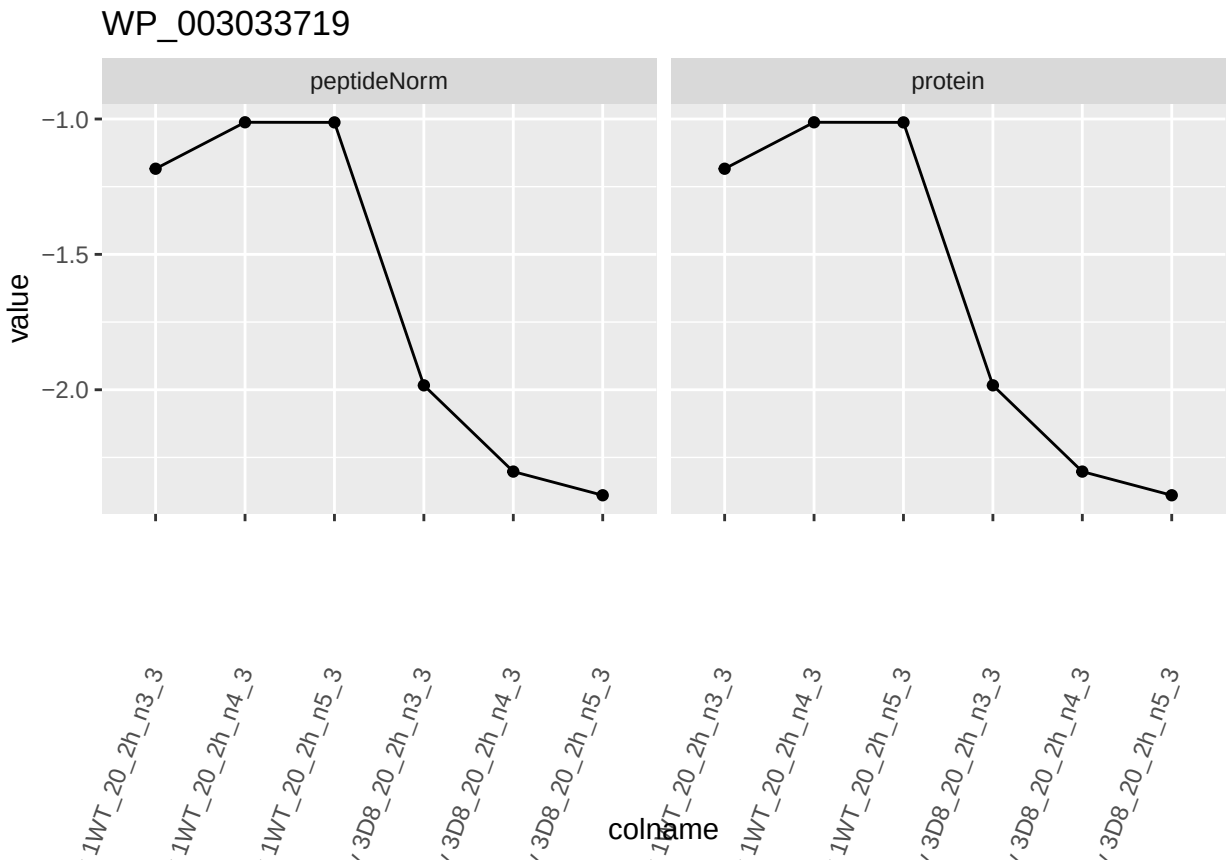

WP_003014552
peptideNorm
protein
peptide intensity (log2)
0
−1
−2
−3
sample
rowname
INSFINNL
SEPLQDFLFELK
TEDNIQNFFK
WP_003014552
genotype
WT
D8

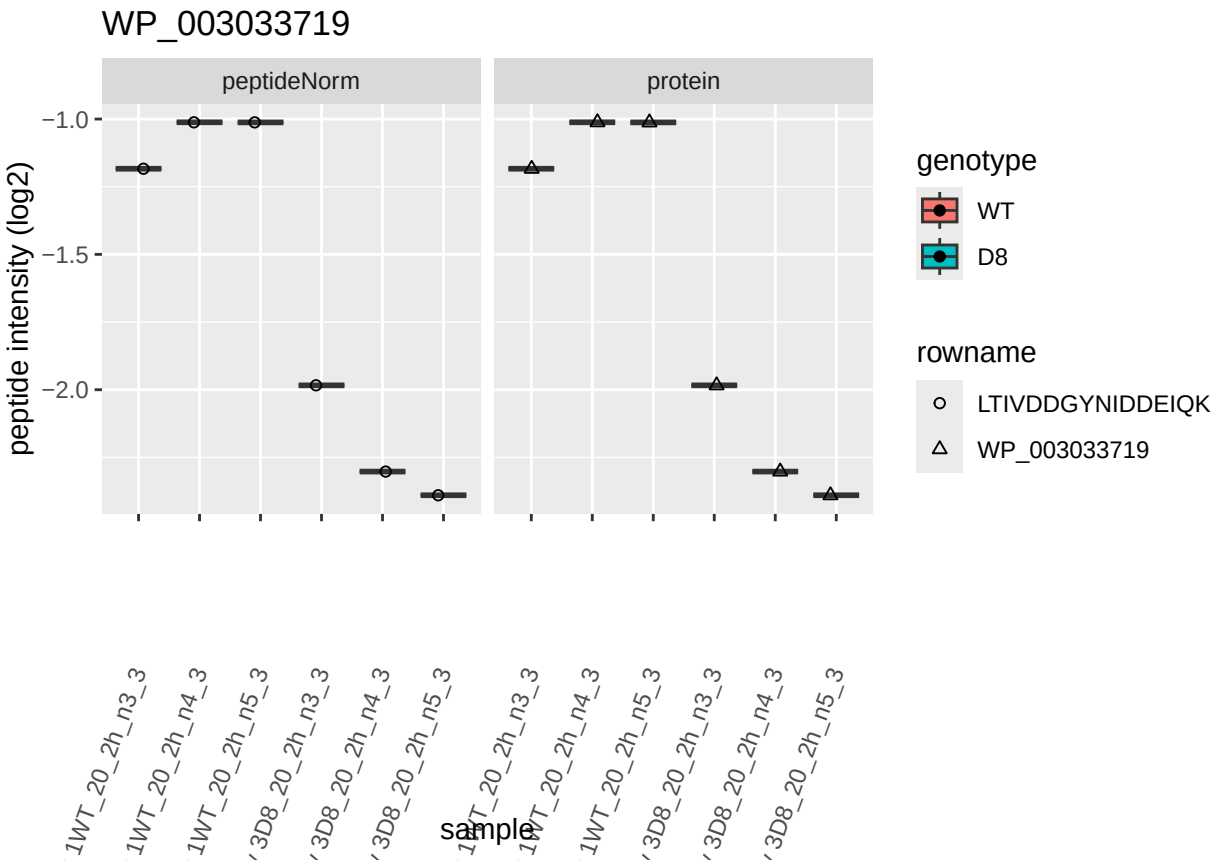

WP_003023392
peptideNorm
protein
value
0.0
−0.5
−1.0
−1.5
−2.0
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
colname

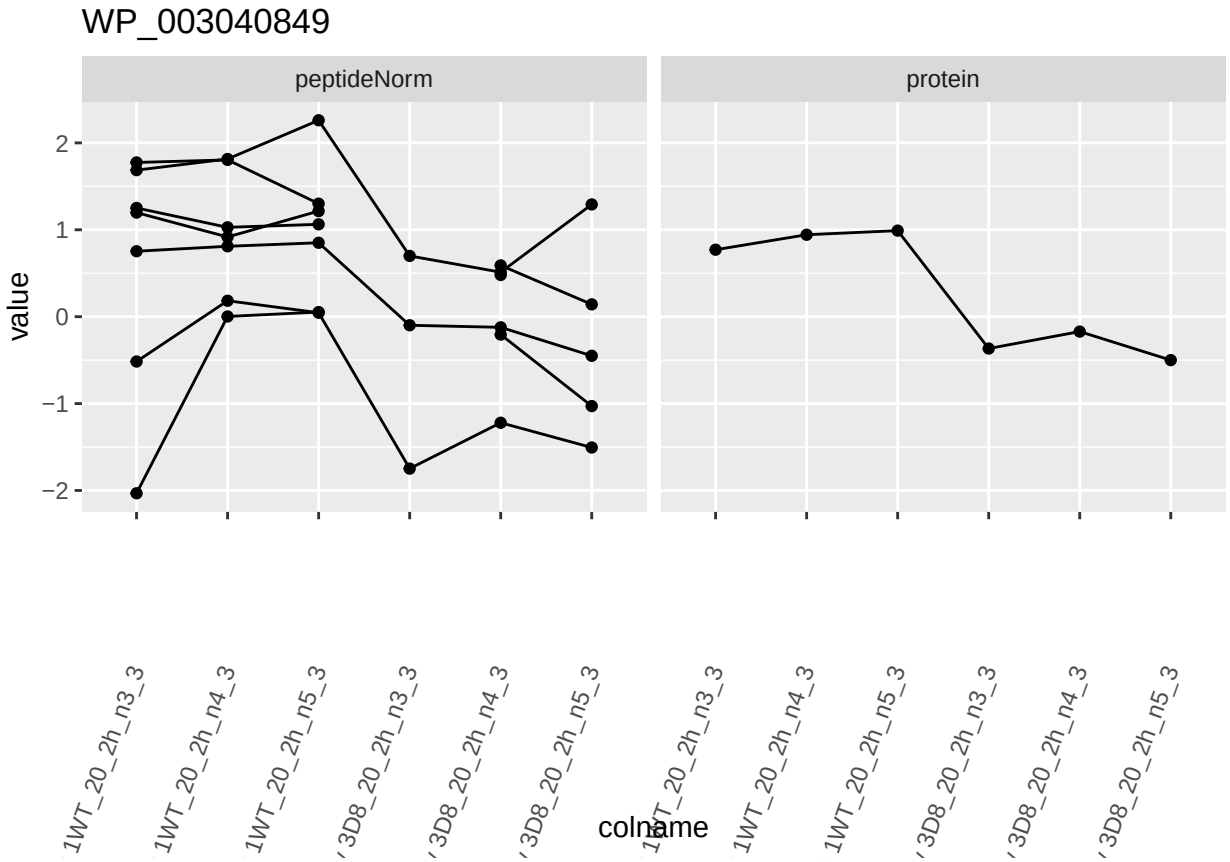

WP_003023392
peptideNorm
protein
peptide intensity (log2)
0.0
−0.5
−1.0
−1.5
−2.0
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
sample
genotype
WT
D8
rowname
APFVDLVFGPQTIHR
FDYLPEPK
GPEVNRPFEDVLAECAILAEQGVK
LQDLLNSNAQIISR
WP_003023392

