Nasonia

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Loading libraries

```
# Install libraries for the first time using BiocManager:
# if (!requireNamespace("BiocManager", quietly = TRUE))
# install.packages("BiocManager")
# BiocManager::install()
# source("https://bioconductor.org/biocLite.R")
# BiocInstaller::biocLite("DESeq")

# load libraries
library(RColorBrewer)
library(matrixStats)
library(ggplot2)
library(edgeR)
library(limma)
library(doParallel)
library(variancePartition)
library(clusterProfiler)
library(GOSemSim)
library(biomaRt)
library(VennDiagram)
library(ggrepel)
library(dplyr)
library(stringr)
library(forcats)
library("knitr")
```

Setting the environment parameters.

```
# package knitr is required to set the working directory
require("knitr")
# set the working directory
opts_knit$set(root.dir = "~/Dropbox (ASU)/NASONIA_take3/DE_limmaVoom")

# Defining colors
viralPalette <- brewer.pal(8, "Set2")
VV_Color <- viralPalette[1] # green
GG_Color <- viralPalette[2] # orange
VG_Color <- viralPalette[3] # blue
GV_Color <- viralPalette[4] # pink
```

```
# Defining shapes
VV_Shape <- c(15) # square
GG_Shape <- c(16) # circle
VG_Shape <- c(17) # triangle
GV_Shape <- c(18) # diamond
```

Importing count and pheno type data to create DGEList object

```
# reading in expression data
# gene count information for each sample
# each column is a sample
# each row is the raw count (expression) for that gene
counts_pseudoNgir <- read.table("clark_counts_VgirRef.txt",
             header = TRUE,
             sep = "\t")
counts_Nvit <-
  read.table("clark_counts.txt", header = TRUE, sep = "\t")
DF <- data.frame(counts_Nvit, counts_pseudoNgir)
colnames = c(
  "SRR1566022",
  "SRR1566023",
  "SRR1566024",
  "SRR1566028",
  "SRR1566029",
  "SRR1566030",
  "SRR2773794",
  "SRR2773795",
  "SRR2773796",
  "SRR2773797",
  "SRR2773799"
)
counts <-
  sapply(colnames, function(x)
    rowMeans(DF [, grep(x, names(DF))]))

head(counts) # inspect the file
```

```
##      SRR1566022 SRR1566023 SRR1566024 SRR1566028 SRR1566029 SRR1566030
## [1,]        8.0       20.0         15       50.0       25.0       32.0
## [2,]        6.0       12.0         11        1.0        1.0        1.0
## [3,]        3.0        7.0          2        1.0        0.0        2.0
## [4,]     3185.5     2238.0       2375     3156.5     3236.5     3088.0
## [5,]       33.0       35.0         76       25.5       31.0       29.0
## [6,]    12318.0     6411.5       6434     6291.5     5357.5     5413.5
##      SRR2773794 SRR2773795 SRR2773796 SRR2773797 SRR2773799
## [1,]       19.0       15.0       41.0       17.0       28.0
## [2,]        4.0       11.0       12.0       12.0        5.0
## [3,]        0.0        6.0       10.0        4.0        9.0
## [4,]     3084.0     4049.0     5775.0     4016.0     4223.5
## [5,]       25.5       38.5       57.5       46.0       70.5
## [6,]     7929.0     7895.5    10922.5    11770.5     8321.0
```

```r
genes <- read.table("genes.txt", header = TRUE, sep = "\t")
# the gene file contains information about the genes
# Geneid, Chr, Start, End, Length
head(genes)
```

```
##              Geneid         Chr Start   End Strand Length
## 1 gene-LOC100679504 NC_045757.1  6142  8476      -   2335
## 2 gene-LOC116415970 NC_045757.1  9540 13384      -   3845
## 3 gene-LOC103315360 NC_045757.1 16491 18228      +   1738
## 4 gene-LOC100117425 NC_045757.1 54820 61373      -   6554
## 5 gene-LOC100678203 NC_045757.1 63374 65444      +   2071
## 6 gene-LOC100117470 NC_045757.1 65587 69067      +   3481
```

```r
pheno <- read.table("clark_pheno.csv", header = TRUE, sep = ",")
# the pheno file contains information about the samples
# sampleID,Strain
head(pheno)
```

```
##     sampleID Strain
## 1 SRR1566022     VV
## 2 SRR1566023     VV
## 3 SRR1566024     VV
## 4 SRR1566028     GG
## 5 SRR1566029     GG
## 6 SRR1566030     GG
```

```r
removals <- c("SRR2773798")
#removals <- c()
samplesToRemove <-
  c(removals) # update depending on comparison being made

SAMPLE_LENGTH <- as.numeric(length(samplesToRemove)) # to call later
half_sample_length <- SAMPLE_LENGTH / 2 # half the sample length
pheno <-
  pheno[!pheno$sampleID %in% samplesToRemove[1:SAMPLE_LENGTH], ] # update 1:16 depending on size of sam


# define colors and shapes for the species
viralPalette <- brewer.pal(8, "Set2")
VV_Color <- viralPalette[1]
GG_Color <- viralPalette[2]
VG_Color <- viralPalette[3]
GV_Color <- viralPalette[4]

VV_Shape <- c(15)
GG_Shape <- c(16)
VG_Shape <- c(17)
GV_Shape <- c(18)

# Create the DGEList object using the counts and genes
dge <- DGEList(counts = counts, genes = genes)
dge$samples$strain <- pheno$Strain
table(dge$samples$strain) # Inspecting the N of samples in each group
```

```
##
```

```
## GG GV VG VV
##  3  2  3  3
```

Filtering the expression data

Normalization of counts using RPKM and FPKM reads/fragments per kilobase of exon per million reads/fragments mapped gene count normalization using gene length accounting for gene length is necessary for comparing expression between different genes within the same sample.

```
# Filtering expression data
fpkm <- rpkm(dge, gene.length = dge$genes$Length)
dim(dge$genes) # N of genes before filtering
```

```
## [1] 15259     6
```

```
# mean fpkm for each strain
VV_mean_fpkm <- apply(as.data.frame(fpkm)
                      [(dge$samples$strain == "VV")],
                      1, mean, na.rm = TRUE)
GG_mean_fpkm <- apply(as.data.frame(fpkm)
                      [(dge$samples$strain == "GG")],
                      1, mean, na.rm = TRUE)
VG_mean_fpkm <- apply(as.data.frame(fpkm)
                      [(dge$samples$strain == "VG")],
                      1, mean, na.rm = TRUE)
GV_mean_fpkm <- apply(as.data.frame(fpkm)
                      [(dge$samples$strain == "GV")],
                      1, mean, na.rm = TRUE)

# Filtering expression data
# the mean fpkm in each strain must be
# greater than 0.5 to be considered expressed
# and kept for downstream analysis
keep <- (VV_mean_fpkm > 0.5 | GG_mean_fpkm > 0.5 |
           VG_mean_fpkm > 0.5 | GV_mean_fpkm > 0.5)

dge <- dge[keep, , keep.lib.sizes = FALSE]
dge <- calcNormFactors(dge, method = "TMM")
keep <- rowSums(dge$counts > 6) >= 2
dge <- dge[keep, , keep.lib.size = FALSE]
dge <- calcNormFactors(dge, method = "TMM")

# N of genes retained after filtering
dim(dge$genes)
```

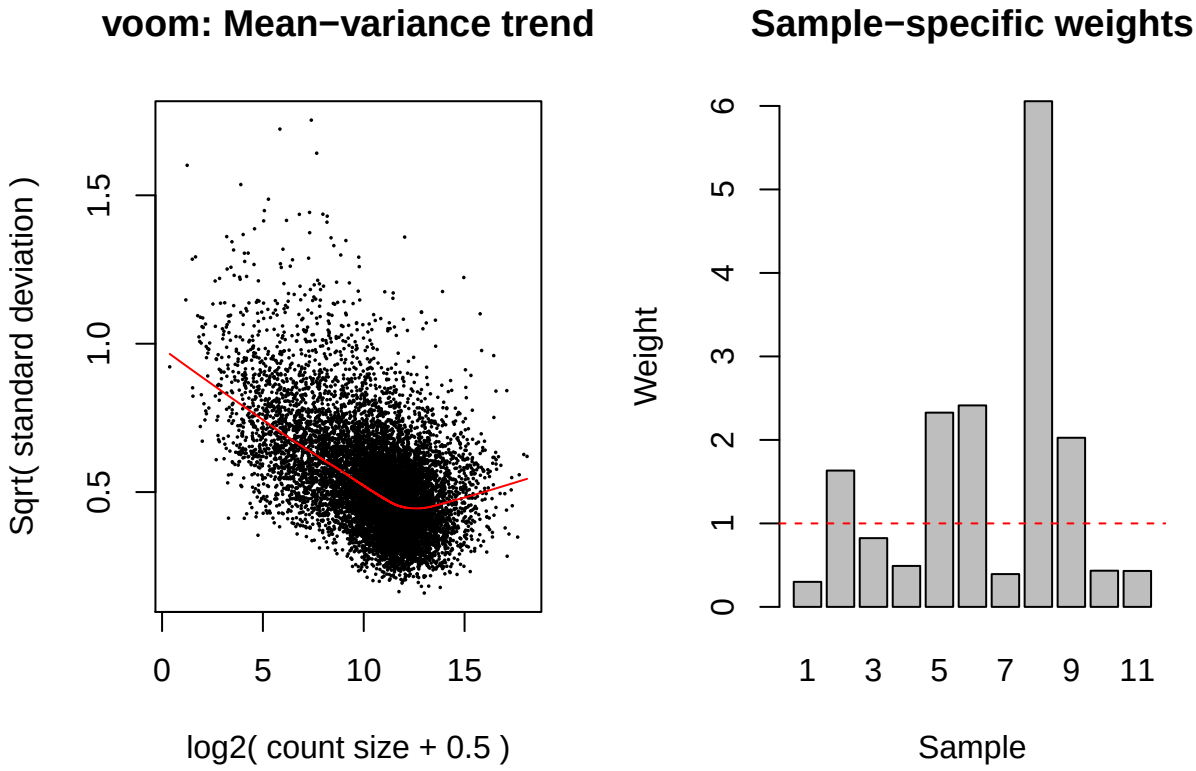
```
## [1] 10319     6
```

voom transformation

```
# create a design model matrix with the variable of interest
design <- model.matrix( ~ 0 + dge$samples$strain)
colnames(design) <-
  gsub("dge\\$samples\\$strain", "", colnames(design))
head(design)
```

```
##   GG GV VG VV
## 1  0  0  0  1
## 2  0  0  0  1
## 3  0  0  0  1
## 4  1  0  0  0
## 5  1  0  0  0
## 6  1  0  0  0
```

```
# run voom with quality weights.
# normalize expression intensities so that the log-ratios
# have similar distributions across a set of samples.
v <- voomWithQualityWeights(dge, design, plot = TRUE)
```



