Project Bioinformatic Resources

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Introduction

The following analysis focus on RNA-seq count data extracted from different cancer datasets from the Cancer Genome Atlas (TCGA). From the original TCGA data 50 cases (tumor samples) and 50 controls (normal samples) were randomly selected.

Analysis

The packages needed for the analysis are the following:

```
library(tidyverse)
library(biomaRt)
library(MotifDb)  # large collection of motifs across different species
library(seqLogo)
library(PWMEnrich)
library(PWMEnrich.Hsapiens.background)
library(GEOquery)
library(oligo)
library(pd.hg.u133.plus.2)
library(hgu133plus2.db)
library(genefilter)
library(limma)
library(pheatmap)
library(stringr)
library(GenomicFeatures)  # to build object with specific information such as genomic coordinates
library(ggplot2)
library(edgeR)  # designed to perform Differential Expression Analysis from RNA-Seq data
library(fgsea)  # computation of enrichment scores and other statistics
library(org.Hs.eg.db)  # database annotation human specific transcript from Ensembl
library(clusterProfiler)  # uses Fisher test, explore gene list of interest against a reference
library(enrichplot)  # build enrich map
library(ggnewscale)
library(DOSE)
library(pathview)  # contains cartoons of pathways and we project on them our genes of interest
library(igraph)
```

Task 1: Load data

In particular, we consider data coming from **Lung adenocarcinoma**.

```
load("./RData/Lung_adenocarcinoma.RData")
```

After loading the data, the following three data-frames are available:

- `raw_counts_df` which contains the raw RNA-seq counts;
- `c_anno_df`, which contains sample name and condition (case and control);
- `r_anno_df`, which contains the ENSEMBL genes ids, the length of the genes and the genes symbols.

Task 2: Filter protein coding genes

To extract only protein coding genes from `raw_counts_df` and `r_anno_df`, we use the `biomaRt` package.

First, we retrieve the information about the protein coding genes from Ensembl.

```
database <- useMart("ensembl")
datasetHuman <- useDataset("hsapiens_gene_ensembl", mart = database)
query <- getBM(attributes = c("ensembl_gene_id", "external_gene_name", "gene_biotype"),
    filters = c("ensembl_gene_id"), values = r_anno_df$ensembl_gene_id, mart = datasetHuman)

query_protein_coding <- query[which(query$gene_biotype == "protein_coding"), ]
```

Then, we filter the data frames containing the raw counts and the annotation to keep only the protein coding genes.

```
indexes_r_anno_df <- which(r_anno_df$ensembl_gene_id %in% query_protein_coding$ensembl_gene_id)
r_anno_df_protein_coding <- r_anno_df[indexes_r_anno_df, ]

indexes_raw_counts_df <- which(rownames(raw_counts_df) %in% query_protein_coding$ensembl_gene_id)
raw_counts_df_protein_coding <- raw_counts_df[indexes_raw_counts_df, ]
```

Task 3: Differential expression analysis

To perform the differential expression analysis, we will use the `edgeR` package.

It is important to remove genes with low signal that will have low statistical power. Since, we want to focus on the transcripts we can use to perform the analysis, we can filter raw counts data using a threshold of raw count > 20 in at least 5 replicates.

```
# count threshold
count_thr <- 20

# number of replicates with more counts than the count threshold
repl_thr <- 5

filter_vec <- apply(raw_counts_df_protein_coding,1, # go through all count matrices by rows
    function(y) max(by(y, c_anno_df$condition, function(x) sum(x>=count_thr))))
    # groups the values on each condition and sum all the values above the count
```

Then, we filter the previously updated data frames.

```
filter_counts_df <- raw_counts_df_protein_coding[filter_vec >= repl_thr, ]
dim(filter_counts_df)  # 17289
```

```
## [1] 17289   100
```

```
filter_anno_df <- r_anno_df_protein_coding[rownames(filter_counts_df), ]
dim(filter_anno_df)  # 17289
```

```
## [1] 17289     3
```

Now we check the library size of each sample (how many reads we have sequenced for each experiment)

```
size_df <- data.frame(sample = colnames(filter_counts_df), read_millions = colSums(filter_counts_df)/1e

ggplot(data = size_df, aes(sample, read_millions)) + geom_bar(stat = "identity",
    fill = "indianred", colour = "indianred", width = 0.7, alpha = 0.7) + coord_flip() +
    theme_bw()
```