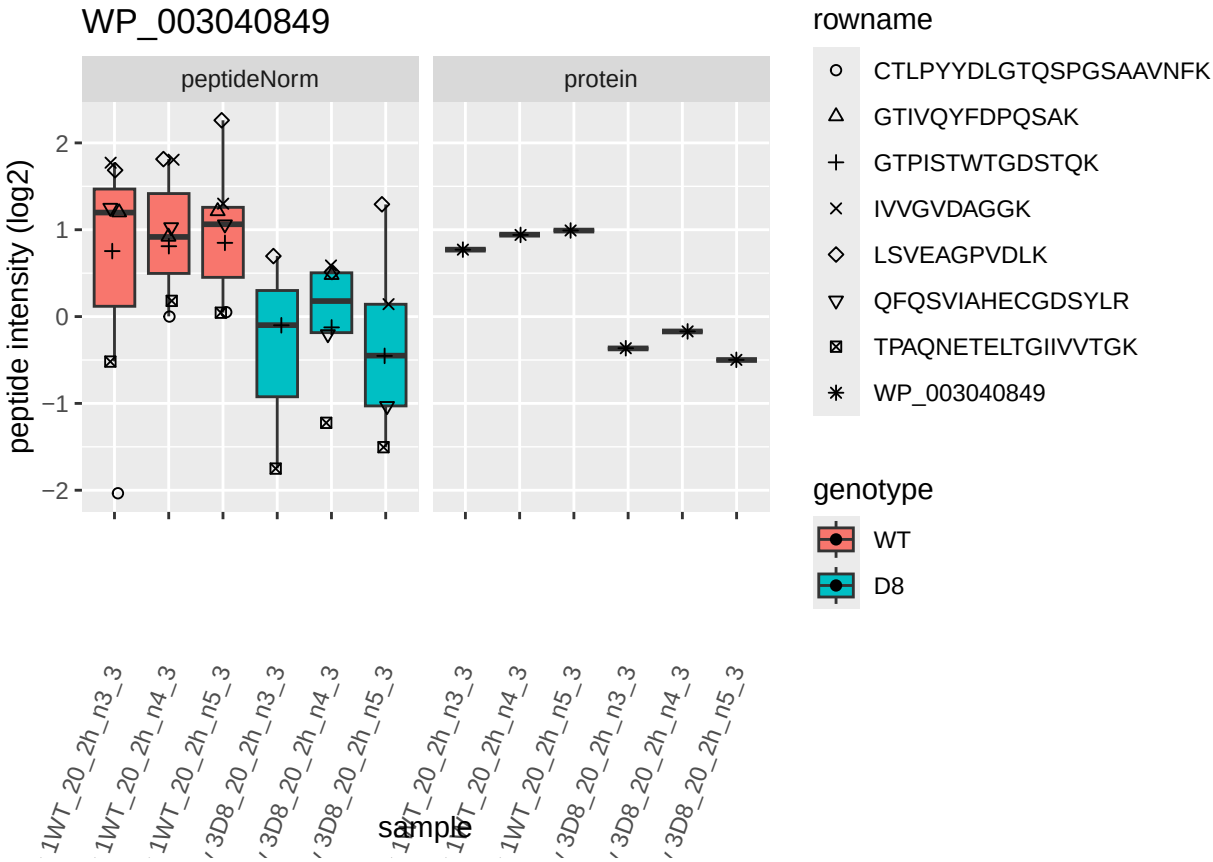

WP_003026016
peptideNorm
protein
value
0
−2
−4
colname
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3


WP_003026016
peptideNorm
protein
peptide intensity (log2)
0
−2
−4
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
sample
rowname
DFDQVAEIITEVK
EFYPEIITTR
FNLLLELLQLPAAPK
ILFPQAR
KDFDQVAEIITEVK
LLTENNATVNSDNFLLAK
PLTELILQALEIHNK
SIPINTLIPVK
WP_003026016
genotype
WT
D8


WP_003033719
peptideNorm
protein
−1.0
−1.5
−2.0
value
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
colname


WP_003033719
peptideNorm
protein
peptide intensity (log2)
−1.0
−1.5
−2.0
genotype
WT
D8
rowname
LTIVDDGYNIDDEIQK
WP_003033719
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
sample


WP_003040849
peptideNorm
protein
value
2
1
0
−1
−2
colname
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3
1WT_20_2h_n3_3
1WT_20_2h_n4_3
1WT_20_2h_n5_3
3D8_20_2h_n3_3
3D8_20_2h_n4_3
3D8_20_2h_n5_3



2 Experimental Design

2.1 Sample size

$$\log_2 \mathrm{FC} = \bar{y}_{p1} - \bar{y}_{p2}$$

$$T_g = \frac{\log_2 \mathrm{FC}}{\mathrm{se}_{\log_2 \mathrm{FC}}}$$

$$T_g = \frac{\widehat{\text{signal}}}{\widehat{\text{Noise}}}$$

If we can assume equal variance in both treatment groups:

$$\mathrm{se}_{\log_2 \mathrm{FC}} = \mathrm{SD}\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

$\rightarrow$ if number of bio-repeats increases we have a higher power!

- cfr. Study of tamoxifen treated Estrogen Recepter (ER) positive breast cancer patients

2.2 Randomized complete block designs

$$\sigma^2 = \sigma^2_{bio} + \sigma^2_{\text{lab}} + \sigma^2_{\text{extraction}} + \sigma^2_{\text{run}} + \dots$$

- Biological: fluctuations in protein level between mice, fluctations in protein level between cells, ...
- Technical: cage effect, lab effect, week effect, plasma extraction, MS-run, ...

2.2.1 Nature methods: Points of significance - Blocking

https://www.nature.com/articles/nmeth.3005.pdf

2.2.2 Mouse example

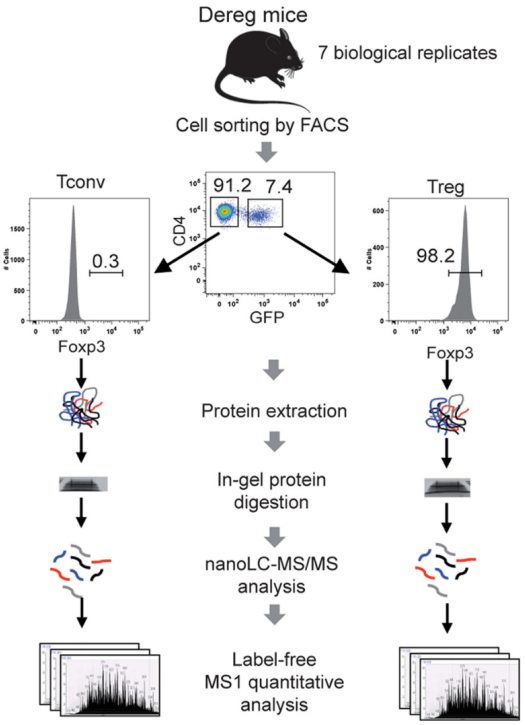


FIG. 1. **Label-free quantitative analysis of conventional and regulatory T cell proteomes.** General analytical workflow based on cell sorting by flow cytometry using the DEREG mouse model and parallel proteomic analysis of Tconv and Treg cell populations by nanoLC-MS/MS and label-free relative quantification.

Duguet et al. (2017) MCP 16(8):1416-1432. doi: 10.1074/mcp.m116.062745

- All treatments of interest are present within block!
- We can estimate the effect of the treatment within block!