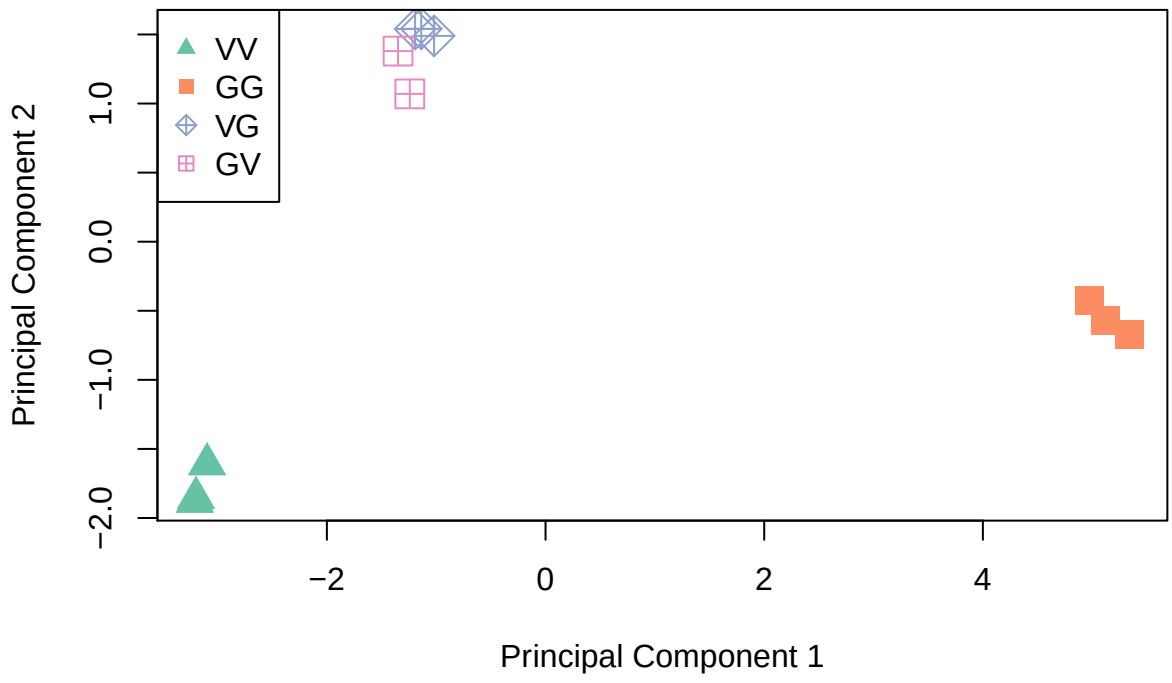

```
# to quantile normalize, add normalize.method="quantile"
```

Multi-dimensional scaling plot

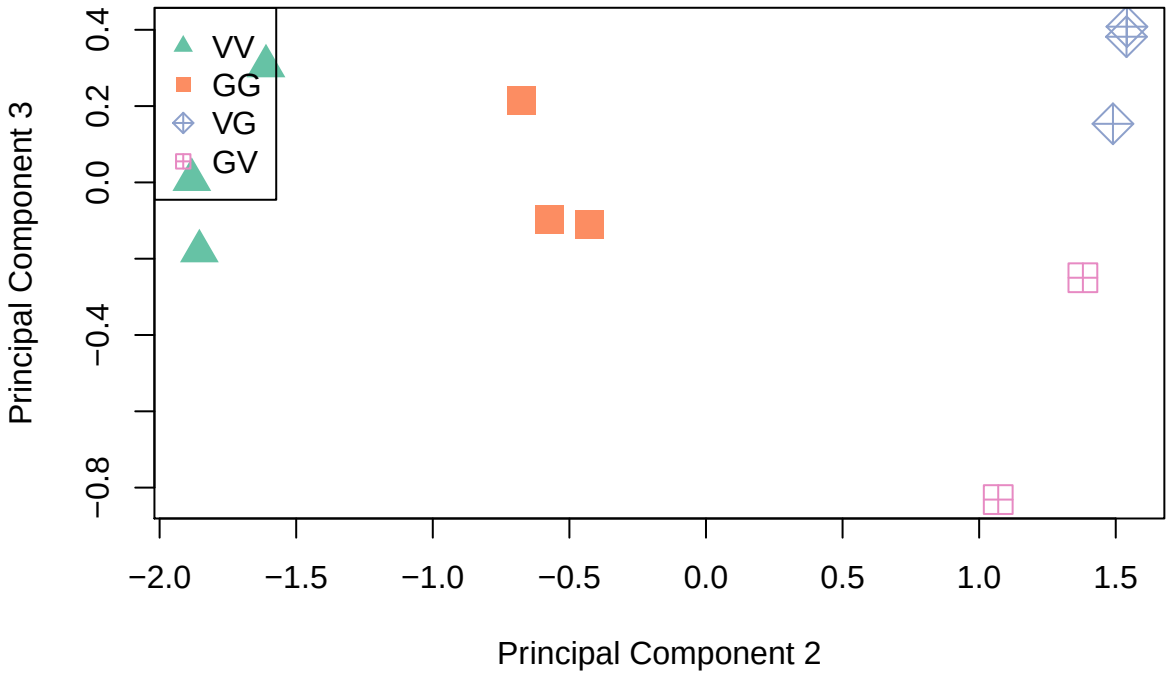
To save figures as png, uncomment all png(filename) and dev.off()

```
# MDS plot of the first two dimensions using top 50 genes.
# The dimensions are in the mds object.
# MDS plot of the first two dimensions using top50 genes. The dimensions are in the mds object.
#png(filename = "figures/clark_AvgREFs_mds_1and2.png",
#    width = 450,
#    height = 450)
mds <-
  plotMDS(
    v,
    top = 50,
    ndim = 10,
```

```
    dim.plot = c(1, 2),
    plot = TRUE,
    cex = 2,
    pch = ifelse(
      v$targets$strain %in% c("VV"),
      17,
      ifelse(
        v$targets$strain %in% c("GG"),
        15,
        ifelse(v$targets$strain %in% c("VG"), 9,  12)
      )
    ),
    col = ifelse(
      v$targets$strain == "VV",
      VV_Color,
      ifelse(
        v$targets$strain == "GG",
        GG_Color,
        ifelse(v$targets$strain == "VG",
               VG_Color, GV_Color)
      )
    ),
    gene.selection = "common"
  )

# Adding legends
legend(
  "topleft",
  pch = c(17, 15, 9, 12),
  col = c(VV_Color, GG_Color, VG_Color, GV_Color),
  legend = c("VV", "GG", "VG", "GV")
)
```



```r
# dev.off()
# dev.off()
# MDS plot of the first two dimensions using top50 genes. The dimensions are in the mds object.
#png(filename = "figures/clark_AvgREFs_mds_2and3.png",
#    width = 450,
#    height = 450)
mds <-
  plotMDS(
    v,
    top = 50,
    ndim = 10,
    dim.plot = c(2, 3),
    plot = TRUE,
    cex = 2,
    pch = ifelse(
      v$targets$strain %in% c("VV"),
      17,
      ifelse(
        v$targets$strain %in% c("GG"),
        15,
        ifelse(v$targets$strain %in% c("VG"), 9,  12)
      )
    ),
    col = ifelse(
      v$targets$strain == "VV",
      VV_Color,
      ifelse(
        v$targets$strain == "GG",
        GG_Color,
        ifelse(v$targets$strain == "VG",
               VG_Color, GV_Color)
      )
    ),
    gene.selection = "common"
  )

# Adding legends
legend(
  "topleft",
  pch = c(17, 15, 9, 12),
  col = c(VV_Color, GG_Color, VG_Color, GV_Color),
  legend = c("VV", "GG", "VG", "GV")
)
```




```
# dev.off()
# dev.off()
```

PCA on all/the most variable genes

Select most variable genes based on the biological coefficient of variance

```
# Voom transformed counts
# Voom transformed counts
voomCounts <- v$E
voomCountsMatrix <- data.matrix(voomCounts, rownames.force = NA)

# Setting the N of genes to use
ntop = length(dge$genes$Geneid)

# Sorting by the coefficient of variance
means <- rowMeans(voomCountsMatrix)
Pvars <- rowVars(voomCountsMatrix)
cv2 <- Pvars / means ^ 2
select <-
  order(cv2, decreasing = TRUE)[seq_len(min(ntop, length(cv2)))]
head(select)
```

```
## [1]    6 6156 3767 6239 4614 7226
```

```
highly_variable_exp <- ((voomCountsMatrix)[select,])
dim(highly_variable_exp)
```

```
## [1] 10319    11
```

```
# Running PCA
pca_exp <- prcomp(t(highly_variable_exp), scale = F, center = T)
# scale a logical value indicating whether the variables should be scaled
# to have unit variance before the analysis takes place.
```

```r
# a logical value indicating whether the variables should be shifted to be zero centered.
head(pca_exp$x)[, 1:3]
```