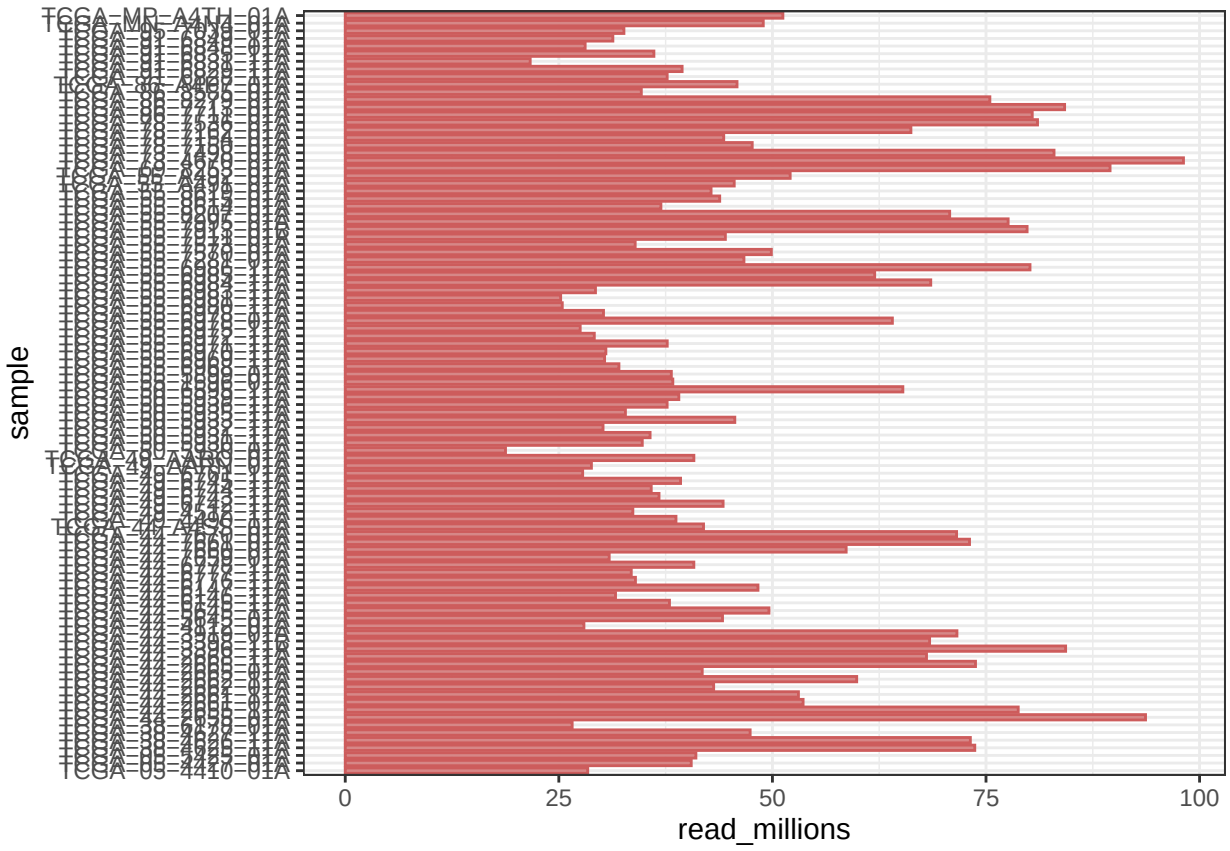


Figure 1: Library size of each sample.

Then, we visualize a boxplot of gene counts.

```
long_counts_df <- gather(as.data.frame(filter_counts_df), key = "sample", value = "read_number")

ggplot(data = long_counts_df, aes(sample, read_number + 1)) + geom_boxplot(colour = "deeppink4",
    fill = "deeppink4", alpha = 0.7) + theme_bw() + scale_y_log10()
```

