# Differential Gene Exprpression and Pathway Analysis

2024-03-13

## BACKGROUND

ACESO Genomics and TIDREC collaboration on the SCOPE project. The example code below is meant to illustrate the process of standard gene expresison and pathway analysis to the enable RNA-Seq analytical capabilities going forward.

## CONTENT:

- PART1 - Loading data, preparation and running of DESeq2 analysis
- PART2 - Plotting data and Gene Set Enrichment Analysis
- PART3 - Exploring results plots
- PART4 - Table1 and metadata summaries

## PART1

Goal: - use the DESeq2 analysis to identify specific enriched or depleted genes - perform gene set enrichment analysis to find relevant pathways

### 1. Setup your environment

```
## Clean
rm(list = ls())
gc()
```

```
##          used (Mb) gc trigger (Mb) limit (Mb) max used (Mb)
## Ncells 478351 25.6    1032027 55.2         NA   669380 35.8
## Vcells 902294  6.9    8388608 64.0     256000  1851870 14.2
```

```
##
## PACKAGES
##

## load basic packages
suppressPackageStartupMessages(suppressWarnings({
  library(data.table);library(parallel);library(tidyr);library(tidyverse)}))


## For plotting
suppressPackageStartupMessages(suppressWarnings({
  library(ggpubr);library(ggbeeswarm);library(RColorBrewer);library(ggdendro);
  library(ggridges);library(ggrepel)}))
```

```
## For clustering
suppressPackageStartupMessages(suppressWarnings({library(pheatmap)}))

## For DESeq analysis
suppressPackageStartupMessages(suppressWarnings({library(DESeq2)}))
#library(sva) <- could be used for batch normalization

## For GSEA
suppressPackageStartupMessages(suppressWarnings({
  library(clusterProfiler);library(msigdb);library(msigdbr);
  library(enrichplot);library(ggupset)}))

##
## DIRECTORIES
##

TAB.DIR <- "/Users/a_PGenzor/Documents/GITHUB/ahjf_scope/results/tables/"
FIG.DIR <- "/Users/a_PGenzor/Documents/GITHUB/ahjf_scope/results/figures/"
#SES.DIR <- "/Users/a_PGenzor/Documents/GITHUB/ahjf_scope/sessions/"

##
## VERSION AND CONTROLS
##

aSeed="1003"
set.seed(aSeed)
version.date = "10MAR24"
```

## 2. Load the data

- Loading count data should be relatively simple since it should all be contained in the single matrix.
- Make sure that the column names in count matrix match the names in your metadata tables or that there is a way to calcualte them

```
##
## gene count table
count_table_path <- "/Users/a_PGenzor/Documents/GITHUB/ahjf_scope/data/from_kimkee/10MAR24/RNASeq_COVID
cnt.dt <- fread(count_table_path)
cnt.dt[1:5,1:3]
```

```
##                              gene_id
## 1:       ENSG00000223764.2|LINC02593
## 2: ENSG00000272438.1|ENSG00000272438
## 3: ENSG00000230699.2|ENSG00000230699
## 4: ENSG00000241180.1|ENSG00000241180
## 5: ENSG00000288531.1|ENSG00000288531
##    02_D0_DKDL230010052-1A_HNG3NDSX7_L1.nonovel.gtf
## 1:                                               10
## 2:                                                0
## 3:                                                0
## 4:                                                0
## 5:                                                9
##    06_D0_DKDL230011035-1A_HC2HKDSX7_L3.nonovel.gtf
## 1:                                                7
## 2:                                                      0
## 3:                                                      9
## 4:                                                      0
## 5:                                                      0
```

```
## splitting complex names into pieces
colnames(cnt.dt)[1:5]
```

```
## [1] "gene_id"
## [2] "02_D0_DKDL230010052-1A_HNG3NDSX7_L1.nonovel.gtf"
## [3] "06_D0_DKDL230011035-1A_HC2HKDSX7_L3.nonovel.gtf"
## [4] "100_D0_DKDL230011026-1A_HC2HKDSX7_L3.nonovel.gtf"
## [5] "11_D0_DKDL230011071-1A_HC2HKDSX7_L4.nonovel.gtf"
```

```
unlist(tstrsplit(colnames(cnt.dt),split="_",keep = 1))
```

```
##   [1] "gene" "02"   "06"   "100"  "11"   "14"   "21"   "26"   "31"   "32"
##  [11] "34"   "35"   "36"   "37"   "39"   "03"   "43"   "45"   "46"   "47"
##  [21] "49"   "51"   "53"   "54"   "56"   "57"   "58"   "60"   "61"   "62"
##  [31] "67"   "68"   "69"   "70"   "72"   "74"   "75"   "77"   "78"   "79"
##  [41] "80"   "81"   "82"   "85"   "86"   "87"   "88"   "89"   "90"   "91"
##  [51] "92"   "93"   "94"   "95"   "98"   "02"   "03"   "06"   "11"   "14"
##  [61] "21"   "32"   "36"   "37"   "39"   "43"   "45"   "47"   "49"   "51"
##  [71] "54"   "56"   "57"   "58"   "60"   "62"   "67"   "75"   "77"   "78"
##  [81] "79"   "80"   "81"   "82"   "85"   "86"   "87"   "88"   "89"   "90"
##  [91] "91"   "92"   "93"   "94"   "98"   "100"  "32"   "49"   "62"   "92"
## [101] "93"   "100"  "32"   "49"   "62"   "92"   "93"
```

```
unlist(tstrsplit(colnames(cnt.dt),split="_",keep = 2))
```

```
##   [1] "id"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"
##  [13] "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"
##  [25] "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"
##  [37] "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"
##  [49] "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D0"  "D28" "D28" "D28" "D28" "D28"
##  [61] "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28"
##  [73] "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28"
##  [85] "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D28" "D3"
##  [97] "D3"  "D3"  "D3"  "D3"  "D3"  "D7"  "D7"  "D7"  "D7"  "D7"  "D7"
```

```
## make new colnames
cnt_new_col_names <- paste(unlist(tstrsplit(colnames(cnt.dt),split="_",keep = 2)),
      unlist(tstrsplit(colnames(cnt.dt),split="_",keep = 1)),
      sep = "_")
cnt_new_col_names <-gsub("id_gene","gene_id",cnt_new_col_names)
colnames(cnt.dt) <- cnt_new_col_names
cnt.dt[1:5,1:5] ## <- ready to use
```

```
##                              gene_id D0_02 D0_06 D0_100 D0_11
## 1:        ENSG00000223764.2|LINC02593    10     7      7    14
## 2: ENSG00000272438.1|ENSG00000272438     0     0      0     0
## 3: ENSG00000230699.2|ENSG00000230699     0     9     17     7
## 4: ENSG00000241180.1|ENSG00000241180     0     0      0     0
## 5: ENSG00000288531.1|ENSG00000288531     9     0     21    30
```

```
##
## metadata
metadata_path <- "/Users/a_PGenzor/Documents/GITHUB/ahjf_scope/data/from_kimkee/10MAR24/RNASeq_COVID/Me
```

```r
meta.dt <- fread(metadata_path)

meta_cols_to_use <- c("IGU_Code","sex","pathogen","disease","time")
meta.clean.dt <- meta.dt[,.SD,.SDcols = meta_cols_to_use]
meta.clean.dt[,"subject":=tstrsplit(IGU_Code,split="_",keep = 1)]
meta.clean.dt[,"seq_id":= paste(time,subject,sep = "_")]
meta.clean.dt
```

```
##       IGU_Code    sex   pathogen disease time subject seq_id
##   1:     02_D0 Female SARS-CoV-2 COVID19   D0      02  D0_02
##   2:    02_D28 Female SARS-CoV-2 COVID19  D28      02 D28_02
##   3:     03_D0 Female SARS-CoV-2 COVID19   D0      03  D0_03
##   4:    03_D28 Female SARS-CoV-2 COVID19  D28      03 D28_03
##   5:     06_D0 Female SARS-CoV-2 COVID19   D0      06  D0_06
##  ---
## 102:     98_D0   Male SARS-CoV-2 COVID19   D0      98  D0_98
## 103:    98_D28   Male SARS-CoV-2 COVID19  D28      98 D28_98
## 104:    100_D0   Male SARS-CoV-2 COVID19   D0     100 D0_100
## 105:    100_D3   Male SARS-CoV-2 COVID19   D3     100 D3_100
## 106:    100_D7   Male SARS-CoV-2 COVID19   D7     100 D7_100
```