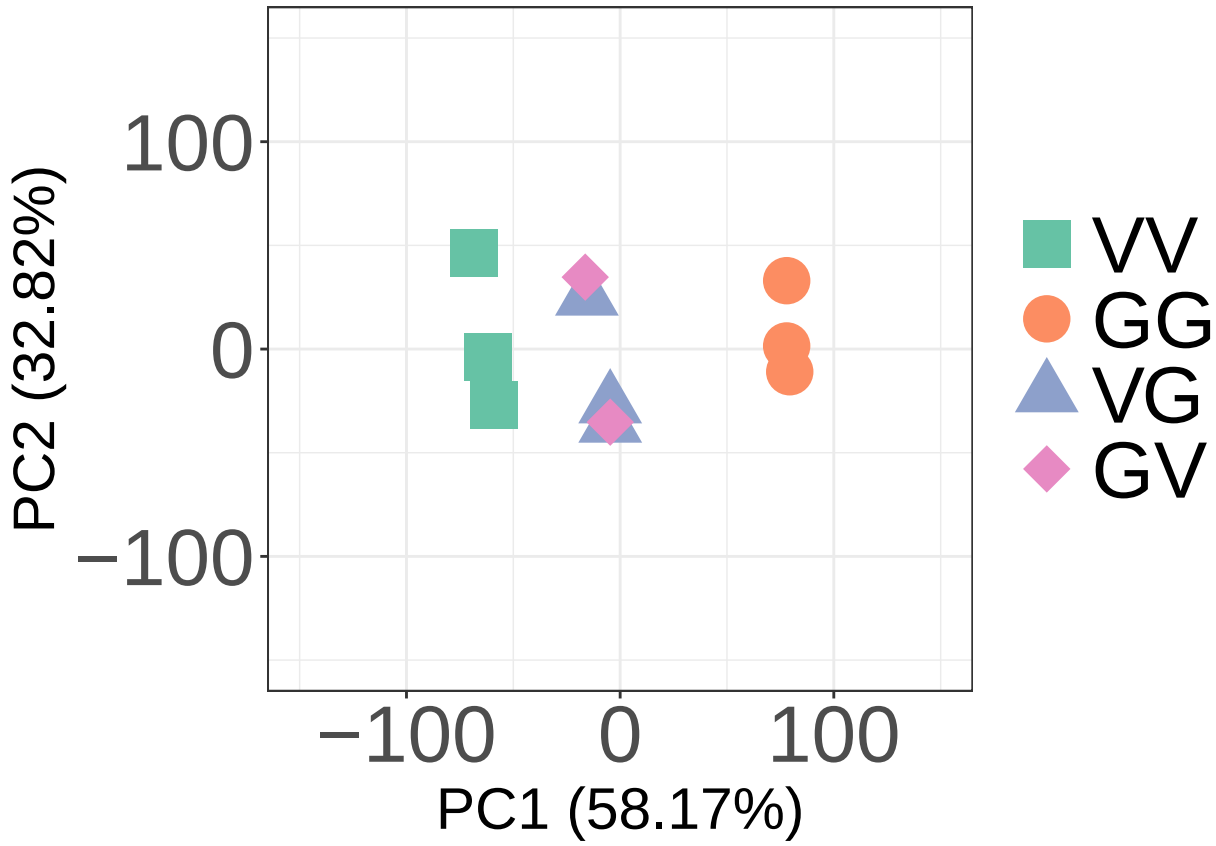
```
##                  PC1       PC2       PC3
## SRR1566022 -68.30250  46.10999 21.621365
## SRR1566023 -62.06973  -3.72869 18.859465
## SRR1566024 -59.02948 -27.10464 41.958028
## SRR1566028  77.93861  32.97086  6.128084
## SRR1566029  77.93213   1.48196 21.930258
## SRR1566030  79.36974 -10.94378 20.171195
```

```r
summary(pca_exp)
```

```
## Importance of components:
##                             PC1     PC2     PC3      PC4      PC5      PC6
## Standard deviation     55.6989 30.3257 26.9249 16.01573 12.05656 11.11384
## Proportion of Variance  0.5644  0.1673  0.1319  0.04666  0.02644  0.02247
## Cumulative Proportion   0.5644  0.7317  0.8635  0.91018  0.93663  0.95910
##                             PC7     PC8     PC9    PC10      PC11
## Standard deviation     9.39631 8.30295 6.41021 5.15180 5.092e-14
## Proportion of Variance 0.01606 0.01254 0.00747 0.00483 0.000e+00
## Cumulative Proportion  0.97516 0.98770 0.99517 1.00000 1.000e+00
```

```r
# Dataframe with the first 10 PCs
dim1_10 <- data.frame(pca_exp$x[, 1:10])
# Adding metadata
pcaWithMetadata <- merge(dim1_10, dge$samples, by = 0, all = TRUE)
pcaWithMetadata$strain <- factor(pcaWithMetadata$strain,
                                 levels = c("VV", "GG", "VG", "GV", NA))

# Plotting
#png(filename = "figures/clark_AvgREFs_PCA.png",
#    width = 650,
#    height = 650)
ggplot(data = pcaWithMetadata, aes(
  x = PC1,
  y = PC2,
  shape = strain,
  color = strain
)) +
  geom_point(size = 8) +
  theme_bw() +
  xlim(-150, 150) +
  ylim(-150, 150) +
  scale_color_manual(values = c(VV_Color, GG_Color,
                                VG_Color, GV_Color,
                                "azure3")) +
  scale_shape_manual(values = c(VV_Shape, GG_Shape,
                                VG_Shape, GV_Shape)) +
  theme(
    plot.title = element_text(size = 12, face = "bold"),
    legend.title = element_text(size = 30),
    legend.text = element_text(size = 30),
    axis.text.x = element_text(size = 30),
    axis.text.y = element_text(size = 30),
```

```
    axis.title.x = element_text(size = 22),
    axis.title.y = element_text(size = 22)
  ) +
  #guides(color = guide_legend(order = 2)) +
  theme(legend.title = element_blank()) +
  xlab("PC1 (58.17%)") +
  ylab("PC2 (32.82%)")
```



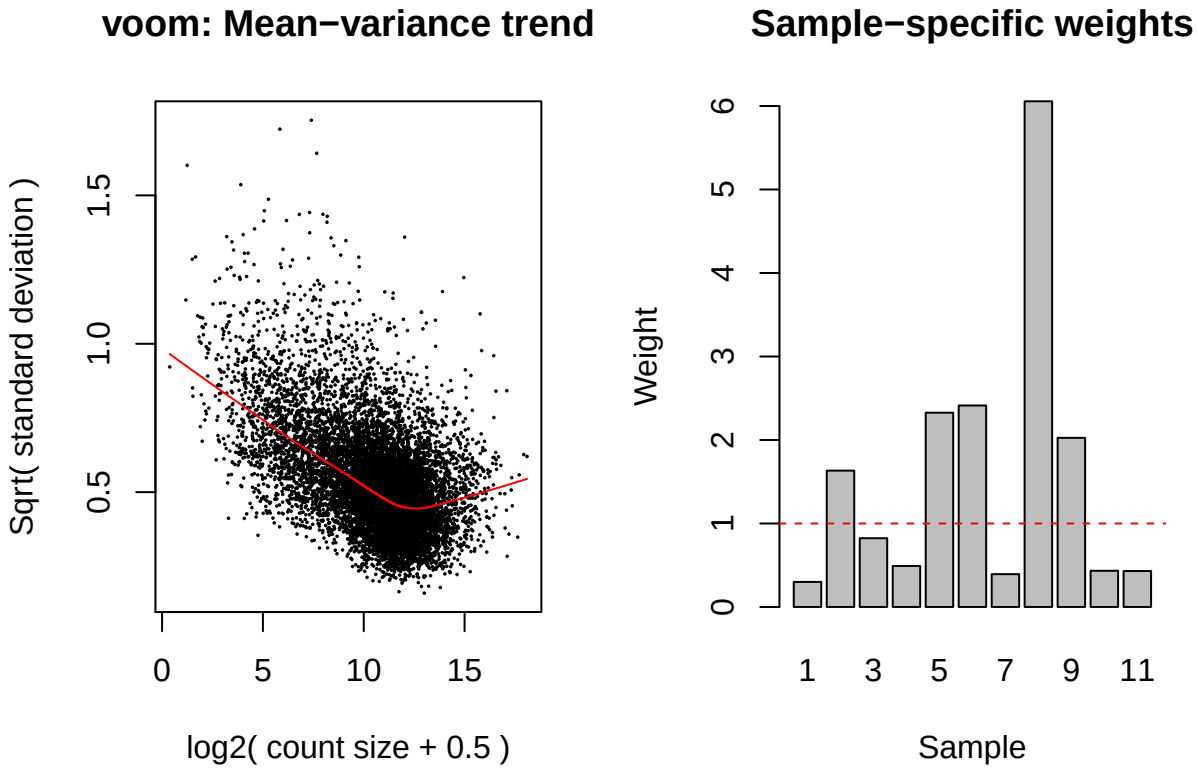

```
# dev.off()
# dev.off()
# samples cluster by strain
```

Differential expression analysis with limma

```
design <- model.matrix( ~ 0 + v$targets$strain)
colnames(design) <-
  gsub("v\\$targets\\$strain", "", colnames(design))
head(design)
```

```
##   GG GV VG VV
## 1  0  0  0  1
## 2  0  0  0  1
## 3  0  0  0  1
## 4  1  0  0  0
## 5  1  0  0  0
## 6  1  0  0  0
```

```r
# Running voom again with the new design matrix.
v <- voomWithQualityWeights(dge, design, plot = TRUE)
```