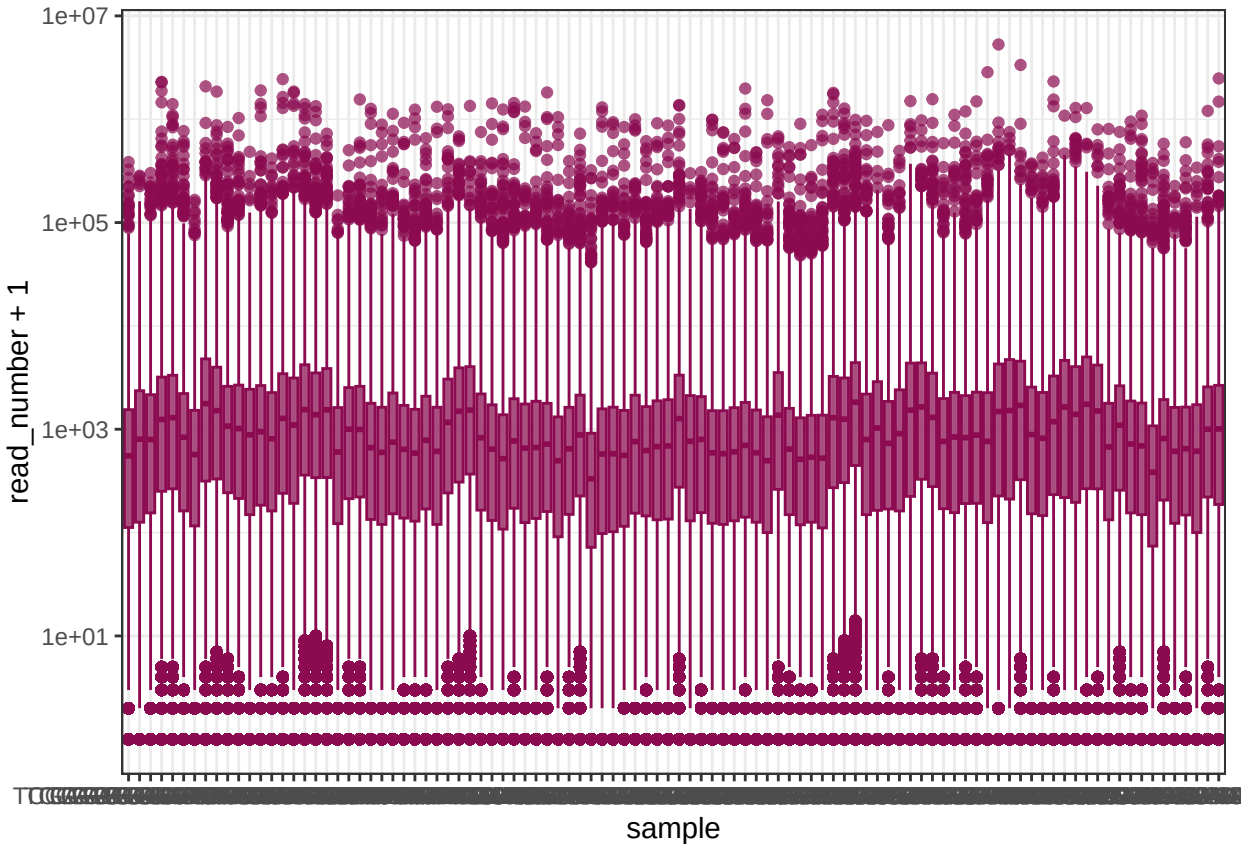


Figure 2: Boxplot of gene counts before normalization.

As we can see from the plots, there is a significant variability across samples in term of library sizes (reads per million). We have to take into account this aspect because the expected size of each count is the product of the relative abundance of that gene in that sample but also of the library size.

As we can see from the boxplot, we need to normalize our data before testing for differential expression. Normalization can be obtained using different methodologies. Among them, TMM (the default method) is a method that consider in the normalization also variables related to the library size.

To perform the DEG analysis, we first need to create a DGRList object containing information about counts, annotation, samples and genes. To do that, we use the function `DGEList` to create the input for the following normalization step. This object contains information about counts, samples and genes.