To illustrate the power of blocking we have subsetted the data of Duguet et al. in a

- completely randomized design with
  - four mice for which we only have measurements on the ordinary T-cells
  - four mice for which we only have measurements on the regulatory T-cells
- randomized complete block design with four mice for which we both have
  - measurements on ordinary T-cells as well as
  - measurements on regulatory T-cells

2.2.3 Data

Click to see code

```
peptidesTable <- fread("https://raw.githubusercontent.com/statOmics/PDA21/data/quantification/mouseTcel
int64 <- which(sapply(peptidesTable,class) == "integer64")
for (j in int64) peptidesTable[[j]] <- as.numeric(peptidesTable[[j]])

peptidesTable2 <- fread("https://raw.githubusercontent.com/statOmics/PDA21/data/quantification/mouseTce
int64 <- which(sapply(peptidesTable2,class) == "integer64")
for (j in int64) peptidesTable2[[j]] <- as.numeric(peptidesTable2[[j]])

peptidesTable3 <- fread("https://raw.githubusercontent.com/statOmics/PDA21/data/quantification/mouseTce
int64 <- which(sapply(peptidesTable3,class) == "integer64")
for (j in int64) peptidesTable3[[j]] <- as.numeric(peptidesTable3[[j]])

quantCols <- grep("Intensity ", names(peptidesTable))
pe <- readQFeatures(
  assayData = peptidesTable,
  fnames = 1,
  quantCols =  quantCols,
  name = "peptideRaw")
rm(peptidesTable)
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8810032 470.6   15503455 828.0         NA 15503455 828.0
## Vcells 45288133 345.6   81348285 620.7      16384 81348285 620.7
```

```
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8810017 470.6   15503455 828.0         NA 15503455 828.0
## Vcells 45285987 345.6   81348285 620.7      16384 81348285 620.7
```

```
quantCols2 <- grep("Intensity ", names(peptidesTable2))
pe2 <- readQFeatures(
  assayData = peptidesTable2,
  fnames = 1,
  quantCols =  quantCols2,
  name = "peptideRaw")
rm(peptidesTable2)
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8721899 465.8   15503455 828.0         NA 15503455 828.0
## Vcells 38681168 295.2   81348285 620.7      16384 81348285 620.7
```

```
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8721898 465.8   15503455 828.0         NA 15503455 828.0
## Vcells 38676885 295.1   81348285 620.7      16384 81348285 620.7
```

```
quantCols3 <- grep("Intensity ", names(peptidesTable3))
pe3 <- readQFeatures(
  assayData = peptidesTable3,
  fnames = 1,
```

```
  quantCols =  quantCols3,
  name = "peptideRaw")
rm(peptidesTable3)
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8721487 465.8   15503455 828.0         NA 15503455 828.0
## Vcells 29992788 228.9   81348285 620.7      16384 81348285 620.7
```

```
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8721412 465.8   15503455 828.0         NA 15503455 828.0
## Vcells 29988230 228.8   81348285 620.7      16384 81348285 620.7
```

```
### Design
colData(pe)$celltype <- substr(
  colnames(pe[["peptideRaw"]]),
  11,
  14) |>
  unlist() |>
  as.factor()

colData(pe)$mouse <- pe[[1]] |>
  colnames() |>
  strsplit(split="[ ]")  |>
  sapply(function(x) x[3]) |>
  as.factor()

colData(pe2)$celltype <- substr(
  colnames(pe2[["peptideRaw"]]),
  11,
  14) |>
  unlist() |>
  as.factor()

colData(pe2)$mouse <- pe2[[1]] |>
  colnames() |>
  strsplit(split="[ ]")  |>
  sapply(function(x) x[3]) |>
  as.factor()

colData(pe3)$celltype <- substr(
  colnames(pe3[["peptideRaw"]]),
  11,
  14) |>
  unlist() |>
  as.factor()

colData(pe3)$mouse <- pe3[[1]] |>
  colnames() |>
  strsplit(split="[ ]")  |>
  sapply(function(x) x[3]) |>
  as.factor()
```

2.2.4 Preprocessing

2.2.4.1 Log-transform Click to see code to log-transfrom the data

- Peptides with zero intensities are missing peptides and should be represent with a `NA` value rather than `0`.

```
pe <- zeroIsNA(pe, "peptideRaw") # convert 0 to NA

pe2 <- zeroIsNA(pe2, "peptideRaw") # convert 0 to NA

pe3 <- zeroIsNA(pe3, "peptideRaw") # convert 0 to NA
```

- Logtransform data with base 2

```
pe <- logTransform(pe, base = 2, i = "peptideRaw", name = "peptideLog")

pe2 <- logTransform(pe2, base = 2, i = "peptideRaw", name = "peptideLog")

pe3 <- logTransform(pe3, base = 2, i = "peptideRaw", name = "peptideLog")
```

2.2.4.2 Filtering Click to see details on filtering

1. Remove peptides that map to multiple proteins

We remove PSMs that could not be mapped to a protein or that map to multiple proteins (the protein identifier contains multiple identifiers separated by a `;`).

```
pe <- filterFeatures(
    pe, ~ Proteins != "" & ## Remove failed protein inference
        !grepl(";", Proteins)) ## Remove protein groups
```

```
## 'Proteins' found in 2 out of 2 assay(s).
```

```
pe2 <- filterFeatures(
    pe2, ~ Proteins != "" & ## Remove failed protein inference
        !grepl(";", Proteins)) ## Remove protein groups
```

```
## 'Proteins' found in 2 out of 2 assay(s).
```

```
pe3 <- filterFeatures(
    pe3, ~ Proteins != "" & ## Remove failed protein inference
        !grepl(";", Proteins)) ## Remove protein groups
```

```
## 'Proteins' found in 2 out of 2 assay(s).
```

2. Remove reverse sequences (decoys) and contaminants

We now remove the contaminants, peptides that map to decoy sequences, and proteins which were only identified by peptides with modifications.

```
pe <- filterFeatures(pe,~Reverse != "+")
```

```
## 'Reverse' found in 2 out of 2 assay(s).
```

```
pe <- filterFeatures(pe,~ Potential.contaminant != "+")
```

```
## 'Potential.contaminant' found in 2 out of 2 assay(s).
```

```
pe2 <- filterFeatures(pe2,~Reverse != "+")
```

```
## 'Reverse' found in 2 out of 2 assay(s).
```

```
pe2 <- filterFeatures(pe2,~ Potential.contaminant != "+")
```

```
## 'Potential.contaminant' found in 2 out of 2 assay(s).
```

```
pe3 <- filterFeatures(pe3,~Reverse != "+")
```

```
## 'Reverse' found in 2 out of 2 assay(s).
```

```
pe3 <- filterFeatures(pe3,~ Potential.contaminant != "+")
```

```
## 'Potential.contaminant' found in 2 out of 2 assay(s).
```

3. Drop peptides that were identified in less than three sample

We keep peptides that were observed at least three times.

```
nObs <- 3
n <- ncol(pe[["peptideLog"]])
pNA <- (n-nObs)/n
pe <- filterNA(pe, pNA = pNA, i = "peptideLog")
nrow(pe[["peptideLog"]])
```

```
## [1] 38782
```

```
n <- ncol(pe2[["peptideLog"]])
pNA <- (n-nObs)/n
pe2 <- filterNA(pe2, pNA = pNA, i = "peptideLog")
nrow(pe2[["peptideLog"]])
```

```
## [1] 37898
```

```
n <- ncol(pe3[["peptideLog"]])
pNA <- (n-nObs)/n
pe3 <- filterNA(pe3, pNA = pNA, i = "peptideLog")
nrow(pe3[["peptideLog"]])
```

```
## [1] 43984
```

2.2.4.3 Normalization

Click to see code to normalize the data

```
pe <- normalize(pe,
                i = "peptideLog",
                name = "peptideNorm",
                method = "center.median")

pe2 <- normalize(pe2,
                i = "peptideLog",
                name = "peptideNorm",
                method = "center.median")


pe3 <- normalize(pe3,
                i = "peptideLog",
                name = "peptideNorm",
                method = "center.median")
```

2.2.4.4 Summarization

Click to see code to summarize the data

```
pe <- aggregateFeatures(pe,
 i = "peptideNorm",
 fcol = "Proteins",
 na.rm = TRUE,
 name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

```
## Aggregated: 1/1
```

```
pe2 <- aggregateFeatures(pe2,
 i = "peptideNorm",
 fcol = "Proteins",
 na.rm = TRUE,
 name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
## Aggregated: 1/1
```

```
pe3 <- aggregateFeatures(pe3,
 i = "peptideNorm",
 fcol = "Proteins",
 na.rm = TRUE,
 name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
## Aggregated: 1/1
```

2.2.4.5 Filtering proteins with too many missing values

We want to have at least two observed protein intensities for each group so we set the minimum number of observed values at 4. We still have to check for the observed proteins if that is the case.

For block design more clever filtering can be used. E.g. we could imply that we have both cell types in at least two animals...

```
nObs <- 4
n <- ncol(pe[["protein"]])
pNA <- (n-nObs)/n
pe<- filterNA(pe, pNA = pNA, i = "protein")

n <- ncol(pe2[["protein"]])
pNA <- (n-nObs)/n
pe2 <- filterNA(pe2, pNA = pNA, i = "protein")

n <- ncol(pe3[["protein"]])
pNA <- (n-nObs)/n
pe3 <- filterNA(pe3, pNA = pNA, i = "protein")
```

2.2.5 Data Exploration: what is impact of blocking?

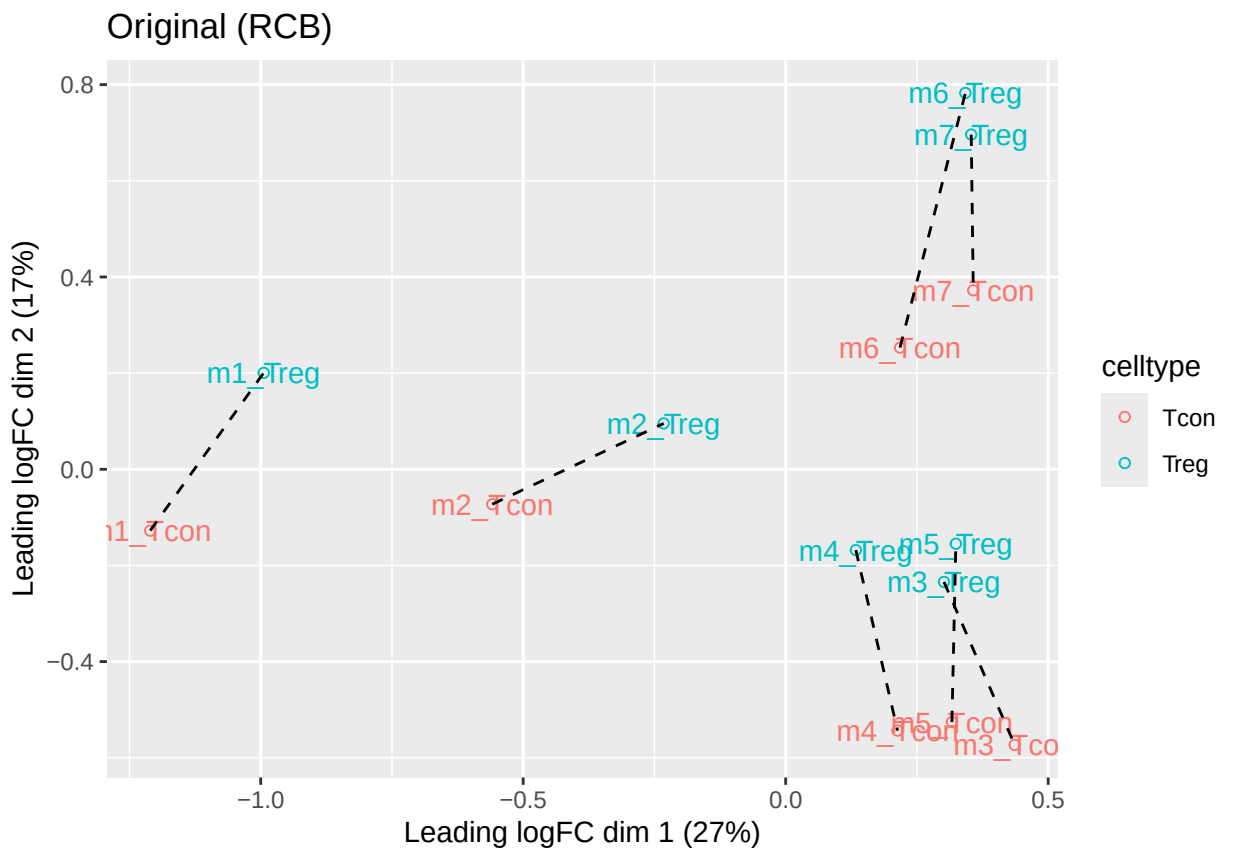
Click to see code