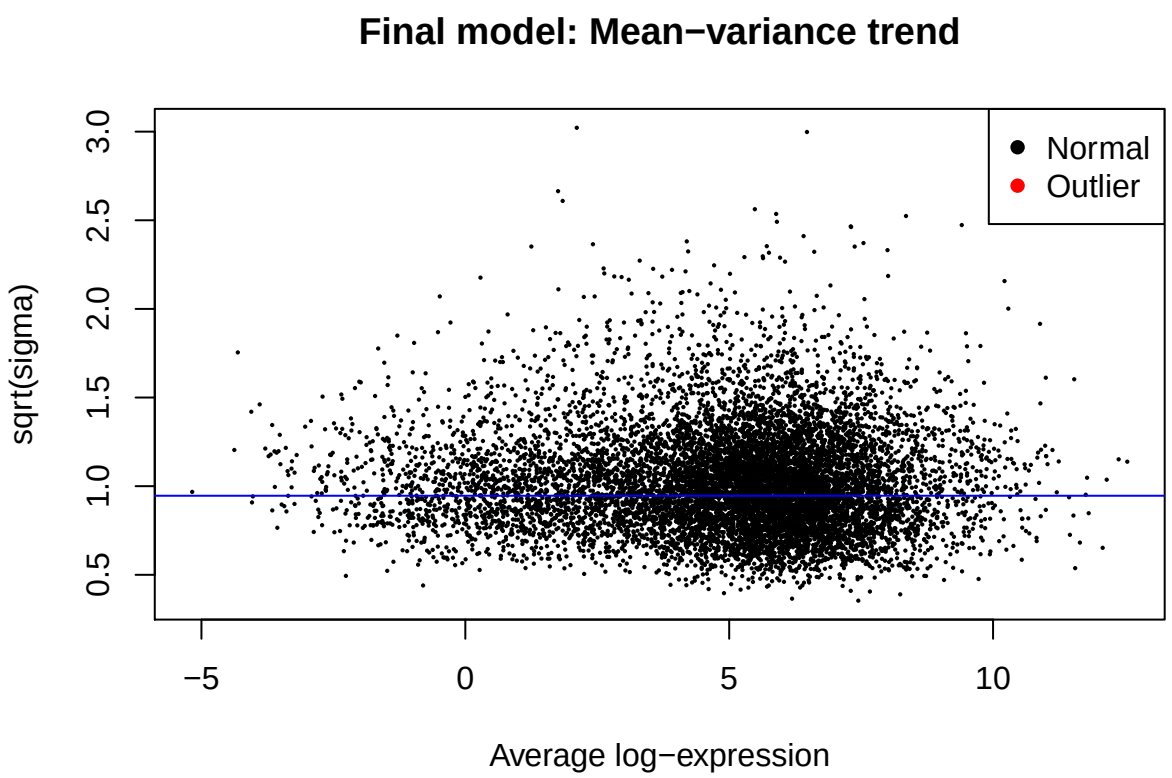



```r
fit <- lmFit(v, design)
# Contrast design for differential expression
# Defining pairwise comparisons
contrasts <- makeContrasts(
  VV_vs_GG = VV - GG,
  VV_vs_VG = VV - VG,
  VV_vs_GV = VV - GV,
  GG_vs_VG = GG - VG,
  GG_vs_GV = GG - GV,
  VG_vs_GV = VG - GV,
  levels = colnames(design)
)
head(contrasts)
```

```
##       Contrasts
## Levels VV_vs_GG VV_vs_VG VV_vs_GV GG_vs_VG GG_vs_GV VG_vs_GV
##     GG       -1        0        0        1        1        0
##     GV        0        0       -1        0       -1       -1
##     VG        0       -1        0       -1        0        1
##     VV        1        1        1        0        0        0
```

```r
# Assigning all comparisons to a vector for later
allComparisons <- colnames(contrasts)

# Running contrast analysis
vfit <- contrasts.fit(fit, contrasts = contrasts)
# Looking at N of DEGs with adj. p <0.01 and log2FC>2
```

```r
sumTable <-
  summary(decideTests(
    vfit,
    adjust.method = "BH",
    p.value = 0.01,
    lfc = 2
  ))

# Computing differential expression based on the empirical Bayes moderation of
# the standard errors towards a common value. Robust = should the estimation of
# the empirical Bayes prior parameters be robustified against outlier sample
# variances?
veBayesFit <- eBayes(vfit, robust = TRUE)
plotSA(veBayesFit, main = "Final model: Mean-variance trend")
```




```r
# Getting the DEGs. Log2FC of 1 is equivalent to linear fold change of 2.
# Getting summary statistics for all genes
# Getting the DEGs. Log2FC of 1 is equivalent to linear fold change of 2.
# Getting summary statistics for all genes
coef = 1
for (i in allComparisons) {
  vTopTableAll <-
    topTable(
      veBayesFit,
      coef = coef,
      n = Inf,
      p.value = 1,
      lfc = 0
    )
```

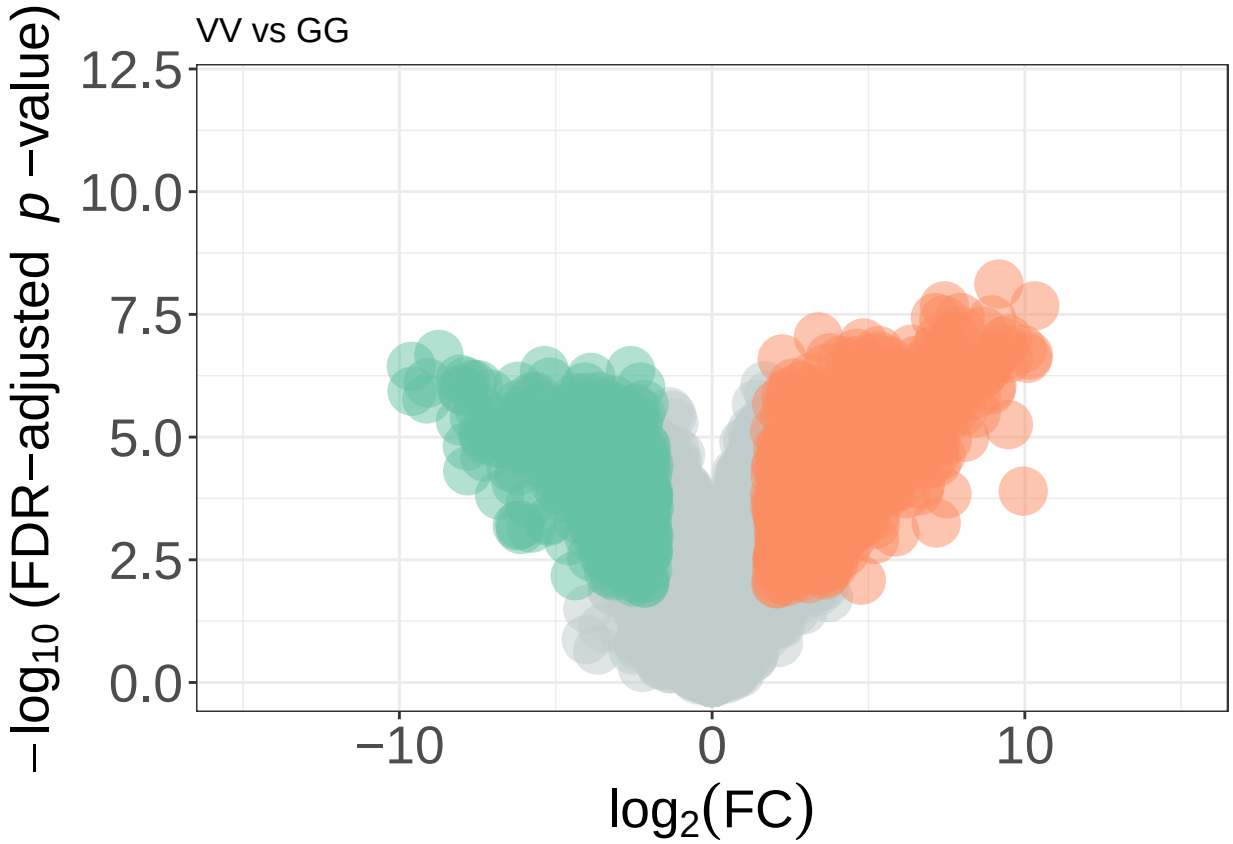
```
path <-
  paste("./DEGs/DEGs_clark_AvgREFs_fpkm05_",
        i,
        "_fdr1_lfc0.txt",
        sep = "")
write.table(vTopTableAll, path, sep = "\t")

# Adj.p<0.05, log2FC>1
vTopTable1 <-
  topTable(
    veBayesFit,
    coef = coef,
    n = Inf,
    p.value = 0.05,
    lfc = 1
  )
path <-
  paste("./DEGs/DEGs_clark_AvgREFs_fpkm05_",
        i,
        "_fdr05_lfc1.txt",
        sep = "")
write.table(vTopTable1, path, sep = "\t")

# Adj.p<0.01, log2FC>0
vTopTable1 <-
  topTable(
    veBayesFit,
    coef = coef,
    n = Inf,
    p.value = 0.01,
    lfc = 0
  )
path <-
  paste("./DEGs/DEGs_clark_AvgREFs_fpkm05_",
        i,
        "_fdr001_lfc0.txt",
        sep = "")
write.table(vTopTable1, path, sep = "\t")

# Adj.p<0.01, log2FC>1
vTopTable2 <-
  topTable(
    veBayesFit,
    coef = coef,
    n = Inf,
    p.value = 0.01,
    lfc = 1
  )
path <-
  paste("./DEGs/DEGs_clark_AvgREFs_fpkm05_",
        i,
        "_fdr001_lfc1.txt",
        sep = "")
```

```
  write.table(vTopTable2, path, sep = "\t")

  # Adj.p<0.01, log2FC>2
  vTopTable3 <-
    topTable(
      veBayesFit,
      coef = coef,
      n = Inf,
      p.value = 0.01,
      lfc = 2
    )
  path <-
    paste("./DEGs/DEGs_clark_AvgREFs_fpkm05_",
          i,
          "_fdr001_lfc2.txt",
          sep = "")
  write.table(vTopTable3, path, sep = "\t")

  coef = coef + 1
}

VV_GG_DEG <-
  rownames(read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_VV_vs_GG_fdr001_lfc2.txt"
  ))
VV_VG_DEG <-
  rownames(read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_VV_vs_VG_fdr001_lfc2.txt"
  ))
VV_GV_DEG <-
  rownames(read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_VV_vs_GV_fdr001_lfc2.txt"
  ))
```