The normalization intra- and inter-sample is done using the function `calcNormFactors` and specifying `method = "TMM"`, which is the weighted trimmed mean of M-values approach.

```
edge_c <- DGEList(counts = filter_counts_df, group = c_anno_df$condition, samples = c_anno_df,
    genes = filter_anno_df)

# computing norm factors for TMM normalization
edge_n <- calcNormFactors(edge_c, method = "TMM")
```

Then, we create a cpm table containing the normalized expression values for each transcript expressed as counts per million (CPM).

```
# create a cpm table (normalized expression values)
cpm_table <- as.data.frame(round(cpm(edge_n), 2))
```

To see the effect of the normalization, we look at the boxplot distribution of gene expression signals after normalization.

```
# look at the boxplot distribution of gene expression signals after
# normalization
long_cpm_df <- gather(cpm_table, key = "sample", value = "CPM")

ggplot(data = long_cpm_df, aes(sample, CPM + 1)) + geom_boxplot(colour = "olivedrab",
    fill = "olivedrab", alpha = 0.7) + theme_bw() + scale_y_log10()
```

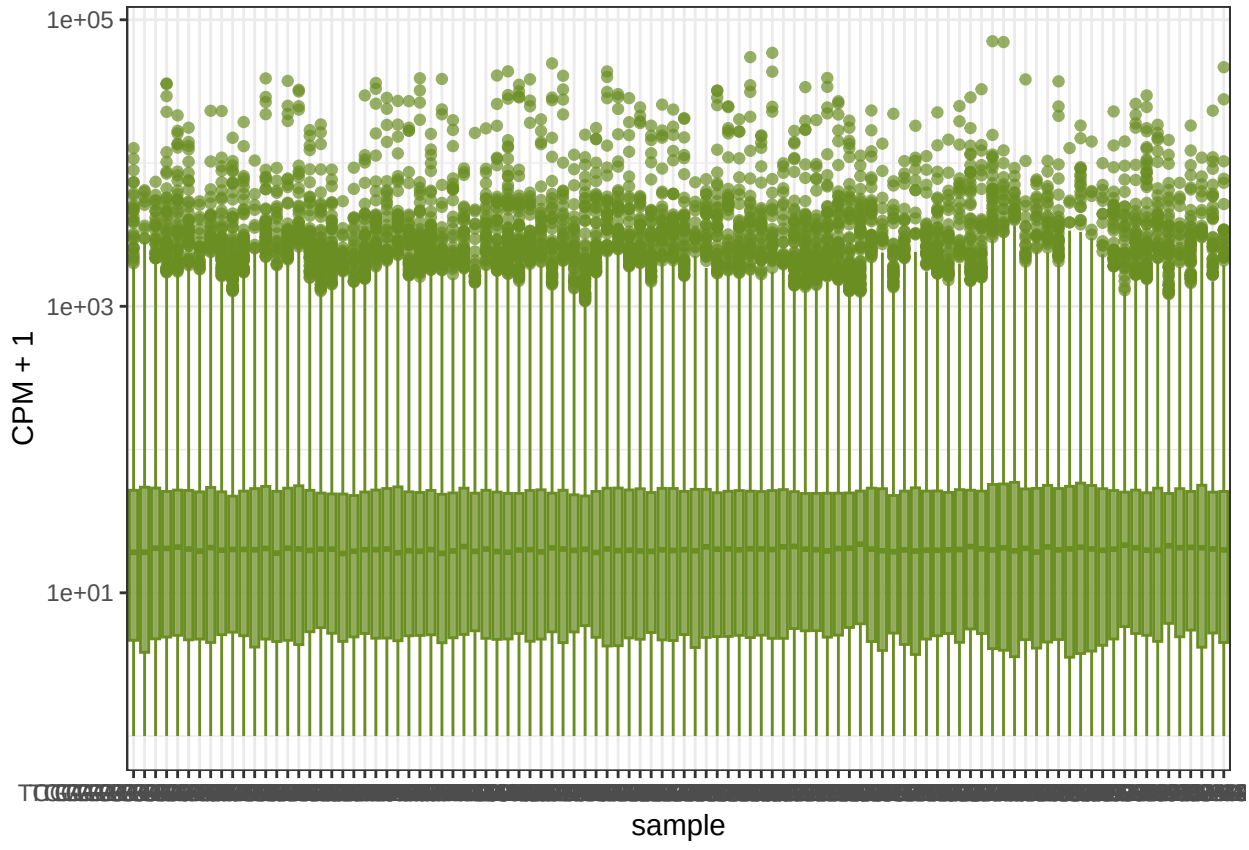


Figure 3: Boxplot distribution of gene expression signals after normalization.

We notice that, with respect to the previous boxplot, after the normalization, the distributions are comparable. That means that now our data is ready to be tested for DE analysis.