## 3. Format the data

Once the data is loaded in a clean way, make sure that you format the data types to ones that can be used by DESeq - eg. matrix instead of table and so on - This is a good place to filter your data to remove uninformative genes - Here you will also be combining the sample information with the metadata so that they correspond to each other during analysis - NOTE: metadata and data alignment is a key for analysis.

```r
##
## Filter raw counts
##

cnt.dt[1:5,1:5]
```

```
##                               gene_id D0_02 D0_06 D0_100 D0_11
## 1:       ENSG00000223764.2|LINC02593    10     7      7    14
## 2: ENSG00000272438.1|ENSG00000272438     0     0      0     0
## 3: ENSG00000230699.2|ENSG00000230699     0     9     17     7
## 4: ENSG00000241180.1|ENSG00000241180     0     0      0     0
## 5: ENSG00000288531.1|ENSG00000288531     9     0     21    30
```

```r
## summarize raw counts
cnt.dt.sumarized <- cnt.dt[,list(max=max(.SD),
                                 min=min(.SD),
                                 mean=mean(unlist(.SD))), by=gene_id]
cnt.dt.sumarized
```

```
##                                   gene_id max min        mean
##     1:       ENSG00000223764.2|LINC02593  38   0  6.40566038
##     2: ENSG00000272438.1|ENSG00000272438   2   0  0.01886792
##     3: ENSG00000230699.2|ENSG00000230699  49   0  7.40566038
##     4: ENSG00000241180.1|ENSG00000241180   0   0  0.00000000
##     5: ENSG00000288531.1|ENSG00000288531  95   0 12.57547170
##    ---
## 61902: ENSG00000275249.1|ENSG00000275249  16   0  2.42452830
## 61903: ENSG00000274792.1|ENSG00000274792  14   0  1.30188679
## 61904: ENSG00000278510.1|ENSG00000278510   6   0  0.35849057
## 61905: ENSG00000277196.4|ENSG00000277196  19   0  1.67924528
## 61906:              ENSG00000277374.1|U1   4   0  0.54716981
```

```
## get gene names that have sufficient expression
## NOTE: this parameter is subjective and you can/should play with your cutoff value
## NOTE: sometimes, it makes more sense to not use min if you think some genes are on/off in subjects
## NOTE: counts are not like TPM, 10 counts per gene may still mean gene is off

cnt.dt.sumarized[mean > 0]
```

```
##                                  gene_id max min        mean
##     1:       ENSG00000223764.2|LINC02593  38   0  6.40566038
##     2: ENSG00000272438.1|ENSG00000272438   2   0  0.01886792
##     3: ENSG00000230699.2|ENSG00000230699  49   0  7.40566038
##     4: ENSG00000288531.1|ENSG00000288531  95   0 12.57547170
##     5:          ENSG00000230368.2|FAM41C  73   0 14.16981132
##    ---
## 61696: ENSG00000275249.1|ENSG00000275249  16   0  2.42452830
## 61697: ENSG00000274792.1|ENSG00000274792  14   0  1.30188679
## 61698: ENSG00000278510.1|ENSG00000278510   6   0  0.35849057
## 61699: ENSG00000277196.4|ENSG00000277196  19   0  1.67924528
## 61700:              ENSG00000277374.1|U1   4   0  0.54716981
```

```
cnt.dt.sumarized[mean > 10]
```

```
##                                  gene_id  max min       mean
##     1: ENSG00000288531.1|ENSG00000288531   95   0   12.57547
##     2:          ENSG00000230368.2|FAM41C   73   0   14.16981
##     3:         ENSG00000187961.15|KLHL17  547  28  200.42453
##     4:        ENSG00000187583.11|PLEKHN1   80   0   33.10377
##     5:          ENSG00000188976.11|NOC2L  836 102  411.50000
##    ---
## 24737: ENSG00000267793.1|ENSG00000267793   75   0   14.43396
## 24738: ENSG00000260197.1|ENSG00000260197  412   0   93.06604
## 24739:          ENSG00000012817.16|KDM5D 7361   0 1614.75472
## 24740: ENSG00000288049.1|ENSG00000288049  170   0   35.42453
## 24741:         ENSG00000198692.10|EIF1AY 1259   0  298.06604
```

```
cnt.dt.sumarized[mean > 50]
```

```
##                                  gene_id   max min       mean
##     1:         ENSG00000187961.15|KLHL17   547  28  200.42453
##     2:          ENSG00000188976.11|NOC2L   836 102  411.50000
##     3: ENSG00000272512.1|ENSG00000272512  1622   0   73.54717
##     4:           ENSG00000188290.11|HES4  2402   0  190.87736
##     5:          ENSG00000187608.10|ISG15 34685  51 1960.95283
##    ---
## 16065:         ENSG00000215580.12|BCORP1   913   0  182.10377
## 16066:         ENSG00000131002.14|TXLNGY  6766   0 1647.10377
## 16067: ENSG00000260197.1|ENSG00000260197   412   0   93.06604
## 16068:          ENSG00000012817.16|KDM5D  7361   0 1614.75472
## 16069:         ENSG00000198692.10|EIF1AY  1259   0  298.06604
```

```
gene_ids_to_include <- cnt.dt.sumarized[mean > 50][["gene_id"]]

## filter data
```

```
cnt.filtered.dt <- cnt.dt[gene_id %in% gene_ids_to_include]
cnt.filtered.dt[1:5,1:5]
```

```
##                                gene_id D0_02 D0_06 D0_100 D0_11
## 1:         ENSG00000187961.15|KLHL17   152   130     49    39
## 2:          ENSG00000188976.11|NOC2L   427   380    111   116
## 3: ENSG00000272512.1|ENSG00000272512   362    46      6    12
## 4:           ENSG00000188290.11|HES4  1337   404      3    20
## 5:          ENSG00000187608.10|ISG15 11707  4699    105   150
```

```
##
## Make sample information table
##

## create a sample information table from cnt table
## NOTE: this will make sure you will always have the right samples present
si.dt <- data.table("seq_id"=colnames(cnt.dt)[-1])
si.dt[,"subject":=tstrsplit(seq_id,split="_",keep = 2)]
si.dt[,"time":=tstrsplit(seq_id,split="_",keep = 1)]
si.dt
```

```
##      seq_id subject time
##   1:  D0_02      02   D0
##   2:  D0_06      06   D0
##   3: D0_100     100   D0
##   4:  D0_11      11   D0
##   5:  D0_14      14   D0
##  ---
## 102:  D7_32      32   D7
## 103:  D7_49      49   D7
## 104:  D7_62      62   D7
## 105:  D7_92      92   D7
## 106:  D7_93      93   D7
```

```
##
## Load metadata and add to the sample information
##

# peak at ready metadata
meta.clean.dt
```

```
##      IGU_Code    sex   pathogen disease time subject seq_id
##   1:    02_D0 Female SARS-CoV-2 COVID19   D0      02  D0_02
##   2:   02_D28 Female SARS-CoV-2 COVID19  D28      02 D28_02
##   3:    03_D0 Female SARS-CoV-2 COVID19   D0      03  D0_03
##   4:   03_D28 Female SARS-CoV-2 COVID19  D28      03 D28_03
##   5:    06_D0 Female SARS-CoV-2 COVID19   D0      06  D0_06
##  ---
## 102:    98_D0   Male SARS-CoV-2 COVID19   D0      98  D0_98
## 103:   98_D28   Male SARS-CoV-2 COVID19  D28      98 D28_98
## 104:   100_D0   Male SARS-CoV-2 COVID19   D0     100 D0_100
## 105:   100_D3   Male SARS-CoV-2 COVID19   D3     100 D3_100
## 106:   100_D7   Male SARS-CoV-2 COVID19   D7     100 D7_100
```

```
# add to si.dt to make a master table (mt)
si.dt
```

```
##      seq_id subject time
##   1:  D0_02      02   D0
##   2:  D0_06      06   D0
##   3: D0_100     100   D0
##   4:  D0_11      11   D0
##   5:  D0_14      14   D0
##  ---
## 102:  D7_32      32   D7
## 103:  D7_49      49   D7
## 104:  D7_62      62   D7
## 105:  D7_92      92   D7
## 106:  D7_93      93   D7
```

```
si.mt.dt <- meta.clean.dt[si.dt,on=.(seq_id=seq_id,time=time,subject=subject)]
si.mt.dt[1:5,]
```

```
##    IGU_Code    sex   pathogen disease time subject seq_id
## 1:    02_D0 Female SARS-CoV-2 COVID19   D0      02  D0_02
## 2:    06_D0 Female SARS-CoV-2 COVID19   D0      06  D0_06
## 3:   100_D0   Male SARS-CoV-2 COVID19   D0     100 D0_100
## 4:    11_D0 Female SARS-CoV-2 COVID19   D0      11  D0_11
## 5:    14_D0   Male SARS-CoV-2 COVID19   D0      14  D0_14
```

```
## filter table to keep only comparison samples
si.mt.comp.dt <- si.mt.dt[time %in% c("D0","D28")]
si.mt.comp.dt[,.N,by=list(disease, time)]
```

```
##        disease time  N
## 1:     COVID19   D0 30
## 2: Non-COVID19   D0 24
## 3:     COVID19  D28 28
## 4: Non-COVID19  D28 12
```

```
si.mt.comp.dt[,.N,by=time]
```

```
##    time  N
## 1:   D0 54
## 2:  D28 40
```

```
##
## Filter count table to keep comparison columns
##

# present columns and their format
colnames(cnt.filtered.dt)
```

```
##   [1] "gene_id" "D0_02"   "D0_06"   "D0_100"  "D0_11"   "D0_14"   "D0_21"
##   [8] "D0_26"   "D0_31"   "D0_32"   "D0_34"   "D0_35"   "D0_36"   "D0_37"
##  [15] "D0_39"   "D0_03"   "D0_43"   "D0_45"   "D0_46"   "D0_47"   "D0_49"
##  [22] "D0_51"   "D0_53"   "D0_54"   "D0_56"   "D0_57"   "D0_58"   "D0_60"
##  [29] "D0_61"   "D0_62"   "D0_67"   "D0_68"   "D0_69"   "D0_70"   "D0_72"
##  [36] "D0_74"   "D0_75"   "D0_77"   "D0_78"   "D0_79"   "D0_80"   "D0_81"
##  [43] "D0_82"   "D0_85"   "D0_86"   "D0_87"   "D0_88"   "D0_89"   "D0_90"
##  [50] "D0_91"   "D0_92"   "D0_93"   "D0_94"   "D0_95"   "D0_98"   "D28_02"
##  [57] "D28_03"  "D28_06"  "D28_11"  "D28_14"  "D28_21"  "D28_32"  "D28_36"
##  [64] "D28_37"  "D28_39"  "D28_43"  "D28_45"  "D28_47"  "D28_49"  "D28_51"
##  [71] "D28_54"  "D28_56"  "D28_57"  "D28_58"  "D28_60"  "D28_62"  "D28_67"
##  [78] "D28_75"  "D28_77"  "D28_78"  "D28_79"  "D28_80"  "D28_81"  "D28_82"
##  [85] "D28_85"  "D28_86"  "D28_87"  "D28_88"  "D28_89"  "D28_90"  "D28_91"
##  [92] "D28_92"  "D28_93"  "D28_94"  "D28_98"  "D3_100"  "D3_32"   "D3_49"
##  [99] "D3_62"   "D3_92"   "D3_93"   "D7_100"  "D7_32"   "D7_49"   "D7_62"
## [106] "D7_92"   "D7_93"
```

```
# wanted columns and their format matching
si.mt.comp.dt[1:5,]
```

```
##    IGU_Code    sex   pathogen disease time subject seq_id
## 1:    02_D0 Female SARS-CoV-2 COVID19   D0      02  D0_02
## 2:    06_D0 Female SARS-CoV-2 COVID19   D0      06  D0_06
## 3:   100_D0   Male SARS-CoV-2 COVID19   D0     100 D0_100
## 4:    11_D0 Female SARS-CoV-2 COVID19   D0      11  D0_11
## 5:    14_D0   Male SARS-CoV-2 COVID19   D0      14  D0_14
```

```
wanted_comp_columns <- si.mt.comp.dt[["seq_id"]]
wanted_comp_columns
```

```
##  [1] "D0_02"  "D0_06"  "D0_100" "D0_11"  "D0_14"  "D0_21"  "D0_26"  "D0_31"
##  [9] "D0_32"  "D0_34"  "D0_35"  "D0_36"  "D0_37"  "D0_39"  "D0_03"  "D0_43"
## [17] "D0_45"  "D0_46"  "D0_47"  "D0_49"  "D0_51"  "D0_53"  "D0_54"  "D0_56"
## [25] "D0_57"  "D0_58"  "D0_60"  "D0_61"  "D0_62"  "D0_67"  "D0_68"  "D0_69"
## [33] "D0_70"  "D0_72"  "D0_74"  "D0_75"  "D0_77"  "D0_78"  "D0_79"  "D0_80"
## [41] "D0_81"  "D0_82"  "D0_85"  "D0_86"  "D0_87"  "D0_88"  "D0_89"  "D0_90"
## [49] "D0_91"  "D0_92"  "D0_93"  "D0_94"  "D0_95"  "D0_98"  "D28_02" "D28_03"
## [57] "D28_06" "D28_11" "D28_14" "D28_21" "D28_32" "D28_36" "D28_37" "D28_39"
## [65] "D28_43" "D28_45" "D28_47" "D28_49" "D28_51" "D28_54" "D28_56" "D28_57"
## [73] "D28_58" "D28_60" "D28_62" "D28_67" "D28_75" "D28_77" "D28_78" "D28_79"
## [81] "D28_80" "D28_81" "D28_82" "D28_85" "D28_86" "D28_87" "D28_88" "D28_89"
## [89] "D28_90" "D28_91" "D28_92" "D28_93" "D28_94" "D28_98"
```

```
## filter raw counts to keep the same samples
cnt.filtered.comp.dt <- cnt.filtered.dt[,.SD,.SDcols = c("gene_id",wanted_comp_columns)]
cnt.filtered.comp.dt[1:5,1:5]
```

```
##                              gene_id D0_02 D0_06 D0_100 D0_11
## 1:         ENSG00000187961.15|KLHL17   152   130     49    39
## 2:          ENSG00000188976.11|NOC2L   427   380    111   116
## 3: ENSG00000272512.1|ENSG00000272512   362    46      6    12
## 4:           ENSG00000188290.11|HES4  1337   404      3    20
## 5:          ENSG00000187608.10|ISG15 11707  4699    105   150
```

```
##
## Format into right types
##date

## counts need to be a matrix where rownames are gene_id
cnt.comp.mat <- as.matrix(x = cnt.filtered.comp.dt, rownames = "gene_id")

## sample information can remain a data table
si.mt.comp.dt[1:5,]
```

```
##    IGU_Code    sex   pathogen disease time subject seq_id
## 1:    02_D0 Female SARS-CoV-2 COVID19   D0      02  D0_02
## 2:    06_D0 Female SARS-CoV-2 COVID19   D0      06  D0_06
## 3:   100_D0   Male SARS-CoV-2 COVID19   D0     100 D0_100
## 4:    11_D0 Female SARS-CoV-2 COVID19   D0      11  D0_11
## 5:    14_D0   Male SARS-CoV-2 COVID19   D0      14  D0_14
```