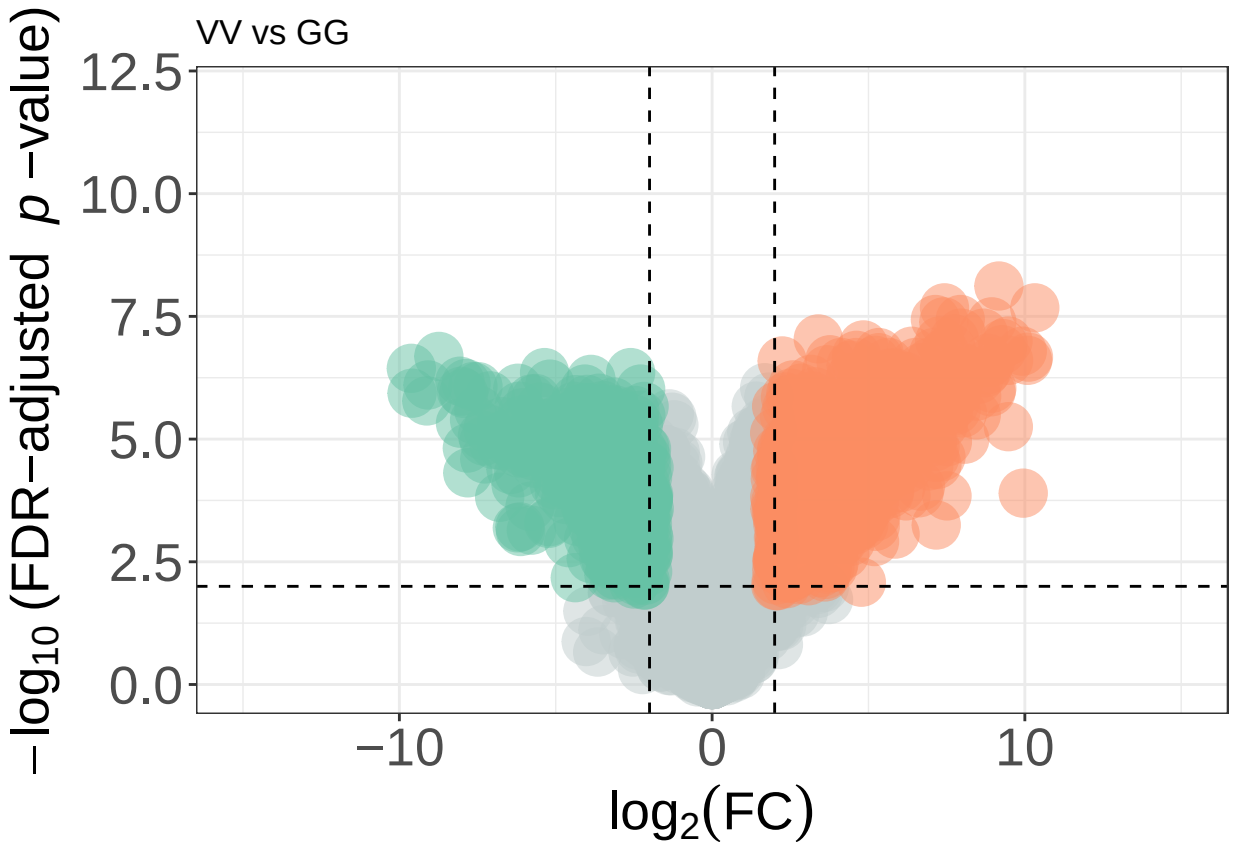
Volcano plots of differential expression analysis for all pairwise comparisions

```
VV_vs_GG_DEG <-
  read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_VV_vs_GG_fdr1_lfc0.txt",
    header = TRUE,
    sep = "\t",
    stringsAsFactors = F
  )
VV_vs_GG_DF <-
  data.frame(VV_vs_GG_DEG$adj.P.Val,
             VV_vs_GG_DEG$logFC,
             VV_vs_GG_DEG$Chr,
             VV_vs_GG_DEG$Geneid)
colnames(VV_vs_GG_DF) <- c("adj.P.Val", "logFC", "Chr", "Geneid")

VV_vs_GG_DF_Sig <-
```

```
  VV_vs_GG_DF[(abs(VV_vs_GG_DF$logFC) >= 2 &
                 VV_vs_GG_DF$adj.P.Val <= 0.01), ]$Geneid

# Finding stain bias genes, assigning color values
nonSig <- subset(VV_vs_GG_DF,!(Geneid %in% VV_vs_GG_DF_Sig))
nonSig <- cbind(nonSig , rep(1, nrow(nonSig)))
colnames(nonSig)[5] <- "Color"

up_VV <-
  subset(
    VV_vs_GG_DF,
    VV_vs_GG_DF$logFC >= 2 &
      VV_vs_GG_DF$adj.P.Val <= 0.01  & (Geneid %in% VV_vs_GG_DF_Sig)
  )
up_VV <- cbind(up_VV , rep(2, nrow(up_VV)))
colnames(up_VV)[5] <- "Color"

up_GG <-
  subset(
    VV_vs_GG_DF,
    VV_vs_GG_DF$logFC <= -2 &
      VV_vs_GG_DF$adj.P.Val <= 0.01  & (Geneid %in% VV_vs_GG_DF_Sig)
  )
up_GG <- cbind(up_GG , rep(3, nrow(up_GG)))
colnames(up_GG)[5] <- "Color"

dfPlot <- rbind(nonSig, up_VV, up_GG)
dfPlot$Color <- as.factor(dfPlot$Color)

# Constructing the plot object.
p <-
  ggplot(data = dfPlot, aes(
    x = logFC,
    y = -log10(adj.P.Val),
    color = Color
  )) +
  geom_point(alpha = 0.5, size = 8) +
  theme_bw() +
  theme(legend.position = "none") +
  xlim(c(-15, 15)) + ylim(c(0, 12)) +
  scale_color_manual(values = c("azure3", GG_Color, VV_Color)) +
  labs(
    title = "VV vs GG",
    x = expression(log[2](FC)),
    y = expression(-log[10] ~ "(FDR-adjusted " ~ italic("p") ~ "-value)")
  ) +
  theme(axis.title.x = element_text(size = 20),
        axis.text.x = element_text(size = 20)) +
  theme(axis.title.y = element_text(size = 20),
        axis.text.y = element_text(size = 20))
# Adding lines for significance thresholds
p
```

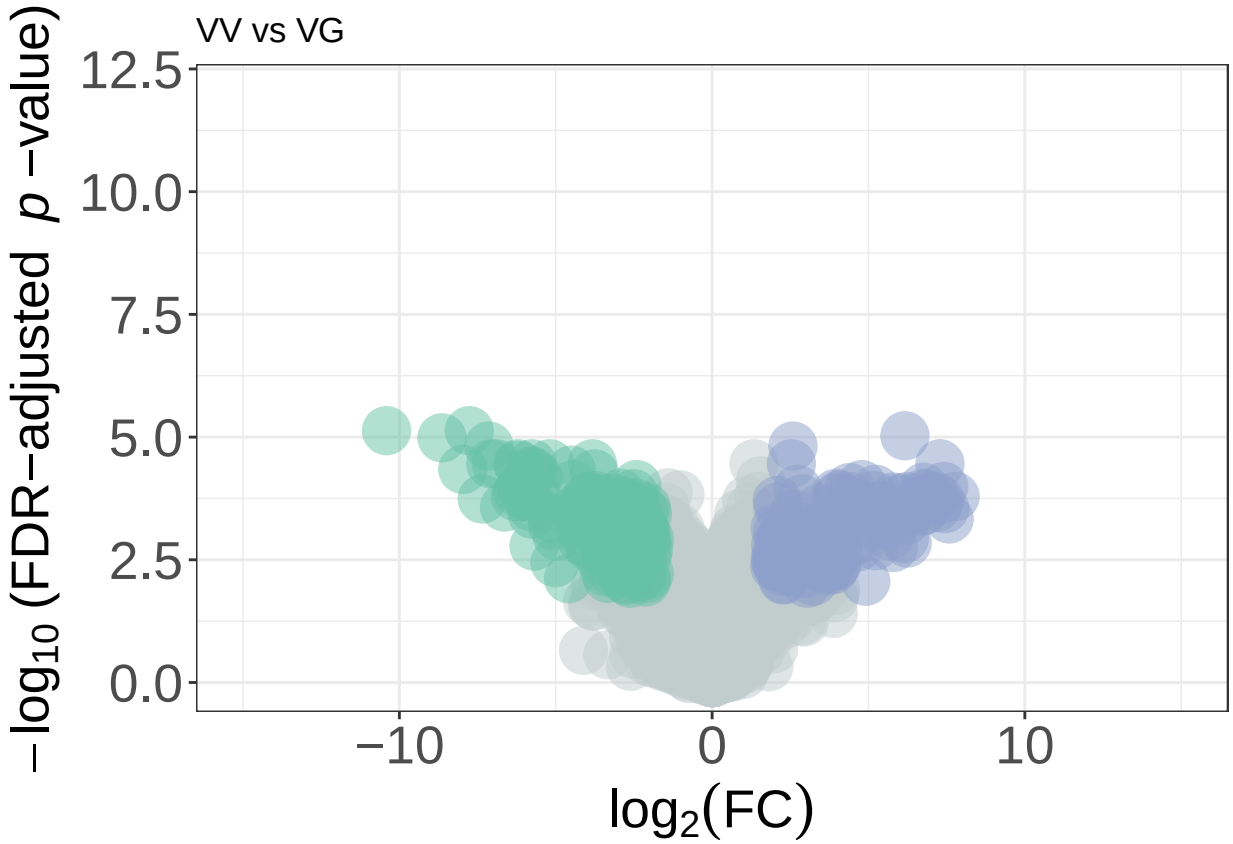



```
#png(filename = "figures/clark_AvgREFs_VV_vs_GG_valcano.png",
#    width = 650,
#    height = 650)
p + geom_hline(yintercept = 2,
               colour = "#000000",
               linetype = "dashed") +
  geom_vline(xintercept = 2,
             colour = "#000000",
             linetype = "dashed") +
  geom_vline(xintercept = -2,
             colour = "#000000",
             linetype = "dashed")
```