We define the experimental design matrix, later needed to calculate the dispersion and fit. This matrix is based on the experimental design, so we define the conditions we want to test (case VS control).

```
design <- model.matrix(~0 + group, data = edge_n$samples)
colnames(design) <- levels(edge_n$samples$group)
rownames(design) <- edge_n$samples$sample
```

Once we normalized the data and the design, we proceed by calculating the dispersion fit.

```
# calculate dispersion and fit with edgeR (necessary for differential
# expression analysis)
edge_d <- estimateDisp(edge_n, design)
edge_f <- glmQLFit(edge_d, design)
```

The `estimateDisp` function tries to estimate the variability at different levels: in the data, inter sample and intra sample. The over dispersion of counts across the samples can be modeled as a Poisson distribution, which can be approximated using a negative binomial distribution by using `glmQLFit` function.

We define the contrasts (conditions to be compared).

```
contro <- makeContrasts("control-case", levels = design)
```

We performed a test through function `glmQLFTest` to determine which genes are differentially expressed. Then, we selected genes based on a 0.01 p-value cutoff and ordered them based on the log2 fold change.

```
# fit the model with generalized linear models
edge_t <- glmQLFTest(edge_f, contrast = contro)
DEGs <- as.data.frame(topTags(edge_t, n = 20000, p.value = 0.01, sort.by = "logFC"))
```

Then, we improve the selection, considering not only the p-value, but also the average expression of the genes (logCPM). We used the logFC value to assign genes to different classes:

- up-regulated genes if $logFC > 1.5$
- down-regulated genes if $logFC < -1.5$
- not significant genes (unchanged) otherwise.

```
DEGs$class <- "="
DEGs$class[which(DEGs$logCPM > 1 & DEGs$logFC > 1.5 & DEGs$FDR < 0.25)] = "+"  # upregulated genes
DEGs$class[which(DEGs$logCPM > 1 & DEGs$logFC < (-1.5) & DEGs$FDR < 0.25)] = "-"  # downregulated genes
DEGs <- DEGs[order(DEGs$logFC, decreasing = T), ]
```

```
head(DEGs)
```

```
##                 ensembl_gene_id external_gene_name length    logFC    logCPM
## ENSG00000179914 ENSG00000179914              ITLN1   8640 6.684901 5.8661078
## ENSG00000108576 ENSG00000108576             SLC6A4  41683 6.084705 6.6338451
## ENSG00000180440 ENSG00000180440             SERTM1  23819 5.663190 3.2093449
## ENSG00000108342 ENSG00000108342               CSF3   2452 5.588520 5.2512424
## ENSG00000034971 ENSG00000034971               MYOC  17271 5.397462 1.5811486
## ENSG00000178084 ENSG00000178084              HTR3C   7626 5.325742 0.5517057
##                          F       PValue          FDR class
## ENSG00000179914  75.57703 6.388727e-14 4.459213e-13     +
## ENSG00000108576 122.16543 3.992071e-19 7.518401e-18     +
```

```
## ENSG00000180440 181.47838 2.383659e-24 1.325115e-22     +
## ENSG00000108342  90.91727 8.943402e-16 8.552128e-15     +
## ENSG00000034971 170.30732 1.866103e-23 8.293843e-22     +
## ENSG00000178084 120.55392 5.781068e-19 1.046585e-17     =
```

```
table(DEGs$class)  # 1193-, 877+, 14949=
```

```
##
##    -    +    =
## 1199  884 9258
```

From the results, we saw that 1193 genes are down-regulated, 877 genes are up-regulated and 14949 genes have no changes in their regulation.

We then create the volcano plot.

```
input_df <- DEGs
xlabel <- "log2 FC case vs control"
ylabel <- "-log10 p-value"

par(fig = c(0, 1, 0, 1), mar = c(4, 4, 1, 2), mgp = c(2, 0.75, 0))
plot(input_df$logFC, -log(input_df$PValue, base = 10), xlab = xlabel, ylab = ylabel,
    col = ifelse(input_df$class == "=", "grey70", "olivedrab4"), pch = 20, frame.plot = TRUE,
    cex = 0.8, main = "Volcano plot")
abline(v = 0, lty = 2, col = "grey20")
```

The volcano plot allows to have a quick visual identification of genes with large fold changes that are also statistically significant.

We also report the heatmap of only up and down-regulated genes.