## 4. Run DESeq2 analysis

- Once the data has been prepared, the DESeq package can be employed and comparative analysis performed. The analysis consists of three simple steps:
  1. Create a DESeq object using the raw counts and metadata from previous section. And specifying the comparison MODEL.
  2. Running the DESeq command.
  3. Retrieval of the result tables for plotting and analysis.

```r
##
## Create a DESeq object
##

## Data
#cnt.comp.mat[1:5,1:5]
#si.mt.comp.dt[1:5]

## load data into deseq object
dds <- DESeqDataSetFromMatrix(countData = cnt.comp.mat,
                              colData = si.mt.comp.dt,
                              design = ~time)
```

```
## Warning in DESeqDataSet(se, design = design, ignoreRank): some variables in
## design formula are characters, converting to factors
```

```r
## add condition to the modeling
dds.sex <- DESeqDataSetFromMatrix(countData = cnt.comp.mat,
                              colData = si.mt.comp.dt,
                              design = ~time+sex)
```

```
## Warning in DESeqDataSet(se, design = design, ignoreRank): some variables in
## design formula are characters, converting to factors
```

```r
##
## Run DESeq Analysis
##

## two modes - with and without sex consideration
dds <- DESeq(dds)
```

```
## estimating size factors

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship

## final dispersion estimates

## fitting model and testing

## -- replacing outliers and refitting for 403 genes
## -- DESeq argument 'minReplicatesForReplace' = 7
## -- original counts are preserved in counts(dds)

## estimating dispersions
## fitting model and testing
```

```r
dds.sex <- DESeq(dds.sex)
```

```
## estimating size factors

## estimating dispersions

## gene-wise dispersion estimates

## mean-dispersion relationship

## final dispersion estimates

## fitting model and testing

## -- replacing outliers and refitting for 273 genes
## -- DESeq argument 'minReplicatesForReplace' = 7
## -- original counts are preserved in counts(dds)

## estimating dispersions

## fitting model and testing
```

```r
##
## View and retrieve the results
##

## Look at results without sex consideration
resultsNames(dds)
```

```
## [1] "Intercept"      "time_D28_vs_D0"
```

```r
res <- results(object = dds, name = "time_D28_vs_D0", alpha = 0.05)
summary(res)
```

```
##
## out of 16068 with nonzero total read count
## adjusted p-value < 0.05
## LFC > 0 (up)       : 931, 5.8%
## LFC < 0 (down)     : 2038, 13%
## outliers [1]       : 0, 0%
## low counts [2]     : 1, 0.0062%
## (mean count < 0)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

```r
## Look at the results with sex consideration
resultsNames(dds.sex)
```

```
## [1] "Intercept"          "time_D28_vs_D0"     "sex_Male_vs_Female"
```

```r
res.sex <- results(object = dds.sex, name = "time_D28_vs_D0", alpha = 0.05)
summary(res.sex)
```

```
##
## out of 16069 with nonzero total read count
## adjusted p-value < 0.05
## LFC > 0 (up)       : 934, 5.8%
## LFC < 0 (down)     : 1926, 12%
## outliers [1]       : 0, 0%
## low counts [2]     : 0, 0%
## (mean count < 2)
## [1] see 'cooksCutoff' argument of ?results
## [2] see 'independentFiltering' argument of ?results
```

```
## export a table of results for each
res.dt <- as.data.table(results(object = dds, name = "time_D28_vs_D0", alpha = 0.05), keep.rownames=TRU
```

```
## Warning in .local(x, row.names, optional, ...): Arguments in '...' ignored
```