```
levels(colData(pe3)$mouse) <- paste0("m",1:7)
mdsObj3 <- plotMDS(assay(pe3[["protein"]]), plot = FALSE)
mdsOrig <- colData(pe3) |>
  as.data.frame() |>
  mutate(mds1 = mdsObj3$x,
         mds2 = mdsObj3$y,
         lab = paste(mouse,celltype,sep="_")) |>
  ggplot(aes(x = mds1, y = mds2, label = lab, color = celltype, group = mouse)) +
  geom_text(show.legend = FALSE) +
  geom_point(shape = 21) +
  geom_line(color = "black", linetype = "dashed") +
  xlab(
    paste0(
      mdsObj3$axislabel,
      " ",
      1,
      " (",
      paste0(
        round(mdsObj3$var.explained[1] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ylab(
    paste0(
      mdsObj3$axislabel,
      " ",
      2,
      " (",
      paste0(
        round(mdsObj3$var.explained[2] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ggtitle("Original (RCB)")

levels(colData(pe)$mouse) <- paste0("m",1:4)
mdsObj <- plotMDS(assay(pe[["protein"]]), plot = FALSE)
mdsRCB <- colData(pe) |>
  as.data.frame() |>
  mutate(mds1 = mdsObj$x,
         mds2 = mdsObj$y,
         lab = paste(mouse,celltype,sep="_")) |>
  ggplot(aes(x = mds1, y = mds2, label = lab, color = celltype, group = mouse)) +
  geom_text(show.legend = FALSE) +
  geom_point(shape = 21) +
  geom_line(color = "black", linetype = "dashed") +
  xlab(
    paste0(
      mdsObj$axislabel,
```

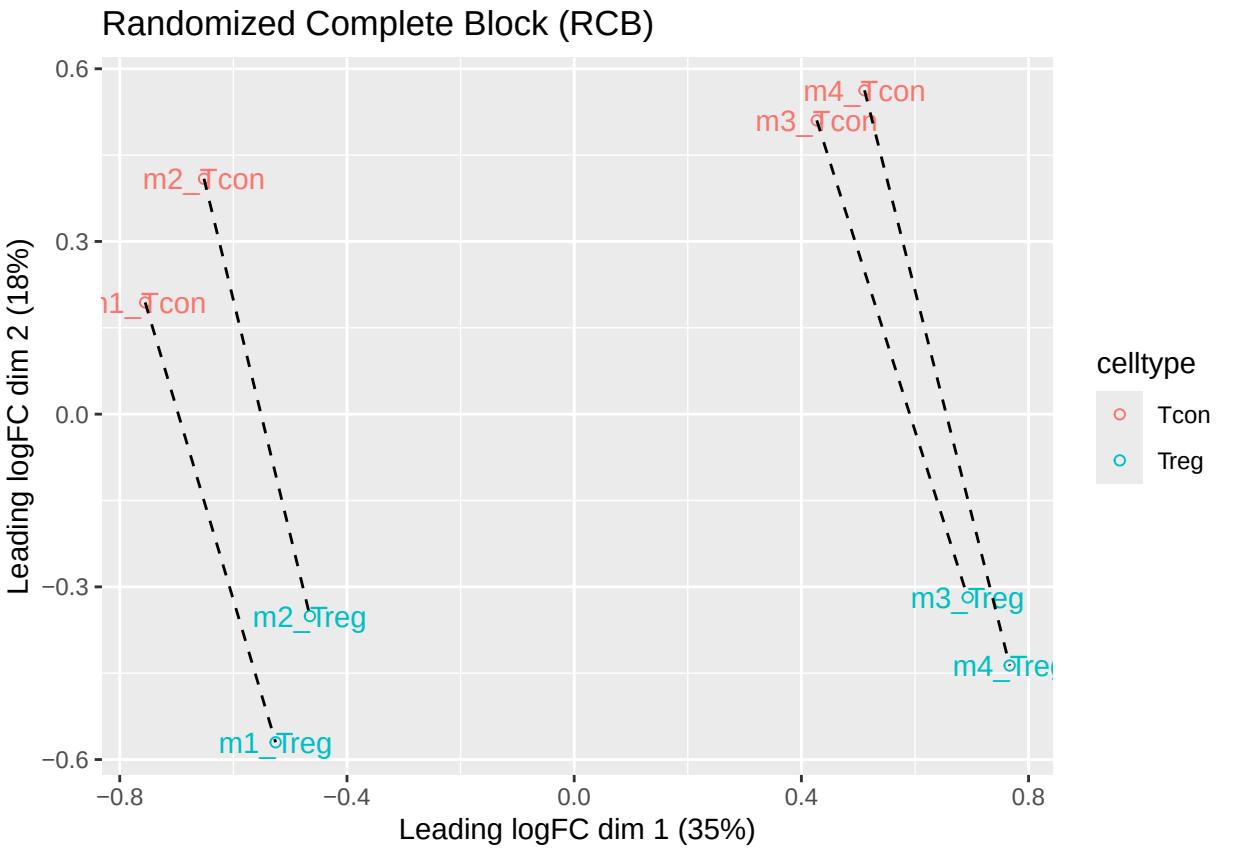
```
      " ",
      1,
      " (",
      paste0(
        round(mdsObj$var.explained[1] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ylab(
    paste0(
      mdsObj$axislabel,
      " ",
      2,
      " (",
      paste0(
        round(mdsObj$var.explained[2] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ggtitle("Randomized Complete Block (RCB)")


levels(colData(pe2)$mouse) <- paste0("m",1:8)
mdsObj2 <- plotMDS(assay(pe2[["protein"]]), plot = FALSE)
mdsCRD <- colData(pe2) |>
  as.data.frame() |>
  mutate(mds1 = mdsObj2$x,
         mds2 = mdsObj2$y,
         lab = paste(mouse,celltype,sep="_")) |>
  ggplot(aes(x = mds1, y = mds2, label = lab, color = celltype, group = mouse)) +
  geom_text(show.legend = FALSE) +
  geom_point(shape = 21) +
  xlab(
    paste0(
      mdsObj$axislabel,
      " ",
      1,
      " (",
      paste0(
        round(mdsObj2$var.explained[1] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ylab(
    paste0(
      mdsObj$axislabel,
      " ",
```

```
      2,
      " (",
      paste0(
        round(mdsObj2$var.explained[2] *100,0),
        "%"
        ),
      ")"
      )
    ) +
  ggtitle("Completely Randomized Design (CRD)")
```

```
mdsOrig
```




```
mdsRCB
```


Randomized Complete Block (RCB)
Leading logFC dim 2 (18%)
Leading logFC dim 1 (35%)
0.6
0.3
0.0
−0.3
−0.6
−0.8
−0.4
0.0
0.4
0.8
m4_Tcon
m3_Tcon
m2_Tcon
m3_Treg
m2_Treg
m1_Treg
celltype
Tcon
Treg

```
mdsCRD
```

- We observe that the leading fold change is according to mouse
- In the second dimension we see a separation according to cell-type
- With the Randomized Complete Block design (RCB) we can remove the mouse effect from the analysis!
- We can isolate the between block variability from the analysis using linear model:
  - Formula in R

$$y \sim \text{celltype} + \text{mouse}$$

  - Formula

$$y_i = \beta_0 + \beta_{\text{Treg}} x_{i,\text{Treg}} + \beta_{m2} x_{i,m2} + \beta_{m3} x_{i,m3} + \beta_{m4} x_{i,m4} + \epsilon_i$$

with

- $x_{i,Treg} = \begin{cases} 1 & \text{Treg} \\ 0 & \text{Tcon} \end{cases}$
- $x_{i,m2} = \begin{cases} 1 & \text{m2} \\ 0 & \text{otherwise} \end{cases}$
- $x_{i,m3} = \begin{cases} 1 & \text{m3} \\ 0 & \text{otherwise} \end{cases}$
- $x_{i,m4} = \begin{cases} 1 & \text{m4} \\ 0 & \text{otherwise} \end{cases}$

- Possible in msqrob2 and MSstats but not possible with Perseus!

2.2.6 Modeling and inference