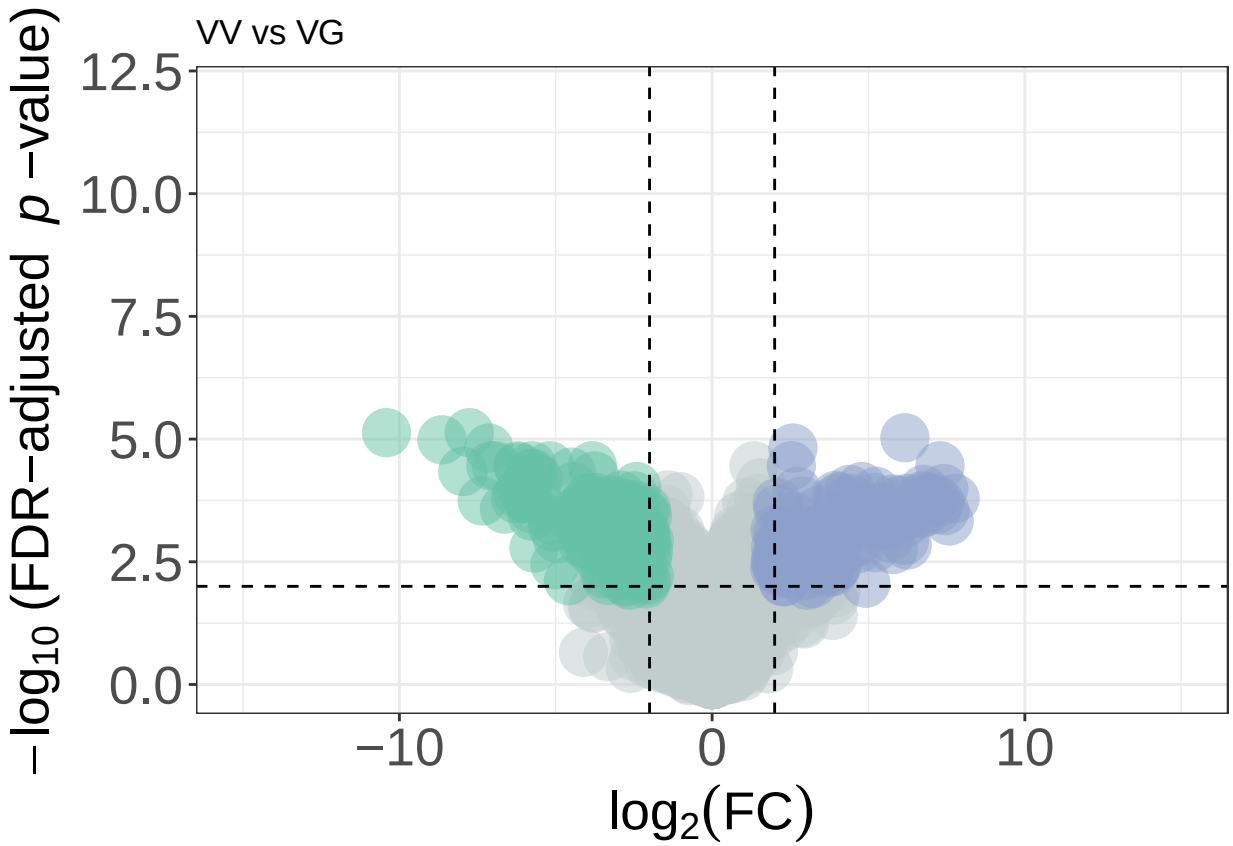



```
# dev.off()
# dev.off()

# VV vs VG
VV_vs_VG_DEG <-
  read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_VV_vs_VG_fdr1_lfc0.txt",
    header = TRUE,
    sep = "\t",
    stringsAsFactors = F
  )
VV_vs_VG_DF <-
  data.frame(VV_vs_VG_DEG$adj.P.Val,
             VV_vs_VG_DEG$logFC,
             VV_vs_VG_DEG$Chr,
             VV_vs_VG_DEG$Geneid)
colnames(VV_vs_VG_DF) <- c("adj.P.Val", "logFC", "Chr", "Geneid")

VV_vs_VG_DF_Sig <-
  VV_vs_VG_DF[(abs(VV_vs_VG_DF$logFC) >= 2 &
                 VV_vs_VG_DF$adj.P.Val <= 0.01), ]$Geneid

# Finding stain bias genes, assigning color values
nonSig <- subset(VV_vs_VG_DF,!(Geneid %in% VV_vs_VG_DF_Sig))
nonSig <- cbind(nonSig , rep(1, nrow(nonSig)))
colnames(nonSig)[5] <- "Color"
```

```r
up_VV <-
  subset(
    VV_vs_VG_DF,
    VV_vs_VG_DF$logFC >= 2 &
      VV_vs_VG_DF$adj.P.Val <= 0.01  & (Geneid %in% VV_vs_VG_DF_Sig)
  )
up_VV <- cbind(up_VV , rep(2, nrow(up_VV)))
colnames(up_VV)[5] <- "Color"

up_VG <-
  subset(
    VV_vs_VG_DF,
    VV_vs_VG_DF$logFC <= -2 &
      VV_vs_VG_DF$adj.P.Val <= 0.01  & (Geneid %in% VV_vs_VG_DF_Sig)
  )
up_VG <- cbind(up_VG , rep(3, nrow(up_VG)))
colnames(up_VG)[5] <- "Color"

dfPlot <- rbind(nonSig, up_VV, up_VG)
dfPlot$Color <- as.factor(dfPlot$Color)

# Constructing the plot object.
p <-
  ggplot(data = dfPlot, aes(
    x = logFC,
    y = -log10(adj.P.Val),
    color = Color
  )) +
  geom_point(alpha = 0.5, size = 8) +
  theme_bw() +
  theme(legend.position = "none") +
  xlim(c(-15, 15)) + ylim(c(0, 12)) +
  scale_color_manual(values = c("azure3", VG_Color, VV_Color)) +
  labs(
    title = "VV vs VG",
    x = expression(log[2](FC)),
    y = expression(-log[10] ~ "(FDR-adjusted " ~ italic("p") ~ "-value)")
  ) +
  theme(axis.title.x = element_text(size = 20),
        axis.text.x = element_text(size = 20)) +
  theme(axis.title.y = element_text(size = 20),
        axis.text.y = element_text(size = 20))
# Adding lines for significance thresholds
p
```



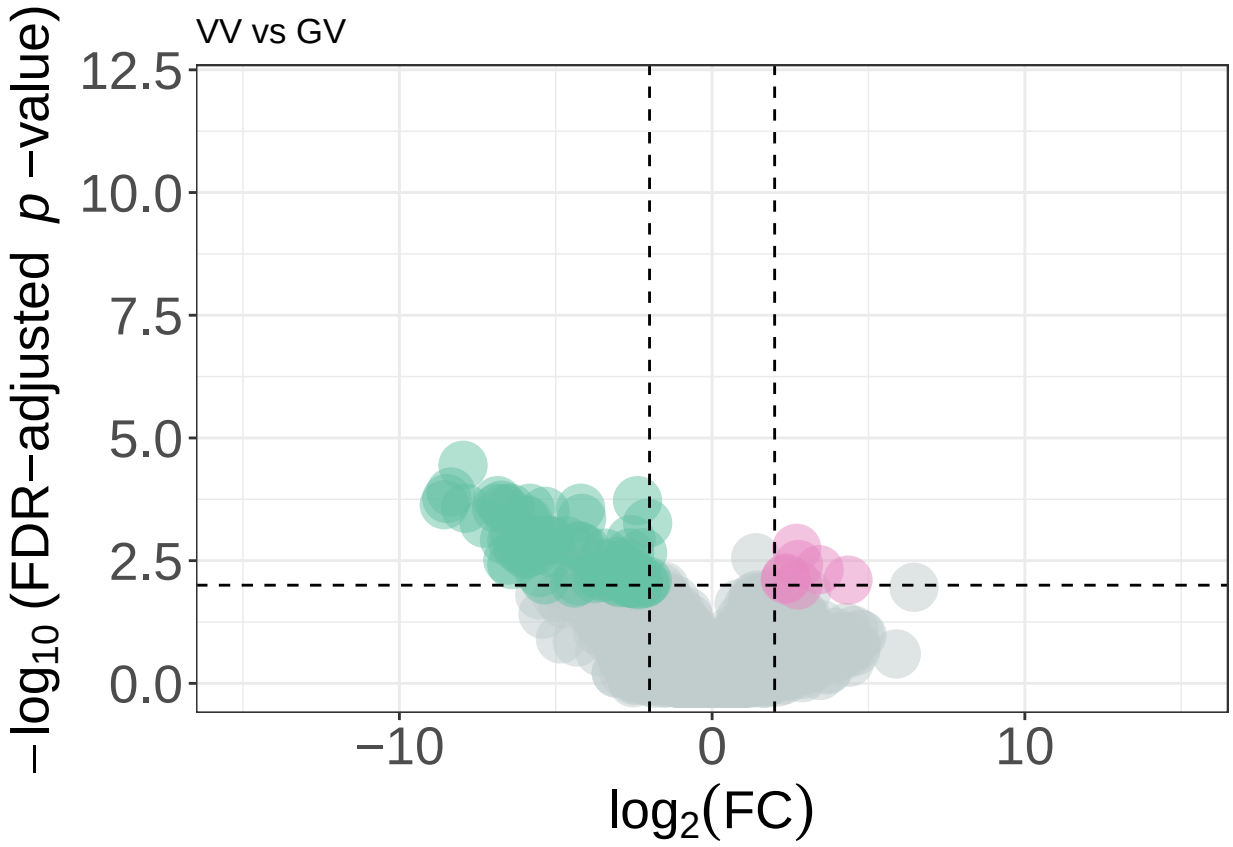

```
#png(filename = "figures/clark_AvgREFs_VV_vs_VG_valcano.png",
#    width = 650,
#    height = 650)
p + geom_hline(yintercept = 2,
               colour = "#000000",
               linetype = "dashed") +
  geom_vline(xintercept = 2,
             colour = "#000000",
             linetype = "dashed") +
  geom_vline(xintercept = -2,
             colour = "#000000",
             linetype = "dashed")
```




```
# dev.off()
# dev.off()

# VV vs GV
VV_vs_GV_DEG <-
  read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_VV_vs_GV_fdr1_lfc0.txt",
    header = TRUE,
    sep = "\t",
    stringsAsFactors = F
  )
VV_vs_GV_DF <-
  data.frame(VV_vs_GV_DEG$adj.P.Val,
             VV_vs_GV_DEG$logFC,
             VV_vs_GV_DEG$Chr,
             VV_vs_GV_DEG$Geneid)
colnames(VV_vs_GV_DF) <- c("adj.P.Val", "logFC", "Chr", "Geneid")

VV_vs_GV_DF_Sig <-
  VV_vs_GV_DF[(abs(VV_vs_GV_DF$logFC) >= 2 &
                 VV_vs_GV_DF$adj.P.Val <= 0.01), ]$Geneid

# Finding stain bias genes, assigning color values
nonSig <- subset(VV_vs_GV_DF,!(Geneid %in% VV_vs_GV_DF_Sig))
nonSig <- cbind(nonSig , rep(1, nrow(nonSig)))
colnames(nonSig)[5] <- "Color"
```

```r
up_VV <-
  subset(
    VV_vs_GV_DF,
    VV_vs_GV_DF$logFC >= 2 &
      VV_vs_GV_DF$adj.P.Val <= 0.01  & (Geneid %in% VV_vs_GV_DF_Sig)
  )
up_VV <- cbind(up_VV , rep(2, nrow(up_VV)))
colnames(up_VV)[5] <- "Color"

up_GV <-
  subset(
    VV_vs_GV_DF,
    VV_vs_GV_DF$logFC <= -2 &
      VV_vs_GV_DF$adj.P.Val <= 0.01  & (Geneid %in% VV_vs_GV_DF_Sig)
  )
up_GV <- cbind(up_GV , rep(3, nrow(up_GV)))
colnames(up_GV)[5] <- "Color"

dfPlot <- rbind(nonSig, up_VV, up_GV)
dfPlot$Color <- as.factor(dfPlot$Color)

# Constructing the plot object.
p <-
  ggplot(data = dfPlot, aes(
    x = logFC,
    y = -log10(adj.P.Val),
    color = Color
  )) +
  geom_point(alpha = 0.5, size = 8) +
  theme_bw() +
  theme(legend.position = "none") +
  xlim(c(-15, 15)) + ylim(c(0, 12)) +
  scale_color_manual(values = c("azure3", GV_Color, VV_Color)) +
  labs(
    title = "VV vs GV",
    x = expression(log[2](FC)),
    y = expression(-log[10] ~ "(FDR-adjusted " ~ italic("p") ~ "-value)")
  ) +
  theme(axis.title.x = element_text(size = 20),
        axis.text.x = element_text(size = 20)) +
  theme(axis.title.y = element_text(size = 20),
        axis.text.y = element_text(size = 20))
# Adding lines for significance thresholds
#png(filename = "figures/clark_AvgREFs_VV_vs_GV_valcano.png",
#    width = 650,
#    height = 650)
p + geom_hline(yintercept = 2,
               colour = "#000000",
               linetype = "dashed") +
  geom_vline(xintercept = 2,
             colour = "#000000",
             linetype = "dashed") +
  geom_vline(xintercept = -2,
```