```
colnames(res.dt) <- gsub("rn","gene_id",colnames(res.dt))

res.sex.dt <- as.data.table(results(object = dds.sex, name = "time_D28_vs_D0", alpha = 0.05), keep.rown
```

```
## Warning in .local(x, row.names, optional, ...): Arguments in '...' ignored
```

```
colnames(res.sex.dt) <- gsub("rn","gene_id",colnames(res.sex.dt))

## RESULT TABLES
res.dt <- res.dt[order(padj,log2FoldChange)]
res.dt
```

```
##                                  gene_id  baseMean log2FoldChange      lfcSE
##     1:        ENSG00000108387.16|SEPTIN4  312.7207  -2.802464e+00 0.27604306
##     2:          ENSG00000165949.13|IFI27 1174.0043  -4.364281e+00 0.44337569
##     3:          ENSG00000184979.11|USP18  830.0480  -3.040946e+00 0.32843781
##     4:        ENSG00000196141.14|SPATS2L  995.0307  -2.607314e+00 0.28203196
##     5:          ENSG00000187608.10|ISG15 2014.3514  -3.131625e+00 0.35052613
##    ---
## 16065:          ENSG00000155903.14|RASA2 3779.8496  -2.503228e-05 0.04456558
## 16066:         ENSG00000257246.2|PHB1P19  104.8990  -4.350574e-05 0.11009555
## 16067:       ENSG00000286219.2|NOTCH2NLC 4280.7756  -9.717332e-06 0.08605497
## 16068:           ENSG00000165195.16|PIGA  497.5808   3.578511e-06 0.05084592
## 16069: ENSG00000269693.1|ENSG00000269693    0.0000   0.000000e+00 0.00000000
##                 stat       pvalue         padj
##     1: -1.015227e+01 3.237387e-24 5.201833e-20
##     2: -9.843303e+00 7.326482e-23 5.886095e-19
##     3: -9.258817e+00 2.067101e-20 9.472012e-17
##     4: -9.244747e+00 2.357982e-20 9.472012e-17
##     5: -8.934070e+00 4.106169e-19 1.319558e-15
##    ---
## 16065: -5.616955e-04 9.995518e-01 9.997385e-01
## 16066: -3.951635e-04 9.996847e-01 9.998092e-01
## 16067: -1.129201e-04 9.999099e-01 9.999438e-01
## 16068:  7.037950e-05 9.999438e-01 9.999438e-01
## 16069:  0.000000e+00 1.000000e+00           NA
```

```
res.sex.dt <- res.sex.dt[order(padj,log2FoldChange)]
res.sex.dt
```

```
##                                  gene_id    baseMean log2FoldChange      lfcSE
##     1:          ENSG00000165949.13|IFI27  1174.00428  -4.321991e+00 0.44438975
##     2:          ENSG00000184979.11|USP18   830.04803  -2.860923e+00 0.31575707
##     3:          ENSG00000187608.10|ISG15  2014.35142  -3.084138e+00 0.34314627
##     4:         ENSG00000142089.17|IFITM3 13186.71862  -2.023653e+00 0.23732131
##     5:          ENSG00000161133.18|USP41    72.44594  -2.609214e+00 0.30872184
##    ---
## 16065:            ENSG00000172336.5|POP7    85.59053  -4.234665e-05 0.09927925
## 16066:          ENSG00000063601.17|MTMR1  1324.63352  -9.389151e-06 0.04372301
## 16067: ENSG00000276136.1|ENSG00000276136   452.38103   2.133293e-05 0.10818643
## 16068:         ENSG00000196417.13|ZNF765   513.27454   2.447131e-05 0.08010933
## 16069:            ENSG00000134548.11|SPX    60.77116   1.316112e-04 0.19918038
##                 stat       pvalue         padj
##     1: -9.7256768058 2.343417e-22 3.765638e-18
##     2: -9.0605202753 1.298298e-19 1.043118e-15
##     3: -8.9878243218 2.521727e-19 1.350721e-15
##     4: -8.5270582503 1.501163e-17 6.030547e-14
##     5: -8.4516667939 2.871732e-17 8.620821e-14
##    ---
## 16065: -0.0004265408 9.996597e-01 9.998427e-01
## 16066: -0.0002147417 9.998287e-01 9.998427e-01
## 16067:  0.0001971868 9.998427e-01 9.998427e-01
## 16068:  0.0003054739 9.997563e-01 9.998427e-01
## 16069:  0.0006607637 9.994728e-01 9.998427e-01
```

## PART2

Goal: - Explore ways of plotting results from DESeq2 analysis - Use the results in Gene Set Enrichemnt Analysis

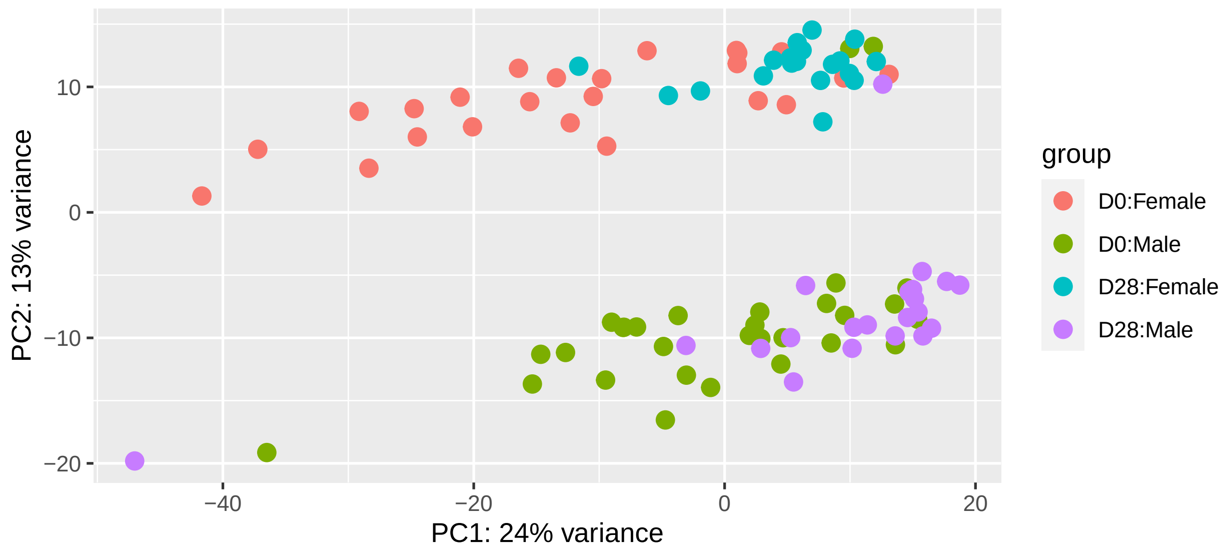
### 1. Plot PCA of the results

- PCA plot is one of the typical plots to evaluate whether there are any patterns in your data
- First, the data is normalized

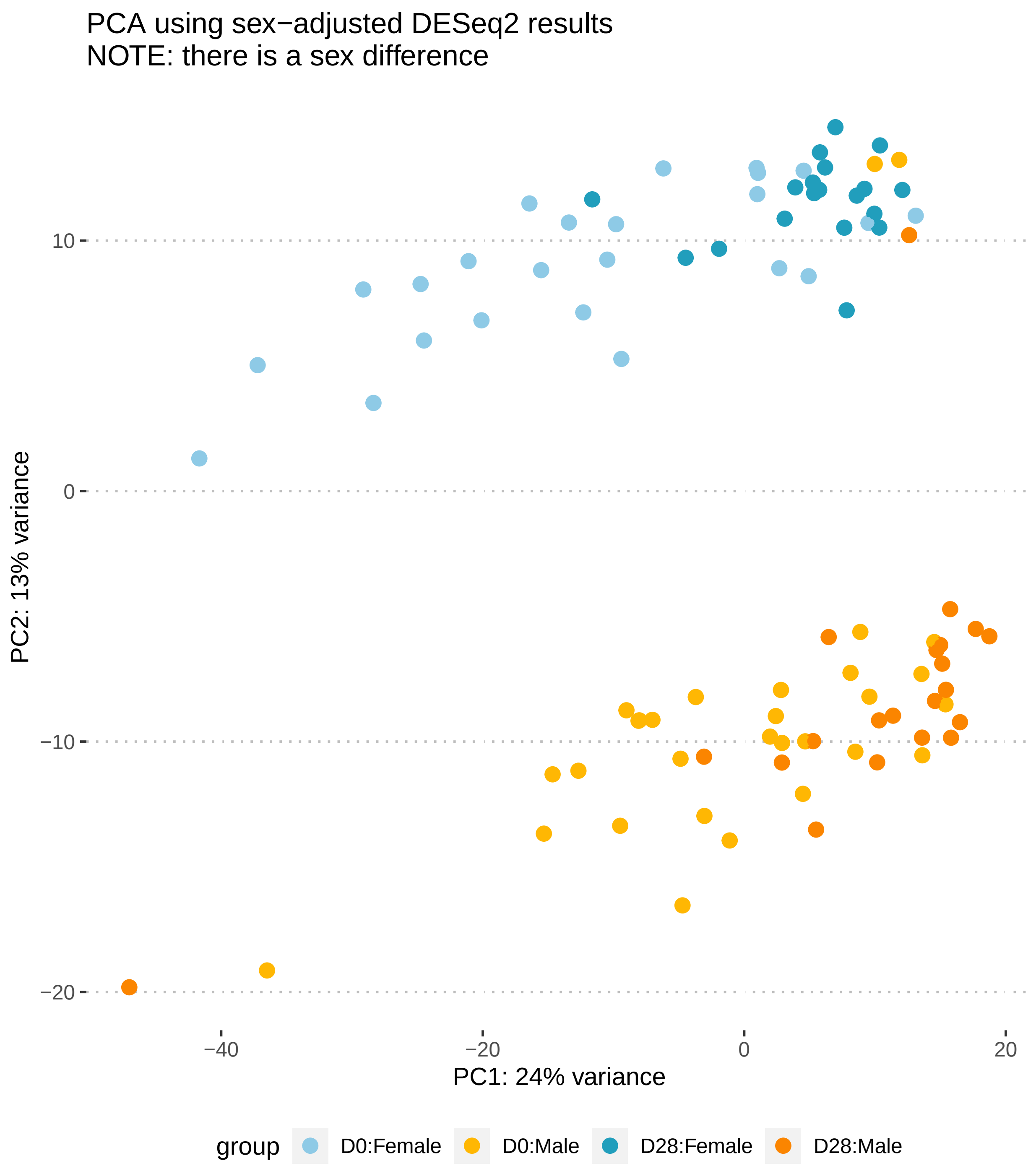
```
# Input data
#dds.sex
#si.mt.comp.dt