```
pe <- msqrob(
  object = pe,
  i = "protein",
  formula = ~ celltype + mouse)
```

2.2.6.1 RCB analysis

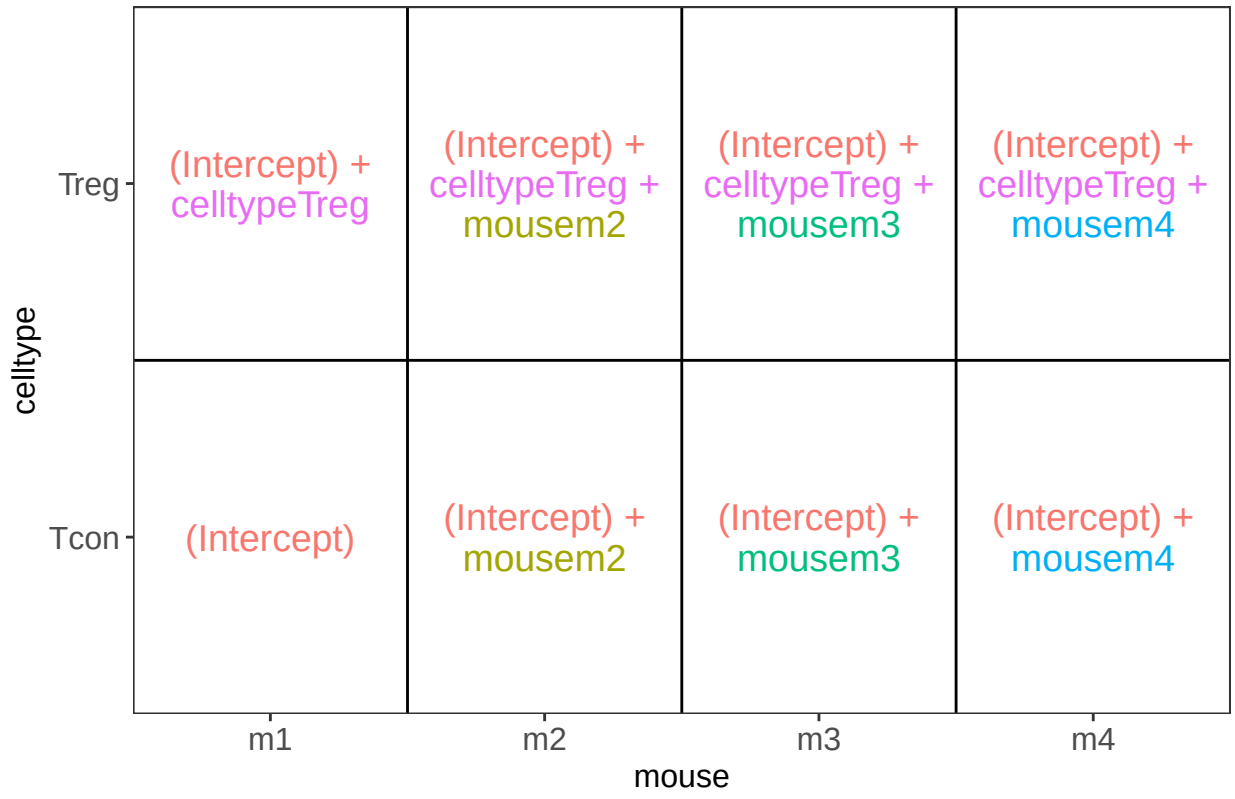
```
pe2 <- msqrob(
  object = pe2,
  i = "protein",
  formula = ~ celltype)
```

2.2.6.2 CRD analysis

2.2.6.3 Estimation, effect size and inference Effect size in RCB

```
library(ExploreModelMatrix)
VisualizeDesign(colData(pe),~ celltype + mouse)$plotlist
```

```
## [[1]]
```



Effect size in CRD

```
VisualizeDesign(colData(pe2),~ celltype)$plotlist
```

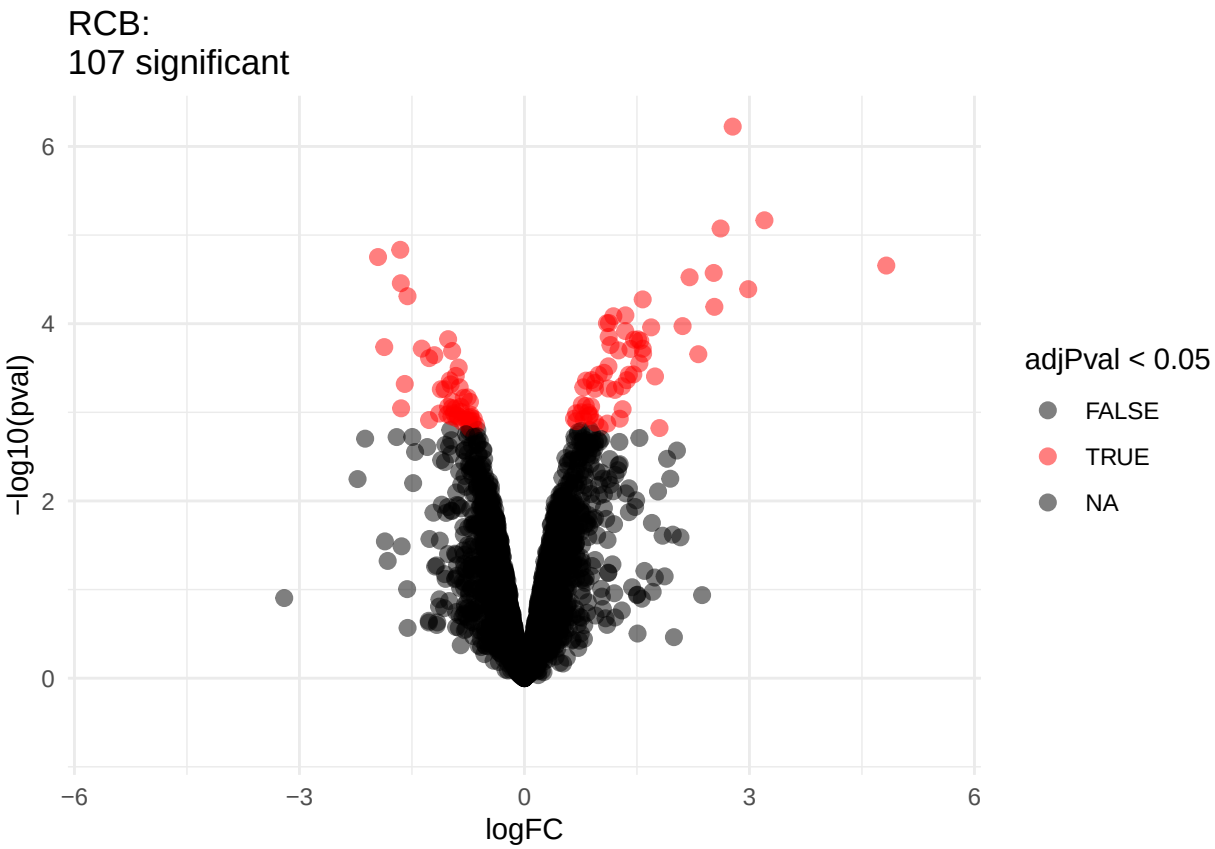
```
## [[1]]
```

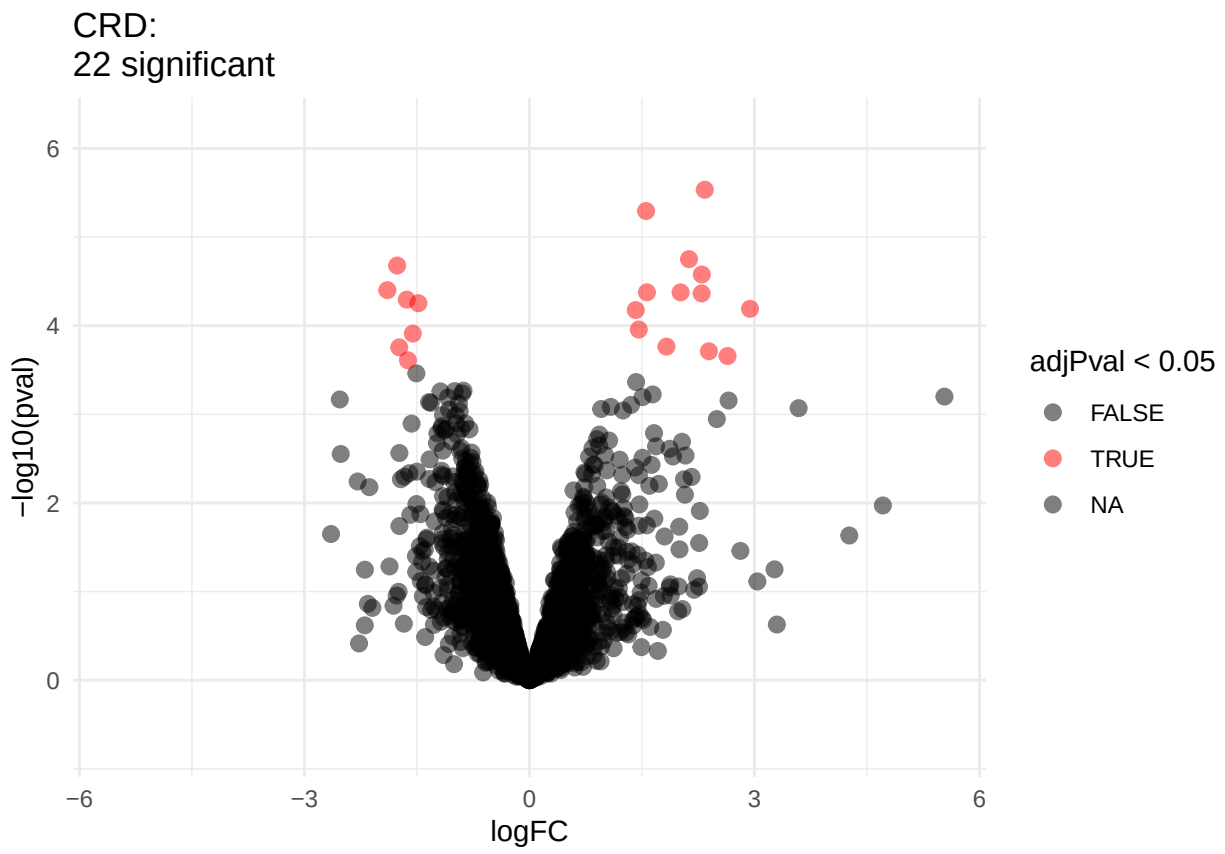
Click to see code for statistical inference