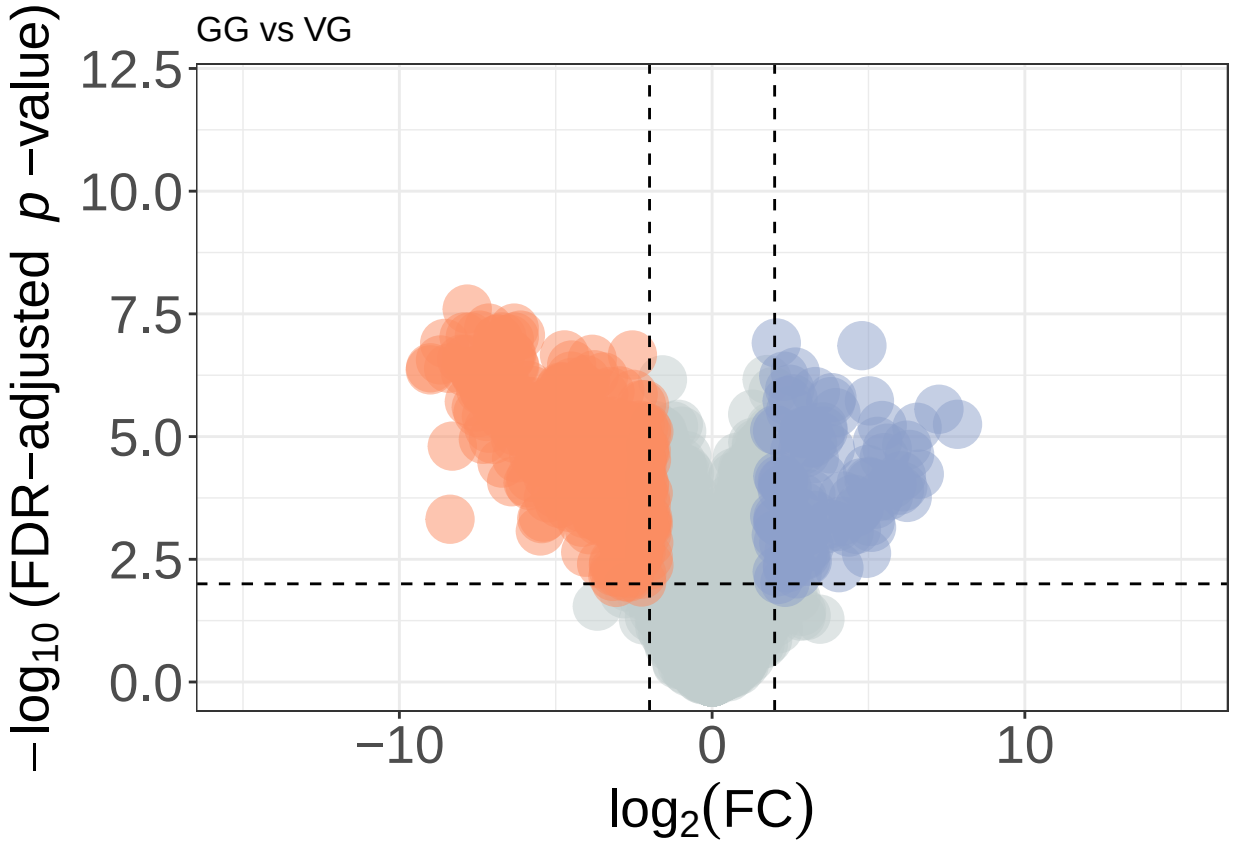
```
            colour = "#000000",
            linetype = "dashed")
```




```
# dev.off()
# dev.off()

# GG vs VG
GG_vs_VG_DEG <-
  read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_GG_vs_VG_fdr1_lfc0.txt",
    header = TRUE,
    sep = "\t",
    stringsAsFactors = F
  )
GG_vs_VG_DF <-
  data.frame(GG_vs_VG_DEG$adj.P.Val,
             GG_vs_VG_DEG$logFC,
             GG_vs_VG_DEG$Chr,
             GG_vs_VG_DEG$Geneid)
colnames(GG_vs_VG_DF) <- c("adj.P.Val", "logFC", "Chr", "Geneid")

GG_vs_VG_DF_Sig <-
  GG_vs_VG_DF[(abs(GG_vs_VG_DF$logFC) >= 2 &
                 GG_vs_VG_DF$adj.P.Val <= 0.01), ]$Geneid

# Finding stain bias genes, assigning color values
nonSig <- subset(GG_vs_VG_DF,!(Geneid %in% GG_vs_VG_DF_Sig))
```

```
nonSig <- cbind(nonSig , rep(1, nrow(nonSig)))
colnames(nonSig)[5] <- "Color"

up_GG <-
  subset(
    GG_vs_VG_DF,
    GG_vs_VG_DF$logFC >= 2 &
      GG_vs_VG_DF$adj.P.Val <= 0.01  & (Geneid %in% GG_vs_VG_DF_Sig)
  )
up_GG <- cbind(up_GG , rep(2, nrow(up_GG)))
colnames(up_GG)[5] <- "Color"

up_VG <-
  subset(
    GG_vs_VG_DF,
    GG_vs_VG_DF$logFC <= -2 &
      GG_vs_VG_DF$adj.P.Val <= 0.01  & (Geneid %in% GG_vs_VG_DF_Sig)
  )
up_VG <- cbind(up_VG , rep(3, nrow(up_VG)))
colnames(up_VG)[5] <- "Color"

dfPlot <- rbind(nonSig, up_GG, up_VG)
dfPlot$Color <- as.factor(dfPlot$Color)

# Constructing the plot object.
p <-
  ggplot(data = dfPlot, aes(
    x = logFC,
    y = -log10(adj.P.Val),
    color = Color
  )) +
  geom_point(alpha = 0.5, size = 8) +
  theme_bw() +
  theme(legend.position = "none") +
  xlim(c(-15, 15)) + ylim(c(0, 12)) +
  scale_color_manual(values = c("azure3", VG_Color, GG_Color)) +
  labs(
    title = "GG vs VG",
    x = expression(log[2](FC)),
    y = expression(-log[10] ~ "(FDR-adjusted " ~ italic("p") ~ "-value)")
  ) +
  theme(axis.title.x = element_text(size = 20),
        axis.text.x = element_text(size = 20)) +
  theme(axis.title.y = element_text(size = 20),
        axis.text.y = element_text(size = 20))
# Adding lines for significance thresholds
#png(filename = "figures/clark_AvgREFs_GG_vs_VG_valcano.png",
#    width = 650,
#    height = 650)
p + geom_hline(yintercept = 2,
               colour = "#000000",
               linetype = "dashed") +
  geom_vline(xintercept = 2,
```