## Stabilize the data using variance stabilizing transformation
vsd.sex <- vst(object = dds.sex)
vsd.sex
```

```
## class: DESeqTransform
## dim: 16069 94
## metadata(1): version
## assays(1): ''
## rownames(16069): ENSG00000187961.15|KLHL17 ENSG00000188976.11|NOC2L ...
##   ENSG00000012817.16|KDM5D ENSG00000198692.10|EIF1AY
## rowData names(27): baseMean baseVar ... replace dispFit
## colnames(94): D0_02 D0_06 ... D28_94 D28_98
## colData names(9): IGU_Code sex ... sizeFactor replaceable
```

```
## Use native DESeq PCA plotting capabilities
#?plotPCA
plotPCA(object = vsd.sex, intgroup = c("time","sex"))
```



```
## Modify the plot by saving into object and adjusting the ggplot parameters within it
## -> https://coolors.co/
## -> above is a great website for color choosing

## number of colors should match the number of conditions
four_colors <- c("#8ecae6","#ffb703","#219ebc","#fb8500")

## Use the ggplot capabilities to make nicer custom plot
pca.plot <- plotPCA(object = vsd.sex, intgroup = c("time","sex"))
pca.plot + theme_pubclean() +
  geom_point(size = 2) +
  ggtitle(paste0("PCA using sex-adjusted DESeq2 results\n",
                 "NOTE: there is a sex difference")) +
  scale_colour_manual(values = four_colors) +
  theme(aspect.ratio = 1,
        legend.position = "bottom")
```



```
##
## PCA on dds (no sex adjustment)
## -> The PCA looks the same, however, the resulting genes are different due to different model [~time+
## Stabilize the data
#vsd <- vst(object = dds)

## Plot similar plot using custom ggplot
#pca.plot <- plotPCA(object = vsd, intgroup = c("time","sex"))
#pca.plot + theme_pubclean() +
#  geom_point(size = 2) +
#  ggtitle(paste0("PCA using NOT-adjusted for sex DESeq2 results\n",
#                 "NOTE: there is a sex difference")) +
#  scale_colour_manual(values = four_colors) +
#  theme(aspect.ratio = 1,
#        legend.position = "right")
```

## 2. Plot Boxplot of the normalized counts

- It can be an important control or piece of data to look at the distribution of expression of a particular gene in your data
- To do so we extract the normalized counts (or use TPM data) and use boxplot that summarized number of imporatant statistics including median, quantiles and outliers

```
## Input
#dds.sex
#si.mt.comp.dt

## Extract normalized count data
# ?counts <- function that extracts normalized data from dds object
ncount.dt <- as.data.table(counts(dds.sex, normalized=TRUE), keep.rownames = TRUE)
colnames(ncount.dt) <- gsub("rn","gene_id",colnames(ncount.dt))
ncount.dt[1:5,1:5]
```

```
##                               gene_id      D0_02      D0_06      D0_100     D0_11
## 1:         ENSG00000187961.15|KLHL17   148.5576  157.58938 122.268608 147.48129
## 2:          ENSG00000188976.11|NOC2L   417.3295  460.64587 276.975826 438.66230
## 3: ENSG00000272512.1|ENSG00000272512   353.8016   55.76239  14.971666  45.37886
## 4:           ENSG00000188290.11|HES4  1306.7203  489.73929   7.485833  75.63143
## 5:          ENSG00000187608.10|ISG15 11441.8660 5696.24984 262.004160 567.23573
```

```
## Re-arrange the table and get gene names
ncount.dtm <- melt.data.table(data = ncount.dt, id.vars = "gene_id",
                              variable.name = "subject",
                              value.name = "ncount")
ncount.dtm[,"gene_name" := tstrsplit(gene_id,split="\\|",keep = 2)]
ncount.dtm
```

```
##                                      gene_id subject     ncount       gene_name
##       1:         ENSG00000187961.15|KLHL17   D0_02   148.5576          KLHL17
##       2:          ENSG00000188976.11|NOC2L   D0_02   417.3295           NOC2L
##       3: ENSG00000272512.1|ENSG00000272512   D0_02   353.8016 ENSG00000272512
##       4:           ENSG00000188290.11|HES4   D0_02  1306.7203            HES4
##       5:          ENSG00000187608.10|ISG15   D0_02 11441.8660           ISG15
##      ---
## 1510482:         ENSG00000215580.12|BCORP1  D28_98   486.9312          BCORP1
## 1510483:          ENSG00000131002.14|TXLNGY  D28_98  3584.4561          TXLNGY
## 1510484: ENSG00000260197.1|ENSG00000260197  D28_98   181.7779 ENSG00000260197
## 1510485:           ENSG00000012817.16|KDM5D  D28_98  3306.3140           KDM5D
## 1510486:          ENSG00000198692.10|EIF1AY  D28_98   617.6069          EIF1AY
```

```
## Combine the normalized counts with metadata
ncount.dtm <- ncount.dtm[si.mt.comp.dt,on=.(subject=seq_id)]
ncount.dtm[1:5]
```