```
L <- makeContrast("celltypeTreg = 0", parameterNames = c("celltypeTreg"))
pe <- hypothesisTest(object = pe, i = "protein", contrast = L)
pe2 <- hypothesisTest(object = pe2, i = "protein", contrast = L)
```

2.2.7 Comparison of results

Click to see code


RCB:
107 significant
6
4
2
0
−log10(pval)
−6
−3
0
3
6
logFC
adjPval < 0.05
FALSE
TRUE
NA



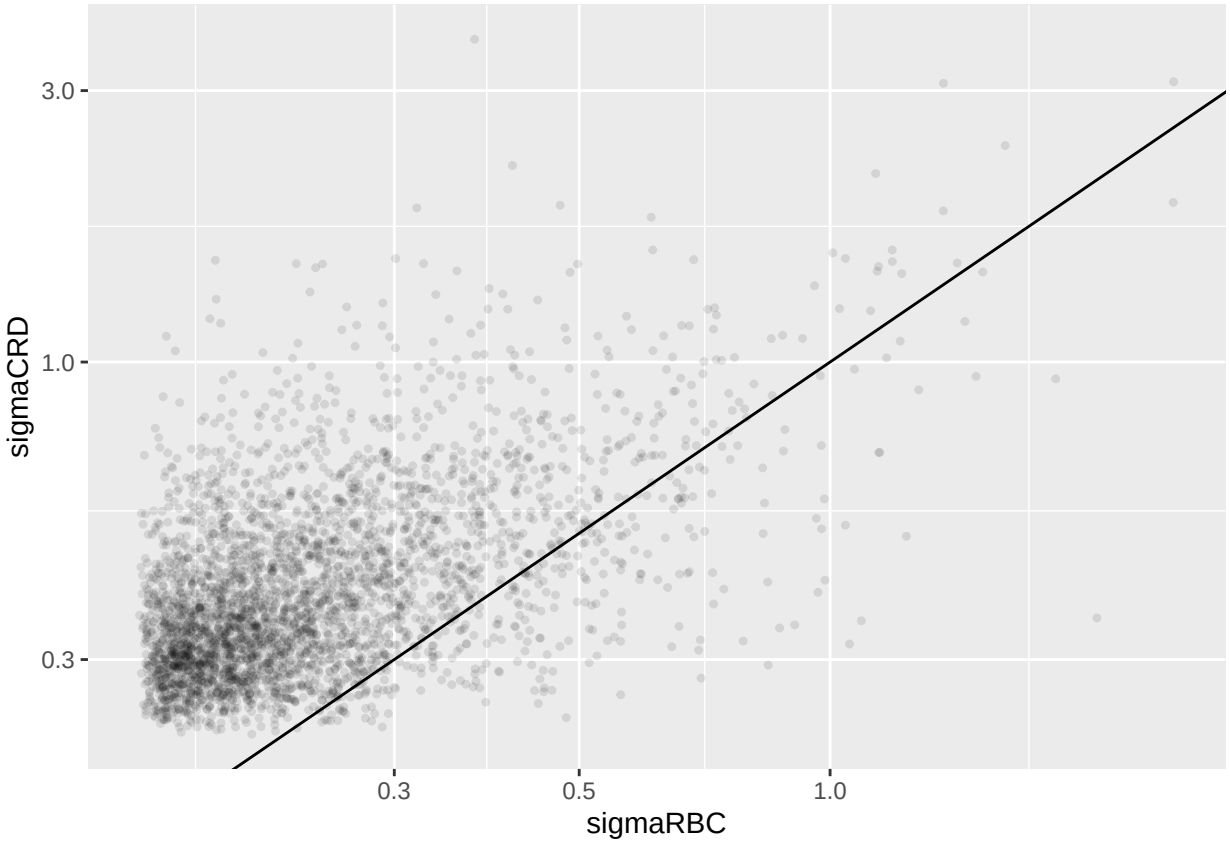
2.2.8 Comparison of standard deviation

Click to see code

```
accessions <- rownames(pe[["protein"]])[rownames(pe[["protein"]])%in%rownames(pe2[["protein"]])]
dat <- data.frame(
sigmaRBC = sapply(rowData(pe[["protein"]])$msqrobModels[accessions], getSigmaPosterior),
sigmaCRD <- sapply(rowData(pe2[["protein"]])$msqrobModels[accessions], getSigmaPosterior)
)

plotRBCvsCRD <- ggplot(data = dat, aes(sigmaRBC, sigmaCRD)) +
    geom_point(alpha = 0.1, shape = 20) +
    scale_x_log10() +
    scale_y_log10() +
    geom_abline(intercept=0,slope=1)
```

```
  plotRBCvsCRD
```

```
## Warning: Removed 351 rows containing missing values or values outside the scale range
## (`geom_point()`).
```



- We clearly observe that the standard deviation of the protein expression in the RCB is smaller for the majority of the proteins than that obtained with the CRD
- Can you think of a reason why it would not be useful to block on a particular factor?

2.3 Pseudo-replication

- The Francisella tularensis that we used before was a subset of the data of Ramond et al.
- The authors have run the sample for each bio-rep in technical triplicate on the mass-spectrometer.

2.3.1 Data

Click to see code to import data

1. We use a peptides.txt file from MS-data quantified with maxquant that contains MS1 intensities summarized at the peptide level.

```
peptidesTable <- fread("https://raw.githubusercontent.com/statOmics/MSqRobSumPaper/refs/heads/master/Fra
int64 <- which(sapply(peptidesTable,class) == "integer64")
for (j in int64) peptidesTable[[j]] <- as.numeric(peptidesTable[[j]])
```

2. Maxquant stores the intensity data for the different samples in columnns that start with Intensity. We can retreive the column names with the intensity data with the code below:

```
quantCols <- grep("Intensity ", names(peptidesTable))
```

4. Read the data and store it in QFeatures object

```
pe <- readQFeatures(
  assayData = peptidesTable,
  fnames = 1,
  quantCols =  quantCols,
  name = "peptideRaw")
```

```
## Checking arguments.
```

```
## Loading data as a 'SummarizedExperiment' object.
```

```
## Formatting sample annotations (colData).
```

```
## Formatting data as a 'QFeatures' object.
```

```
## Setting assay rownames.
```

```
rm(peptidesTable)
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8806173 470.4   15503455 828.0         NA 15503455 828.0
## Vcells 34787570 265.5   81348285 620.7      16384 81348285 620.7
```

```
gc()
```

```
##            used  (Mb) gc trigger  (Mb) limit (Mb) max used  (Mb)
## Ncells  8806067 470.3   15503455 828.0         NA 15503455 828.0
## Vcells 34780907 265.4   81348285 620.7      16384 81348285 620.7
```

5. Update data with information on design

```
colData(pe)$genotype <- pe[[1]] |>
  colnames() |>
  substr(12,13) |>
  as.factor() |>
  relevel("WT")

colData(pe)$biorep <- pe[[1]] |>
  colnames() |>
  substr(22,22)

colData(pe)$biorep <- paste(colData(pe)$genotype,colData(pe)$biorep,sep="_")

pe |> colData()
```

```
## DataFrame with 18 rows and 2 columns
##                           genotype      biorep
##                           <factor> <character>
## Intensity 1WT_20_2h_n3_1       WT        WT_3
## Intensity 1WT_20_2h_n3_2       WT        WT_3
## Intensity 1WT_20_2h_n3_3       WT        WT_3
## Intensity 1WT_20_2h_n4_1       WT        WT_4
## Intensity 1WT_20_2h_n4_2       WT        WT_4
## ...                           ...         ...
## Intensity 3D8_20_2h_n4_2       D8        D8_4
## Intensity 3D8_20_2h_n4_3       D8        D8_4
## Intensity 3D8_20_2h_n5_1       D8        D8_5
## Intensity 3D8_20_2h_n5_2       D8        D8_5
```

```
## Intensity 3D8_20_2h_n5_3       D8        D8_5
```

2.3.2 Preprocessing

Click to see code to log-transfrom the data

1. Log transform

- Peptides with zero intensities are missing peptides and should be represent with a `NA` value rather than `0`.

```
pe <- zeroIsNA(pe, "peptideRaw") # convert 0 to NA
```

- Logtransform data with base 2

```
pe <- logTransform(pe, base = 2, i = "peptideRaw", name = "peptideLog")
```

2. Filtering

- We remove PSMs that could not be mapped to a protein or that map to multiple proteins (the protein identifier contains multiple identifiers separated by a `;`).

```
pe <- filterFeatures(
    pe, ~ Proteins != "" & ## Remove failed protein inference
        !grepl(";", Proteins)) ## Remove protein groups
```

```
## 'Proteins' found in 2 out of 2 assay(s).
```

- Remove reverse sequences (decoys) and contaminants. Note that this is indicated by the column names Reverse and depending on the version of maxQuant with Potential.contaminants or Contaminants.