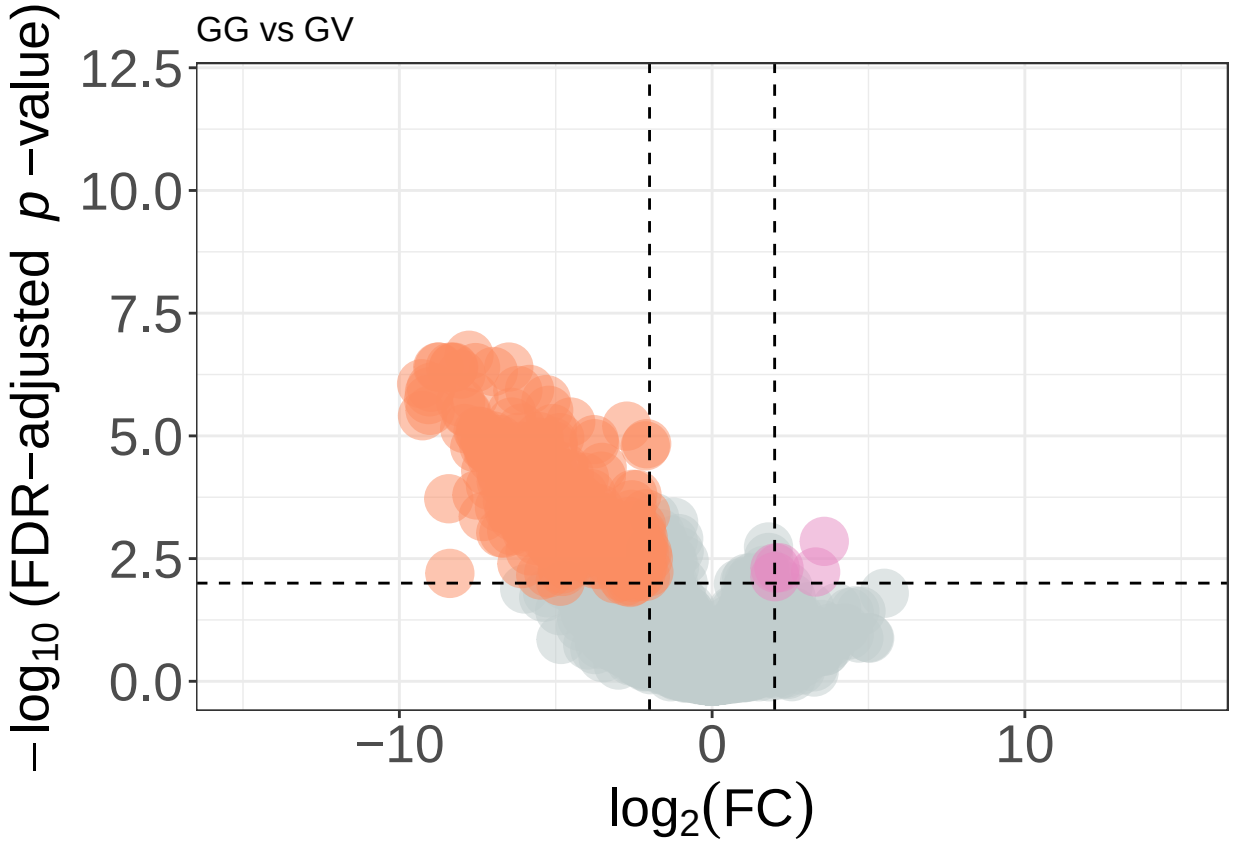
```
            colour = "#000000",
            linetype = "dashed") +
  geom_vline(xintercept = -2,
            colour = "#000000",
            linetype = "dashed")
```




```
# dev.off()
# dev.off()

# GG vs GV
GG_vs_GV_DEG <-
  read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_GG_vs_GV_fdr1_lfc0.txt",
    header = TRUE,
    sep = "\t",
    stringsAsFactors = F
  )
GG_vs_GV_DF <-
  data.frame(GG_vs_GV_DEG$adj.P.Val,
             GG_vs_GV_DEG$logFC,
             GG_vs_GV_DEG$Chr,
             GG_vs_GV_DEG$Geneid)
colnames(GG_vs_GV_DF) <- c("adj.P.Val", "logFC", "Chr", "Geneid")

GG_vs_GV_DF_Sig <-
  GG_vs_GV_DF[(abs(GG_vs_GV_DF$logFC) >= 2 &
                 GG_vs_GV_DF$adj.P.Val <= 0.01), ]$Geneid
```

```r
# Finding stain bias genes, assigning color values
nonSig <- subset(GG_vs_GV_DF,!(Geneid %in% GG_vs_GV_DF_Sig))
nonSig <- cbind(nonSig , rep(1, nrow(nonSig)))
colnames(nonSig)[5] <- "Color"

up_GG <-
  subset(
    GG_vs_GV_DF,
    GG_vs_GV_DF$logFC >= 2 &
      GG_vs_GV_DF$adj.P.Val <= 0.01  & (Geneid %in% GG_vs_GV_DF_Sig)
  )
up_GG <- cbind(up_GG , rep(2, nrow(up_GG)))
colnames(up_GG)[5] <- "Color"

up_GV <-
  subset(
    GG_vs_GV_DF,
    GG_vs_GV_DF$logFC <= -2 &
      GG_vs_GV_DF$adj.P.Val <= 0.01  & (Geneid %in% GG_vs_GV_DF_Sig)
  )
up_GV <- cbind(up_GV , rep(3, nrow(up_GV)))
colnames(up_GV)[5] <- "Color"

dfPlot <- rbind(nonSig, up_GG, up_GV)
dfPlot$Color <- as.factor(dfPlot$Color)

# Constructing the plot object.
p <-
  ggplot(data = dfPlot, aes(
    x = logFC,
    y = -log10(adj.P.Val),
    color = Color
  )) +
  geom_point(alpha = 0.5, size = 8) +
  theme_bw() +
  theme(legend.position = "none") +
  xlim(c(-15, 15)) + ylim(c(0, 12)) +
  scale_color_manual(values = c("azure3", GV_Color, GG_Color)) +
  labs(
    title = "GG vs GV",
    x = expression(log[2](FC)),
    y = expression(-log[10] ~ "(FDR-adjusted " ~ italic("p") ~ "-value)")
  ) +
  theme(axis.title.x = element_text(size = 20),
        axis.text.x = element_text(size = 20)) +
  theme(axis.title.y = element_text(size = 20),
        axis.text.y = element_text(size = 20))
# Adding lines for significance thresholds
#png(filename = "figures/clark_AvgREFs_GG_vs_GV_valcano.png",
#    width = 650,
#    height = 650)
p + geom_hline(yintercept = 2,
               colour = "#000000",
```

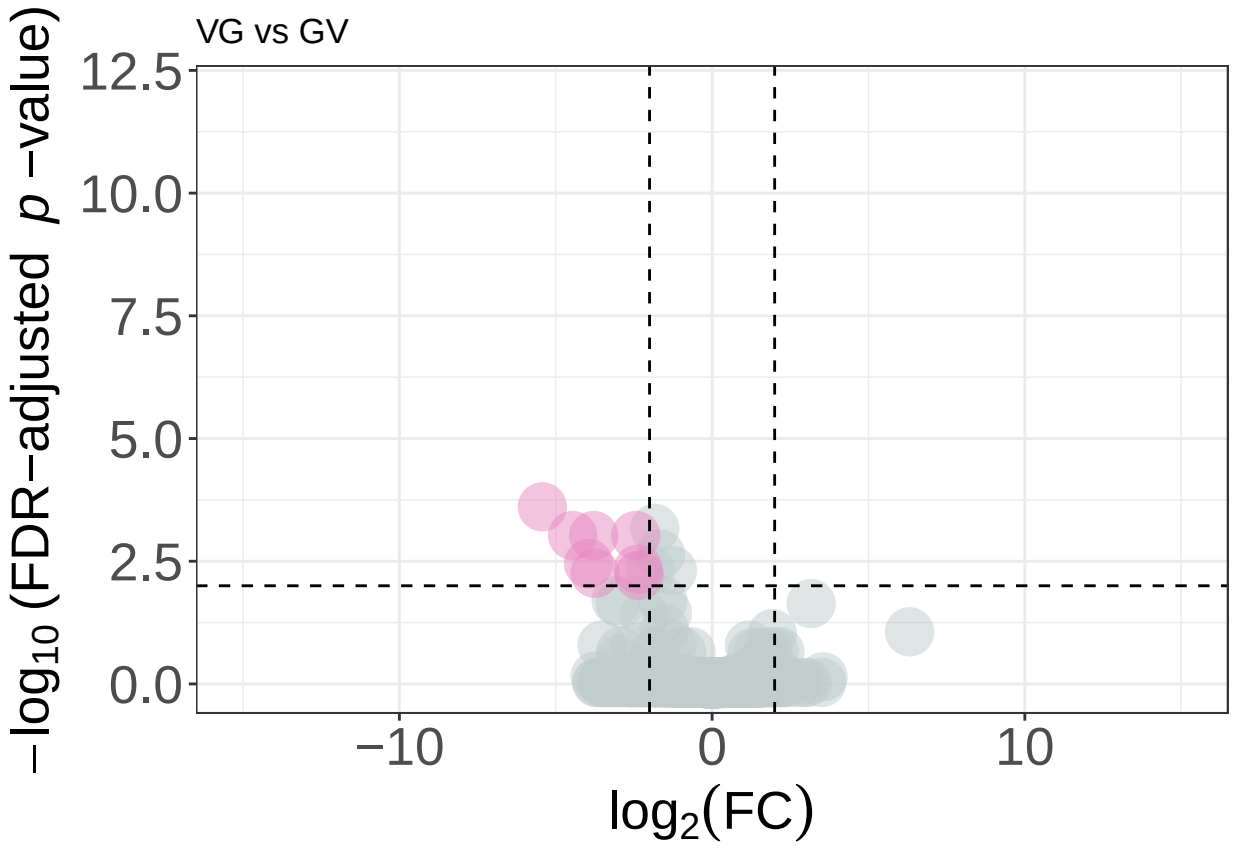
```
              linetype = "dashed") +
  geom_vline(xintercept = 2,
             colour = "#000000",
             linetype = "dashed") +
  geom_vline(xintercept = -2,
             colour = "#000000",
             linetype = "dashed")
```




```
# dev.off()
# dev.off()

# VG vs GV
VG_vs_GV_DEG <-
  read.table(
    "./DEGs/DEGs_clark_AvgREFs_fpkm05_VG_vs_GV_fdr1_lfc0.txt",
    header = TRUE,
    sep = "\t",
    stringsAsFactors = F
  )
VG_vs_GV_DF <-
  data.frame(VG_vs_GV_DEG$adj.P.Val,
             VG_vs_GV_DEG$logFC,
             VG_vs_GV_DEG$Chr,
             VG_vs_GV_DEG$Geneid)
colnames(VG_vs_GV_DF) <- c("adj.P.Val", "logFC", "Chr", "Geneid")

VG_vs_GV_DF_Sig <-
```

```
  VG_vs_GV_DF[(abs(VG_vs_GV_DF$logFC) >= 2 &
                 VG_vs_GV_DF$adj.P.Val <= 0.01), ]$Geneid

# Finding stain bias genes, assigning color values
nonSig <- subset(VG_vs_GV_DF,!(Geneid %in% VG_vs_GV_DF_Sig))
nonSig <- cbind(nonSig , rep(1, nrow(nonSig)))
colnames(nonSig)[5] <- "Color"

up_VG <-
  subset(
    VG_vs_GV_DF,
    VG_vs_GV_DF$logFC >= 2 &
      VG_vs_GV_DF$adj.P.Val <= 0.01  & (Geneid %in% VG_vs_GV_DF_Sig)
  )
up_VG <- cbind(up_VG , rep(2, nrow(up_VG)))
colnames(up_VG)[5] <- "Color"

up_GV <-
  subset(
    VG_vs_GV_DF,
    VG_vs_GV_DF$logFC <= -2 &
      VG_vs_GV_DF$adj.P.Val <= 0.01  & (Geneid %in% VG_vs_GV_DF_Sig)
  )
up_GV <- cbind(up_GV , rep(3, nrow(up_GV)))
colnames(up_GV)[5] <- "Color"

dfPlot <- rbind(nonSig, up_VG, up_GV)
dfPlot$Color <- as.factor(dfPlot$Color)

# Constructing the plot object.
p <-
  ggplot(data = dfPlot, aes(
    x = logFC,
    y = -log10(adj.P.Val),
    color = Color
  )) +
  geom_point(alpha = 0.5, size = 8) +
  theme_bw() +
  theme(legend.position = "none") +
  xlim(c(-15, 15)) + ylim(c(0, 12)) +
  scale_color_manual(values = c("azure3", GV_Color, VG_Color)) +
  labs(
    title = "VG vs GV",
    x = expression(log[2](FC)),
    y = expression(-log[10] ~ "(FDR-adjusted " ~ italic("p") ~ "-value)")
  ) +
  theme(axis.title.x = element_text(size = 20),
        axis.text.x = element_text(size = 20)) +
  theme(axis.title.y = element_text(size = 20),
        axis.text.y = element_text(size = 20))
# Adding lines for significance thresholds
#png(filename = "figures/clark_AvgREFs_VG_vs_GV_valcano.png",
#    width = 650,
```

```
#    height = 650)
p + geom_hline(yintercept = 2,
               colour = "#000000",
               linetype = "dashed") +
  geom_vline(xintercept = 2,
             colour = "#000000",
             linetype = "dashed") +
  geom_vline(xintercept = -2,
             colour = "#000000",
             linetype = "dashed")
```




```
# dev.off()
# dev.off()
```