```
##                              gene_id subject     ncount       gene_name
## 1:         ENSG00000187961.15|KLHL17   D0_02   148.5576          KLHL17
## 2:          ENSG00000188976.11|NOC2L   D0_02   417.3295           NOC2L
## 3: ENSG00000272512.1|ENSG00000272512   D0_02   353.8016 ENSG00000272512
## 4:           ENSG00000188290.11|HES4   D0_02  1306.7203            HES4
## 5:          ENSG00000187608.10|ISG15   D0_02 11441.8660           ISG15
##    IGU_Code    sex   pathogen disease time i.subject
## 1:    02_D0 Female SARS-CoV-2 COVID19   D0        02
## 2:    02_D0 Female SARS-CoV-2 COVID19   D0        02
## 3:    02_D0 Female SARS-CoV-2 COVID19   D0        02
## 4:    02_D0 Female SARS-CoV-2 COVID19   D0        02
## 5:    02_D0 Female SARS-CoV-2 COVID19   D0        02
```

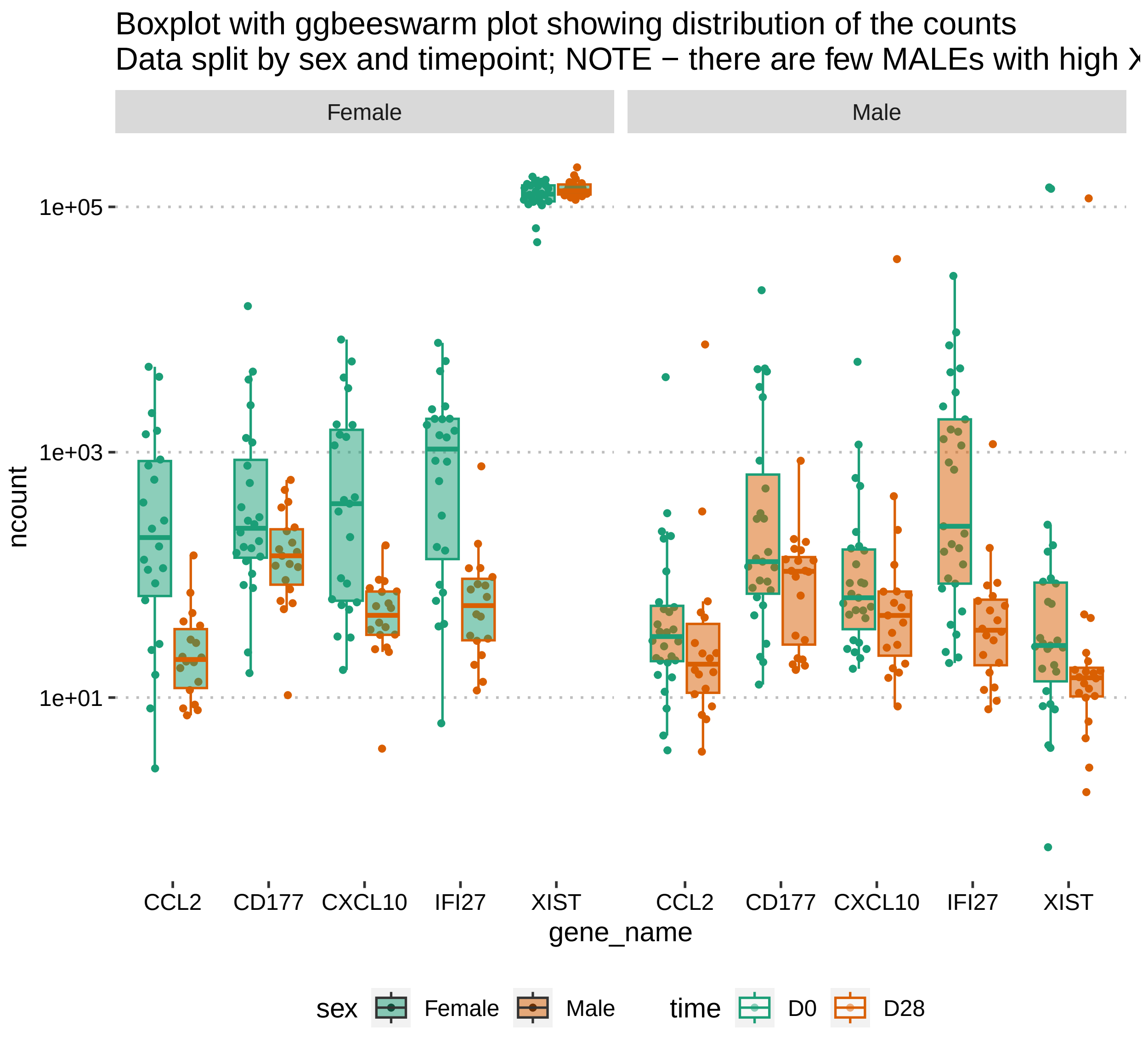
```
## select genes of interest
goi <- c("IFI27","CCL2","CD177","XIST","CXCL10")

## subset the count table
ncount.goi.dtm <- ncount.dtm[gene_name %in% goi]
ncount.goi.dtm
```

```
##                         gene_id subject       ncount gene_name IGU_Code    sex
##   1: ENSG00000169245.6|CXCL10   D0_02   4062.85445    CXCL10    02_D0 Female
##   2: ENSG00000165949.13|IFI27   D0_02    581.52475     IFI27    02_D0 Female
##   3:  ENSG00000108691.10|CCL2   D0_02   1400.54617      CCL2    02_D0 Female
##   4: ENSG00000204936.10|CD177   D0_02     78.18820     CD177    02_D0 Female
##   5:  ENSG00000229807.13|XIST   D0_02 142701.28745      XIST    02_D0 Female
##  ---
## 466: ENSG00000169245.6|CXCL10  D28_98     73.00318    CXCL10   98_D28   Male
## 467: ENSG00000165949.13|IFI27  D28_98      8.03035     IFI27   98_D28   Male
## 468:  ENSG00000108691.10|CCL2  D28_98     16.79073      CCL2   98_D28   Male
## 469: ENSG00000204936.10|CD177  D28_98     20.44089     CD177   98_D28   Male
## 470:  ENSG00000229807.13|XIST  D28_98     44.53194      XIST   98_D28   Male
##        pathogen disease time i.subject
##   1: SARS-CoV-2 COVID19   D0        02
##   2: SARS-CoV-2 COVID19   D0        02
##   3: SARS-CoV-2 COVID19   D0        02
##   4: SARS-CoV-2 COVID19   D0        02
##   5: SARS-CoV-2 COVID19   D0        02
##  ---
## 466: SARS-CoV-2 COVID19  D28        98
## 467: SARS-CoV-2 COVID19  D28        98
## 468: SARS-CoV-2 COVID19  D28        98
## 469: SARS-CoV-2 COVID19  D28        98
## 470: SARS-CoV-2 COVID19  D28        98
```

```
## Boxplot with all the points
ggplot() + theme_pubclean() +
```

```r
  # plots all the points
  geom_quasirandom(data = ncount.goi.dtm,
                   aes(x = gene_name, y = ncount,
                       fill = sex, colour = time),
                   dodge.width = 0.8, size = 1) +
  geom_boxplot(data = ncount.goi.dtm,
               aes(x = gene_name, y = ncount,
                   fill = sex, colour = time),
               alpha = 0.5, outlier.shape = NA) +
  ggtitle(paste0("Boxplot with ggbeeswarm plot showing distribution of the counts\n",
                 "Data split by sex and timepoint; NOTE - there are few MALEs with high XIST expression
  scale_colour_brewer(palette = "Dark2") +
  scale_fill_brewer(palette = "Dark2") +
  # use wrap to conviniently re-arrange results
  facet_wrap(~sex) +
  scale_y_log10() +
  theme(aspect.ratio = 1.5,
        axis.text = element_text(colour = "black"),
        legend.position = "bottom")
```

```
## Warning: Transformation introduced infinite values in continuous y-axis
## Transformation introduced infinite values in continuous y-axis

## Warning: Removed 26 rows containing non-finite values (`stat_boxplot()`).

## Warning: Removed 26 rows containing missing values (`geom_point()`).
```



```
## NOTE: PRACTICE - Try identifying and plotting sex-specific
##      genes that could help distinguish genetic sex
```

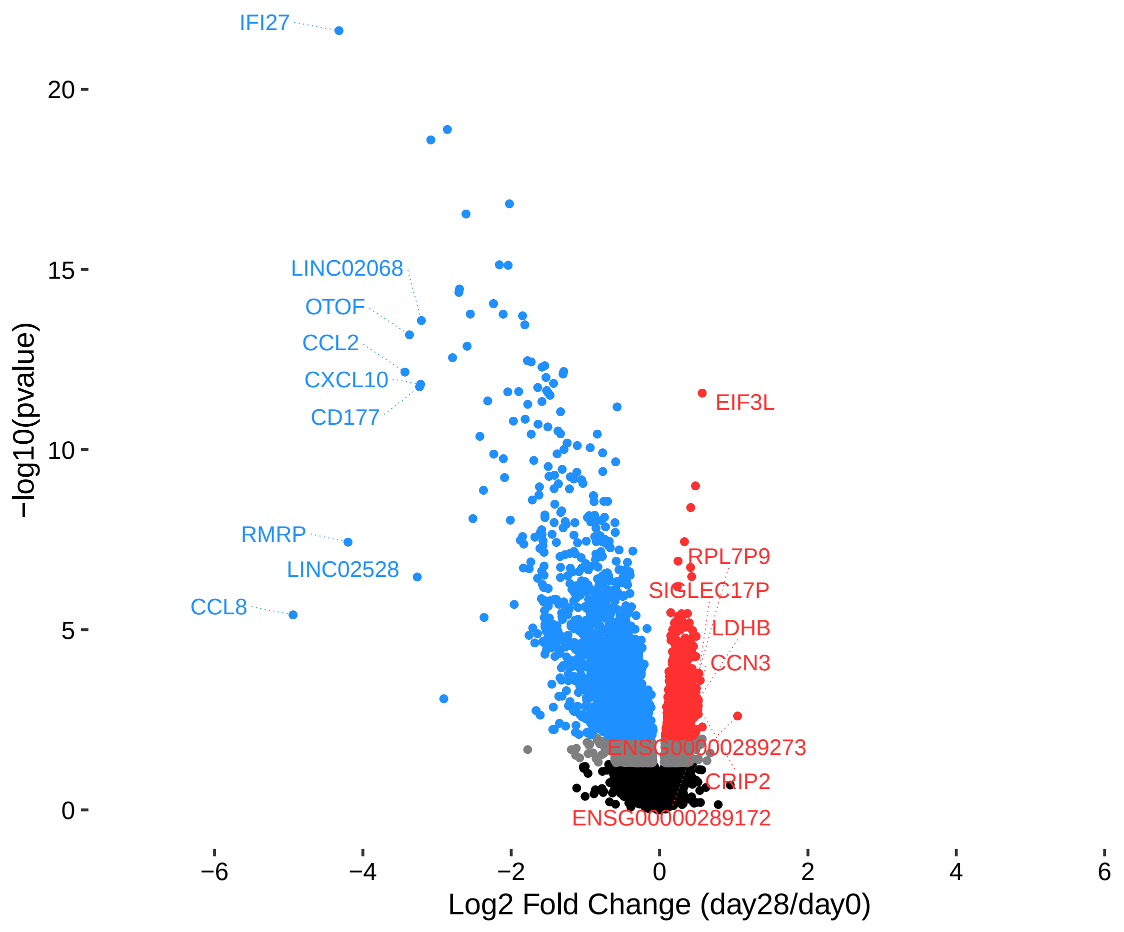
## 3. Volcano plot

- Volcanos are a common way to show overall change in gene expression in comparison of two conditions
- They combine statistical information with directional expression change information
- It is also nice to highlight few genes of interest on these plots