```
pe <- filterFeatures(pe,~Reverse != "+")
```

```
## 'Reverse' found in 2 out of 2 assay(s).
```

```
pe <- filterFeatures(pe,~ Contaminant != "+")
```

```
## 'Contaminant' found in 2 out of 2 assay(s).
```

- Drop peptides that were identified in less than three sample. We tolerate the following proportion of NAs: pNA = (n-3)/n.

```
nObs <- 3
n <- ncol(pe[["peptideLog"]])
pNA <- (n-nObs)/n
pe <- filterNA(pe, pNA = pNA, i = "peptideLog")
nrow(pe[["peptideLog"]])
```

```
## [1] 7244
```

We keep 7244 peptides upon filtering.

3. Normalization by median centering

```
pe <- normalize(pe,
                i = "peptideLog",
                name = "peptideNorm",
                method = "center.median")
```

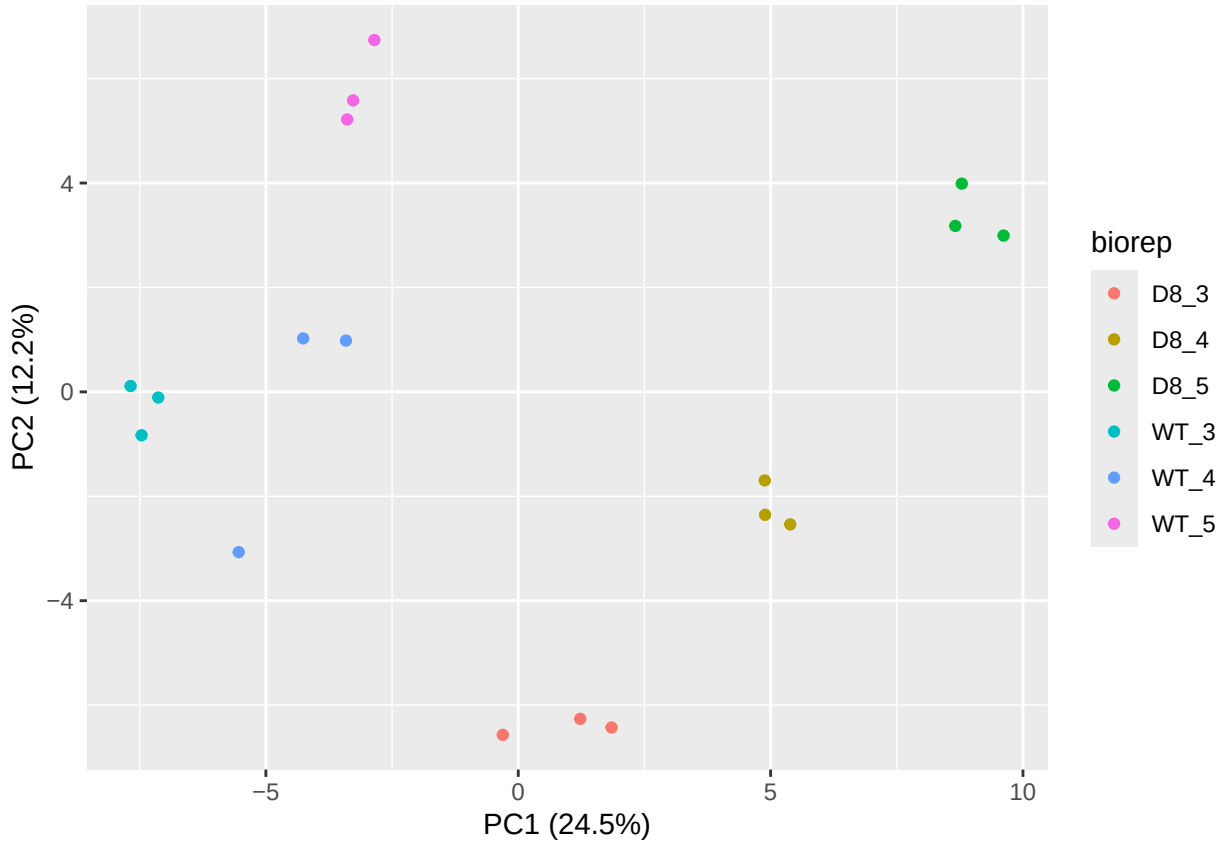
4. Summarization. We use the standard sumarisation in aggregateFeatures, which is a robust summarisation method.

```
pe <- aggregateFeatures(pe,
    i = "peptideNorm",
    fcol = "Proteins",
    na.rm = TRUE,
    name = "protein")
```

```
## Your quantitative and row data contain missing values. Please read the
## relevant section(s) in the aggregateFeatures manual page regarding the
## effects of missing values on data aggregation.
```

```
## Aggregated: 1/1
```

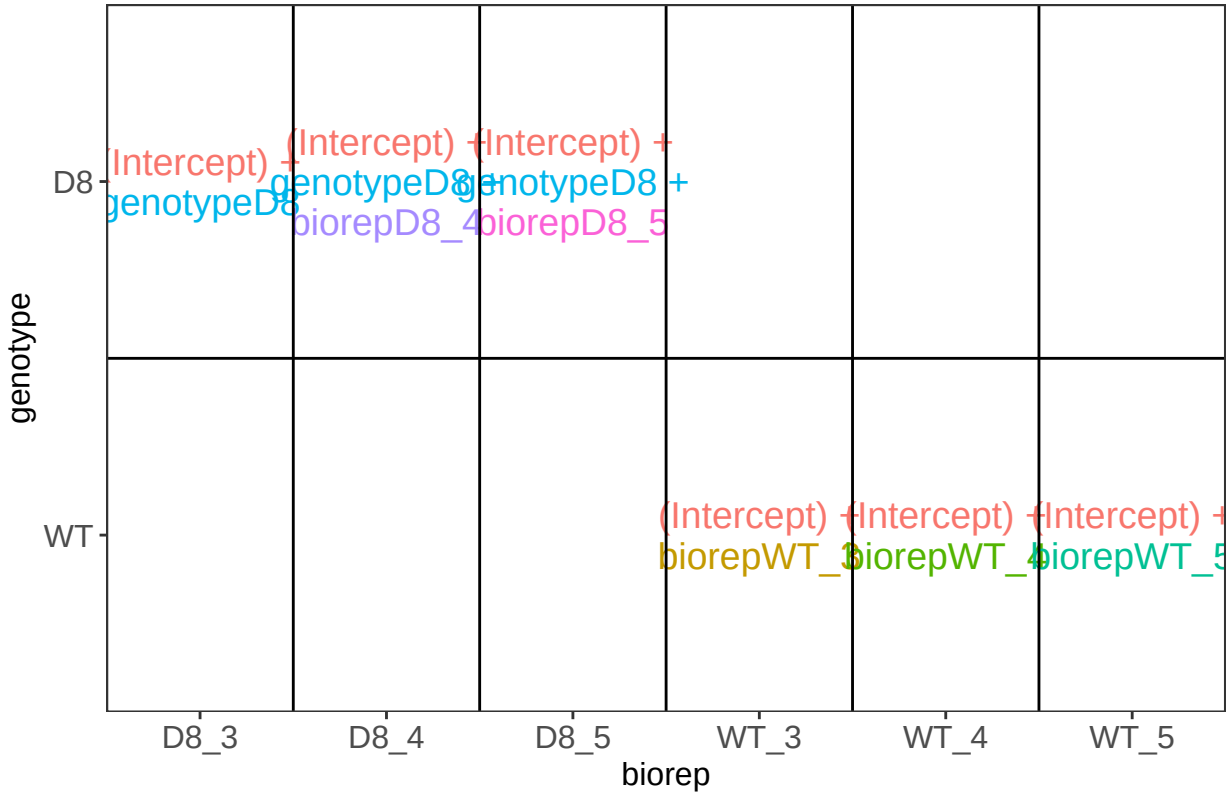
```
msqrob2gui:::plotPCA(pe, "protein", "biorep")
```



- Response?
- Experimental unit?
- Observational unit?
- Factors?