```r
## Input data
#res.sex.dt

## Sig. up and down
## NOTE: ideally by padj value and can also be done by pvalue
res.up.dt <- res.sex.dt[padj <= 0.05][log2FoldChange > 0][order(-log2FoldChange)][1:10]
res.dn.dt <- res.sex.dt[padj <= 0.05][log2FoldChange < 0][order(log2FoldChange)][1:10]

## Volcano
ggplot() + theme_pubclean() +
  # plot non-significant points
  geom_point(data = res.sex.dt[pvalue > 0.05],
             aes(x = log2FoldChange, y = -log10(pvalue)),
             size=1, colour = "black") +

  # plot points by significant pvalue
  geom_point(data = res.sex.dt[pvalue <= 0.05],
             aes(x = log2FoldChange, y = -log10(pvalue)),
             size=1, colour = "grey50") +

  # plot only top significant - INCREASED
  geom_point(data = res.sex.dt[padj <= 0.05][log2FoldChange > 0],
             aes(x = log2FoldChange, y = -log10(pvalue)),
             size=1, colour = "firebrick1") +

  # add labels
  geom_text_repel(data = res.up.dt,
             aes(x = log2FoldChange, y = -log10(pvalue),
                 label = unlist(tstrsplit(gene_id,split="\\|",keep = 2))),
             size=3, colour = "firebrick1", segment.linetype = "dotted",
             nudge_x = 1,
             direction = "y",
             force = 2,
             force_pull = NA,
             vjust=1,
             hjust=1,
             segment.size = 0.2) +

  # plot only top signficant - DECREASED
  geom_point(data = res.sex.dt[padj <= 0.05][log2FoldChange < 0],
             aes(x = log2FoldChange, y = -log10(pvalue)),
             size=1, colour = "dodgerblue") +

  # add labels
  geom_text_repel(data = res.dn.dt,
             aes(x = log2FoldChange, y = -log10(pvalue),
                 label = unlist(tstrsplit(gene_id,split="\\|",keep = 2))),
             size=3, colour = "dodgerblue", segment.linetype = "dotted",
             nudge_x = -1,
             force = 3,
             force_pull = NA,
             vjust=0,
             direction = "y",
             segment.size = 0.2) +

  # add scales and extras
  scale_x_continuous(limits = c(-7,7), breaks = seq(-10,10,2)) +
  ggtitle(paste0("Volcano showing results of DGE analysis comparing d28 versus d0\n",
                 "red/blue indicate genes significantly changed in this comparison\n",
                 "many inflamatory markers decrease by 28 days")) +
  xlab("Log2 Fold Change (day28/day0)") +
  theme(aspect.ratio = 0.75,
        panel.grid.major.y = element_blank(),
        axis.text = element_text(colour = "black"))
```

```
## Warning: Removed 2 rows containing missing values (`geom_point()`).

## Warning: Removed 1 rows containing missing values (`geom_point()`).

## Warning: Removed 1 rows containing missing values (`geom_text_repel()`).

## Warning: Removed 1 rows containing missing values (`geom_point()`).

## Warning: Removed 1 rows containing missing values (`geom_text_repel()`).

## Warning: ggrepel: 1 unlabeled data points (too many overlaps). Consider
## increasing max.overlaps
```

Volcano showing results of DGE analysis comparing d28 versus d0
red/blue indicate genes significantly changed in this comparison
many inflamatory markers decrease by 28 days



## 4. Gene Set Enrichemnt Analysis (GSEA)

- After identification of diferentially changing genes, it is very informative to determine whether these changes amount to any systemic / pathway-specific changes
- It is much easier to interpret results of DGE in terms of pathway incread of single gene - such analysis has more significance
- To do this we extract significantly changing genes from DESeq results
  - When there are too few *padj* significant genes it is possible to do GSEA with genes that pass *pvalue* significance
  - If there are not signifcant genes even by pvalue, analysis really looses significance

```
## input data
#res.sex.dt

## First order the results by the log2FolChange from increasing to decreasing
res.sex.dt <- res.sex.dt[order(-log2FoldChange)]
res.sex.dt
```

```
##                                  gene_id    baseMean log2FoldChange     lfcSE
##     1: ENSG00000269693.1|ENSG00000269693    16.47864     24.8272814 2.9422018
##     2: ENSG00000289273.1|ENSG00000289273    51.07667      1.0517758 0.3475690
##     3:           ENSG00000253755.1|IGHGP    52.29388      0.9532807 0.7521117
##     4:      ENSG00000261796.1|ISY1-RAB43    65.39494      0.7907526 2.1478173
##     5:        ENSG00000184702.20|SEPTIN5    96.50035      0.6822781 0.3072850
##    ---
## 16065:           ENSG00000108691.10|CCL2   266.02706     -3.4328214 0.4781748
## 16066:            ENSG00000269900.3|RMRP 19150.27418     -4.2001709 0.7630199
## 16067:          ENSG00000165949.13|IFI27  1174.00428     -4.3219911 0.4443898
## 16068:            ENSG00000108700.5|CCL8    69.89154     -4.9407004 1.0697586
## 16069: ENSG00000215472.10|RPL17-C18orf32    15.85758    -10.6915338 2.6299579
##              stat       pvalue         padj
##     1:  8.4383340 3.218926e-17 8.620821e-14
##     2:  3.0260916 2.477372e-03 2.112998e-02
##     3:  1.2674723 2.049865e-01 3.949952e-01
##     4:  0.3681657 7.127497e-01 8.347187e-01
##     5:  2.2203429 2.639550e-02 1.031742e-01
##    ---
## 16065: -7.1790102 7.021789e-13 4.339736e-10
## 16066: -5.5046674 3.698659e-08 4.571827e-06
## 16067: -9.7256768 2.343417e-22 3.765638e-18
## 16068: -4.6185189 3.864887e-06 1.996941e-04
## 16069: -4.0652870 4.797338e-05 1.265820e-03
```

```
## Make sure gene_name is available, if not extract it to new column
res.sex.dt[,"gene_name" := tstrsplit(gene_id,split="\\|",keep = 2)]
res.sex.dt
```

```
##                                  gene_id    baseMean log2FoldChange     lfcSE
##     1: ENSG00000269693.1|ENSG00000269693    16.47864     24.8272814 2.9422018
##     2: ENSG00000289273.1|ENSG00000289273    51.07667      1.0517758 0.3475690
##     3:           ENSG00000253755.1|IGHGP    52.29388      0.9532807 0.7521117
##     4:      ENSG00000261796.1|ISY1-RAB43    65.39494      0.7907526 2.1478173
##     5:        ENSG00000184702.20|SEPTIN5    96.50035      0.6822781 0.3072850
##    ---
## 16065:           ENSG00000108691.10|CCL2   266.02706     -3.4328214 0.4781748
## 16066:            ENSG00000269900.3|RMRP 19150.27418     -4.2001709 0.7630199
## 16067:          ENSG00000165949.13|IFI27  1174.00428     -4.3219911 0.4443898
## 16068:            ENSG00000108700.5|CCL8    69.89154     -4.9407004 1.0697586
## 16069: ENSG00000215472.10|RPL17-C18orf32    15.85758    -10.6915338 2.6299579
##              stat       pvalue         padj       gene_name
##     1:  8.4383340 3.218926e-17 8.620821e-14 ENSG00000269693
##     2:  3.0260916 2.477372e-03 2.112998e-02 ENSG00000289273
##     3:  1.2674723 2.049865e-01 3.949952e-01           IGHGP
##     4:  0.3681657 7.127497e-01 8.347187e-01      ISY1-RAB43
##     5:  2.2203429 2.639550e-02 1.031742e-01         SEPTIN5
##    ---
## 16065: -7.1790102 7.021789e-13 4.339736e-10            CCL2
## 16066: -5.5046674 3.698659e-08 4.571827e-06            RMRP
## 16067: -9.7256768 2.343417e-22 3.765638e-18           IFI27
## 16068: -4.6185189 3.864887e-06 1.996941e-04            CCL8
## 16069: -4.0652870 4.797338e-05 1.265820e-03  RPL17-C18orf32
```

```r
## check if gene name is unique - duplicates cannot move further in analysis
summary(duplicated(res.sex.dt[["gene_name"]])) # <- there are 49 duplicates here
```

```
##    Mode   FALSE    TRUE
## logical   16020      49
```

```r
##
## Select the genes to use for GSEA
##

## Sets cutoff of significance
res_cutoff <- 0.05

## Since there are more than few hundred sig. genes by padj use those
nrow(res.sex.dt[padj<res_cutoff])
```

```
## [1] 2860
```

```r
## Extract log2FoldChange for these genes into a vector
geneVec <- res.sex.dt[padj <= res_cutoff][["log2FoldChange"]]

## Add names for each gene to the vector
names(geneVec) <- res.sex.dt[padj <= res_cutoff][["gene_name"]]

## Check if vector names are duplicated
summary(duplicated(names(geneVec))) ## YES <- remove duplicates
```

```
##    Mode   FALSE    TRUE
## logical    2847      13
```

```r
## Find the duplicates and their names
geneVec[duplicated(names(geneVec))]
```

```
##      P2RY8    SLC25A6         U2         U2         U2         U2         U2
##  0.2167833  0.1981591 -0.6324921 -0.6435656 -0.6443804 -0.6516084 -0.6577563
##         U2         U2         U2         U2   5_8S_rRNA  5_8S_rRNA
## -0.6651420 -0.6855793 -0.6930374 -0.7116059 -0.7417669 -0.7784530
```

```r
dup_names <- names(geneVec[duplicated(names(geneVec))])
geneVec[names(geneVec) %in% dup_names]
```

```
##    SLC25A6      P2RY8      P2RY8    SLC25A6         U2         U2         U2
##  0.2623454  0.2170988  0.2167833  0.1981591 -0.5367835 -0.6324921 -0.6435656
##         U2         U2         U2         U2         U2         U2         U2
## -0.6443804 -0.6516084 -0.6577563 -0.6651420 -0.6855793 -0.6930374 -0.7116059
##  5_8S_rRNA  5_8S_rRNA  5_8S_rRNA
## -0.7300136 -0.7417669 -0.7784530
```

```
## For now remove duplicates BUT - work to avoid having duplicate gene names
geneVec <- geneVec[!duplicated(names(geneVec))]


##
## Use the msigdbr package to load gene lists to compare with
##

msig.H.dt <- as.data.table(msigdbr(species = "Homo sapiens", category = "H"))
msig.H.dt[1:5,]
```

```
##    gs_cat gs_subcat               gs_name gene_symbol entrez_gene
## 1:      H           HALLMARK_ADIPOGENESIS       ABCA1          19
## 2:      H           HALLMARK_ADIPOGENESIS       ABCB8       11194
## 3:      H           HALLMARK_ADIPOGENESIS       ACAA2       10449
## 4:      H           HALLMARK_ADIPOGENESIS       ACADL          33
## 5:      H           HALLMARK_ADIPOGENESIS       ACADM          34
##       ensembl_gene human_gene_symbol human_entrez_gene human_ensembl_gene gs_id
## 1: ENSG00000165029             ABCA1                19    ENSG00000165029 M5905
## 2: ENSG00000197150             ABCB8             11194    ENSG00000197150 M5905
## 3: ENSG00000167315             ACAA2             10449    ENSG00000167315 M5905
## 4: ENSG00000115361             ACADL                33    ENSG00000115361 M5905
## 5: ENSG00000117054             ACADM                34    ENSG00000117054 M5905
##     gs_pmid gs_geoid gs_exact_source gs_url
## 1: 26771021
## 2: 26771021
## 3: 26771021
## 4: 26771021
## 5: 26771021
##                                                          gs_description
## 1: Genes up-regulated during adipocyte differentiation (adipogenesis).
## 2: Genes up-regulated during adipocyte differentiation (adipogenesis).
## 3: Genes up-regulated during adipocyte differentiation (adipogenesis).
## 4: Genes up-regulated during adipocyte differentiation (adipogenesis).
## 5: Genes up-regulated during adipocyte differentiation (adipogenesis).
```

```
## Extract only gene_name and pathway name columns
msig.H.t2g <- msig.H.dt[,.SD,.SDcols = c("gs_name","gene_symbol")]
msig.H.t2g
```

```
##                              gs_name gene_symbol
##    1:          HALLMARK_ADIPOGENESIS       ABCA1
##    2:          HALLMARK_ADIPOGENESIS       ABCB8
##    3:          HALLMARK_ADIPOGENESIS       ACAA2
##    4:          HALLMARK_ADIPOGENESIS       ACADL
##    5:          HALLMARK_ADIPOGENESIS       ACADM
##   ---
## 8205: HALLMARK_XENOBIOTIC_METABOLISM        UPB1
## 8206: HALLMARK_XENOBIOTIC_METABOLISM        UPP1
## 8207: HALLMARK_XENOBIOTIC_METABOLISM        VNN1
## 8208: HALLMARK_XENOBIOTIC_METABOLISM         VTN
## 8209: HALLMARK_XENOBIOTIC_METABOLISM         XDH
```

```
##
## Run GSEA
## => Much of the options can found online in the ClusterProfiler manual
##

agsea <- clusterProfiler::GSEA(geneList = geneVec,
                               TERM2GENE = msig.H.t2g,
                               minGSSize = 5, # minimum number of genes to match pathway
                               eps = 0,
                               pvalueCutoff = 1, # this way all pathways are returned
                               pAdjustMethod = "BH", # many other methods are out there
                               by = 'fgsea',
                               seed = TRUE)
```

```
## preparing geneSet collections...
```

```
## GSEA analysis...
```

```
## leading edge analysis...
```

```
## done...
```

```
agsea.dt <- as.data.table(x = agsea)

## Clean up and organize the results table
agsea.dt <- agsea.dt[order(p.adjust)]
agsea.dt[,"sig" := p.adjust<=0.05]
agsea.dt[,"updown" := ifelse(NES<0,"down","up")]
agsea.dt[["ID"]] <- gsub("HALLMARK_","",agsea.dt[["ID"]])
agsea.dt[["ID"]] <- tolower(gsub("_"," ",agsea.dt[["ID"]]))
agsea.dt[["ID"]] <- factor(agsea.dt[["ID"]], levels = agsea.dt[["ID"]])
agsea.dt[["Description"]] <- NULL
agsea.dt[1:4]
```

```
##                           ID setSize enrichmentScore       NES       pvalue
## 1: interferon alpha response      54      -0.6741302 -2.071121 4.925396e-09
## 2: interferon gamma response      88      -0.6114107 -1.930563 6.492678e-09
## 3:     inflammatory response      64      -0.4748478 -1.471669 8.397184e-03
## 4:       pancreas beta cells       7      -0.7123578 -1.540125 1.977316e-02
##        p.adjust       qvalue rank                   leading_edge
## 1: 1.558243e-07 1.469396e-07  620 tags=70%, list=22%, signal=56%
## 2: 1.558243e-07 1.469396e-07  620 tags=57%, list=22%, signal=46%
## 3: 1.343550e-01 1.266944e-01  851 tags=53%, list=30%, signal=38%
## 4: 2.372779e-01 2.237489e-01  377 tags=57%, list=13%, signal=50%
##
## 1:                                                                     SAMD9L/UBE2L6/NUB1/HELZ2/IFI
## 2: SAMD9L/C1R/UBE2L6/VAMP5/MX2/HELZ2/IFI30/TOR1B/LAP3/CDKN1A/TRAFD1/SOCS3/EIF2AK2/MX1/IFIT3/PLSCR1/F
## 3:
## 4:
##      sig updown
## 1:  TRUE   down
## 2:  TRUE   down
## 3: FALSE   down
## 4: FALSE   down
```

```
## FINAL RESULT
agsea.dt[1:4]
```

```
##                            ID setSize enrichmentScore       NES       pvalue
## 1: interferon alpha response      54      -0.6741302 -2.071121 4.925396e-09
## 2: interferon gamma response      88      -0.6114107 -1.930563 6.492678e-09
## 3:     inflammatory response      64      -0.4748478 -1.471669 8.397184e-03
## 4:       pancreas beta cells       7      -0.7123578 -1.540125 1.977316e-02
##        p.adjust       qvalue rank                   leading_edge
## 1: 1.558243e-07 1.469396e-07  620 tags=70%, list=22%, signal=56%
## 2: 1.558243e-07 1.469396e-07  620 tags=57%, list=22%, signal=46%
## 3: 1.343550e-01 1.266944e-01  851 tags=53%, list=30%, signal=38%
## 4: 2.372779e-01 2.237489e-01  377 tags=57%, list=13%, signal=50%
##
## 1:                                                                            SAMD9L/UBE2L6/NUB1/HELZ2/IFI
## 2: SAMD9L/C1R/UBE2L6/VAMP5/MX2/HELZ2/IFI30/TOR1B/LAP3/CDKN1A/TRAFD1/SOCS3/EIF2AK2/MX1/IFIT3/PLSCR1/F
## 3:
## 4:
##      sig updown
## 1:  TRUE   down
## 2:  TRUE   down
## 3: FALSE   down
## 4: FALSE   down
```

## PART 3

Goal: - Explore ways of plotting results from GSEA and DESeq - Other ways of visulizing data

```
ggp.d28.gset.bar <- ggplot() + theme_pubclean() +
  geom_bar(data = agsea.dt,
           aes(x = NES, y = ID, fill = updown),
           stat = "identity") +
   geom_bar(data = agsea.dt[p.adjust <= 0.05],
            aes(x = NES, y = ID), colour = "black",fill=NA,
           stat = "identity") +
  scale_fill_manual(values = c("#427AA1","#CE8D99")) +
  theme(aspect.ratio = 3,
        legend.position = "right",
        axis.text.y = element_text()); ggp.d28.gset.bar
```