```
VisualizeDesign(colData(pe),~ genotype + biorep)$plotlist
```

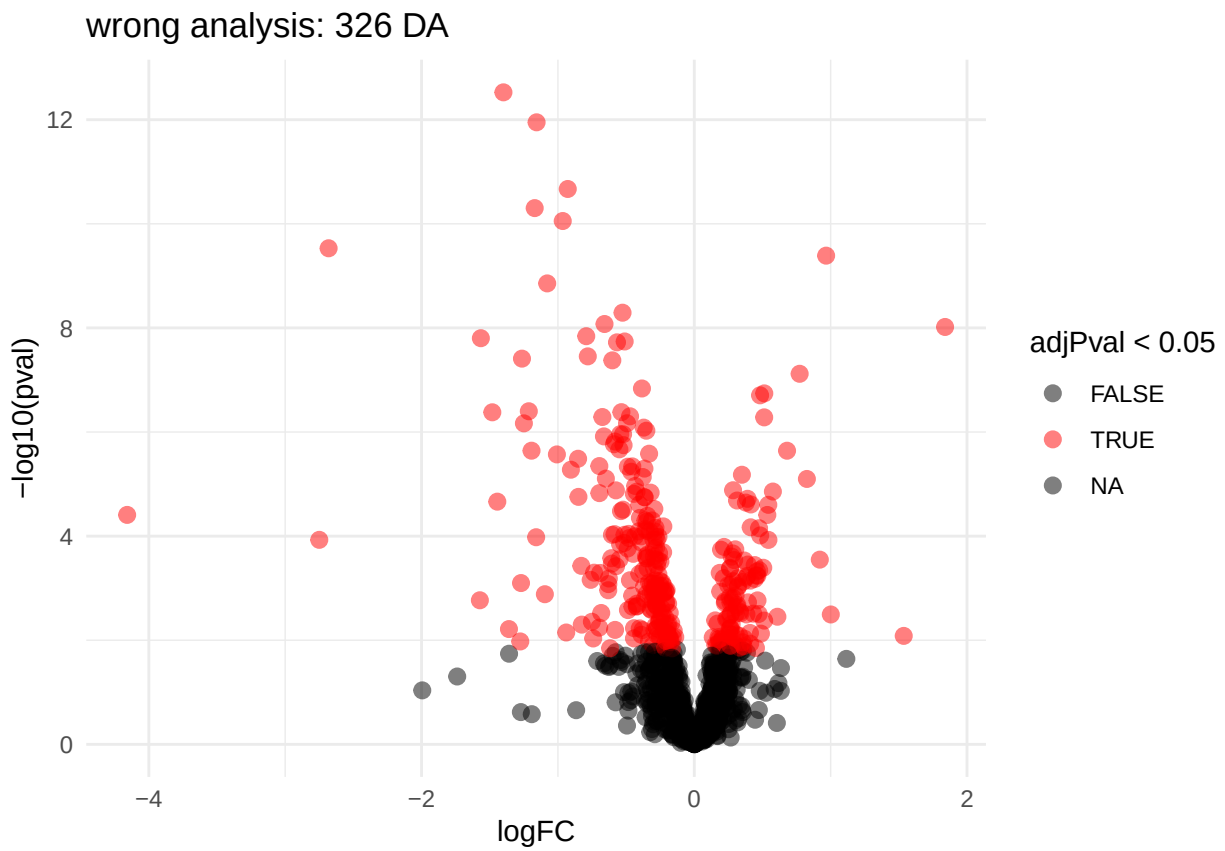
```
## [[1]]
```



→ Pseudo-replication, randomisation to bio-repeat and each bio-repeat measured in technical triplicate. → If we would analyse the data using a linear model based on each measured intensity, we would act as if we had sampled 18 bio-repeats. → Effect of interest has to be assessed between bio-repeats. So block analysis is not possible (if we condition on bio-repeat, we condition on the genotype)!

2.3.3 Wrong analysis

```
pe <- msqrob(object = pe, i = "protein", formula = ~genotype, modelColumnName = "wrong", overwrite = TRU
L <- makeContrast("genotypeD8 = 0", parameterNames = c("genotypeD8"))
pe <- hypothesisTest(object = pe, i = "protein", contrast = L, modelColumn = "wrong",   resultsColumnNa

volcanoWrong <- ggplot(
    rowData(pe[["protein"]])$wrong_genotypeD8,
    aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)
) +
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal() +
    ggtitle(paste0("wrong analysis: ",sum(rowData(pe[["protein"]])$wrong_genotypeD8$adjPval <0.05, na.r

volcanoWrong
```



Note, that the analysis where we ignore that we have multiple technical repeats for each bio-repeat returns many significant DA proteins because we act as if we have much more independent observations.

2.3.4 Correct analysis

- Mixed Models can model the correlation structure in the data
- They can acknowledge that protein expression values from runs from the same biological repeat are more alike than protein expression values from runs of different biological repeats.
- Mixed models can also be used when inference between and within blocks is needed.
- For the francisella example the formula in msqrob2 becomes: (formula: ~ genotype + (1|biorep)).

$$\begin{cases} y_{ir} & = & \beta_0 + \beta_{wt} X_{wt,i} + b_i + \epsilon_{ir} \\ b_i & \sim & N(0, \sigma_b^2) \\ \epsilon_{ir} & \sim & N(0, \sigma_\epsilon^2) \end{cases}$$

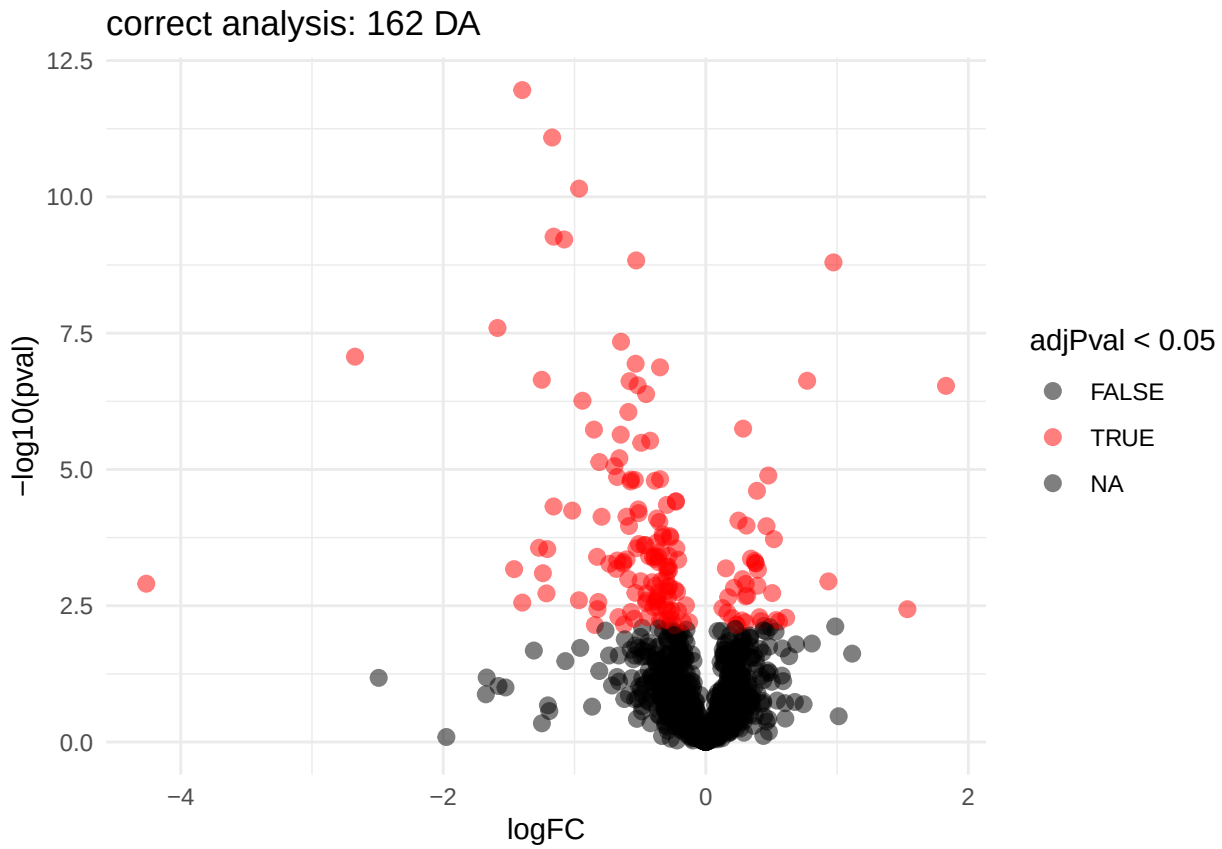
```
pe <- msqrob(object = pe, i = "protein", formula = ~genotype + (1|biorep), overwrite = TRUE)
```

```
## Warning: 'experiments' dropped; see 'drops()'
```

```
L <- makeContrast("genotypeD8 = 0", parameterNames = c("genotypeD8"))
pe <- hypothesisTest(object = pe, i = "protein", contrast = L, overwrite = TRUE)

volcanoMixed <- ggplot(
    rowData(pe[["protein"]])$genotypeD8,
    aes(x = logFC, y = -log10(pval), color = adjPval < 0.05)
) +
```

```
    geom_point(cex = 2.5) +
    scale_color_manual(values = alpha(c("black", "red"), 0.5)) +
    theme_minimal() +
    ggtitle(paste0("correct analysis: ",sum(rowData(pe[["protein"]])$genotypeD8$adjPval <0.05, na.rm=TRU
volcanoMixed
```

```
## Warning: Removed 46 rows containing missing values or values outside the scale range
## (`geom_point()`).
```



- Note, that the statistical inference with mixed models is still a bit too liberal because it:
  - hypothesis tests are only valid asymptotically (large number of biorepeats).
  - in small samples the between biorepeat variance is sometimes estimated to be zero due to estimation uncertainty and for these proteins the model still acts as if all 18 protein expression values are independent.
- Mixed models are also very useful to analyse data from large labeled experiments (Vandenbulcke and Clement 2025).
- But, they are beyond the scope of the lecture series.

CONSULT BIOSTATISTICIAN IN CASE OF EXPERIMENTS WITH PSEUDO-REPLICATES, TECHNICAL REPEATS, COMPLEX DESIGNS, ...

3 Software & code

- Our R/Bioconductor package msqrob2 can be used in R markdown scripts or with GUI/shinyApps QFeaturesGUI (+) and msqrob2gui (+: forked from the UCLouvain-CBIO lab).

- GUIs are intended as a introduction to the key concepts of proteomics data analysis for users who have no experience in R.
- However, learning how to code data analyses in R markdown scripts is key for open en reproducible science and for reporting your proteomics data analyses and interpretation in a reproducible way.
- More information on our tools can be found in our papers (L. J. Goeminne, Gevaert, and Clement 2016), (L. J. E. Goeminne et al. 2020), (Sticker et al. 2020) and (Vandenbulcke and Clement 2025). Please refer to our work when using our tools.

References

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Sticker, A., L. Goeminne, L. Martens, and L. Clement. 2020. "Robust Summarization and Inference in Proteome-wide Label-free Quantification." *Mol Cell Proteomics* 19 (7): 1209–19.

Vandenbulcke, C., S. Vanderaa, and L. Clement. 2025. "msqrob2TMT: Robust Linear Mixed Models for Inferring Differential Signals in Tandem Mass Tag-Based Proteomics." *Molecular & Cellular Proteomics* 24 (3): e10101–1. https://doi.org/10.1016/j.mcpro.2025.00101-X.