To create an annotated heatmap focusing only on up- and down-regulated genes we need first of all a matrix in which we select genes with class "+" or "-. Moreover, we also need to assign a color to each sample. In this case, we assign green to the case condition and beige to the control, and save the corresponding color into the variable `cols`. In this way, we can then set the `ColSideColors` parameter in order to have a color code to recognize the sample condition.

```
# plot only up and down regulated genes
cols <- c(ifelse(c_anno_df$condition == "case", "chartreuse4", "burlywood3"))
pal <- c("blue", "white", "red")
pal <- colorRampPalette(pal)(100)
heatmap(as.matrix(cpm_table[which(rownames(cpm_table) %in% DEGs$ensembl_gene_id[which(DEGs$class !=
    "=")]), ]), ColSideColors = cols, cexCol = 0.5, margins = c(4, 4), col = pal,
    cexRow = 0.2)
```

On the top of the heatmap, we can see the dendrogram indicating how distant our samples are, while on the left the dendrogram related to genes. The dendogram is built by hierarchical clustering, a method based on the concept of dissimilarity.

To simplify the later analysis, we save the list of differentially expressed genes in a text file.

```
up_DEGs <- DEGs[which(DEGs$class == "+"), ]
down_DEGs <- DEGs[which(DEGs$class == "-"), ]
```

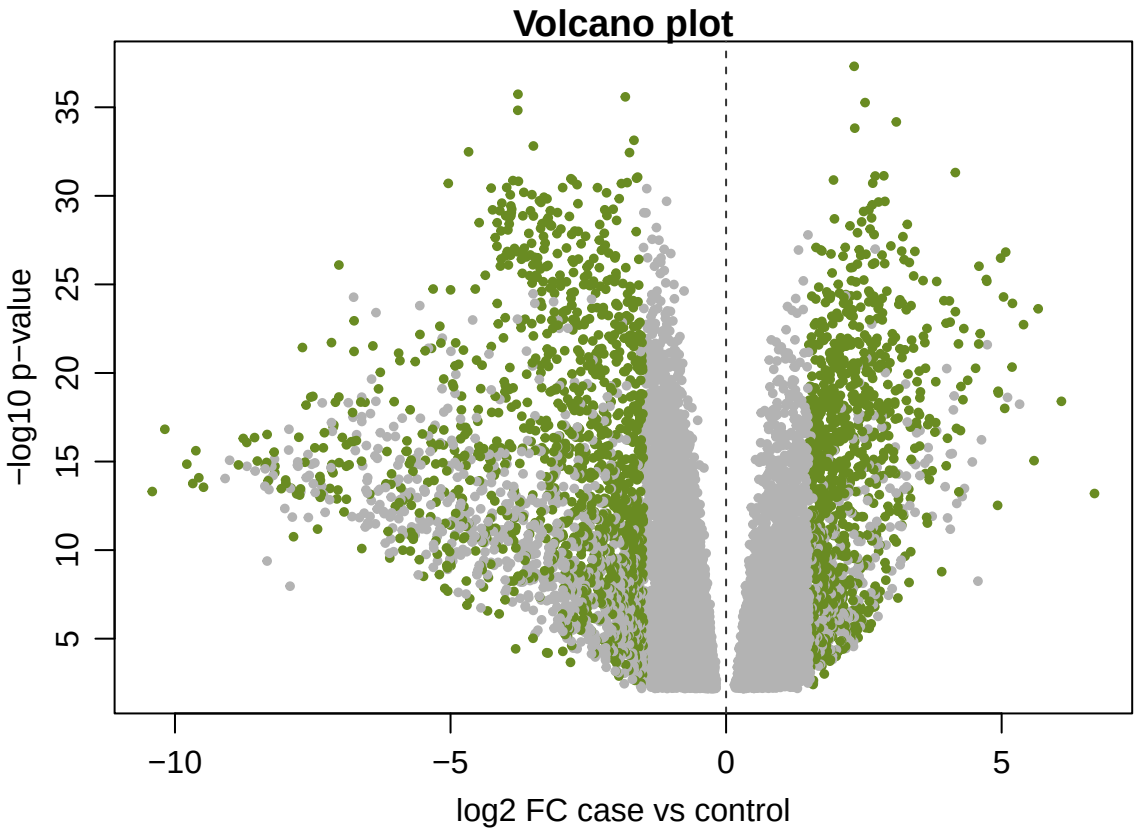


Figure 4: Volcano plot

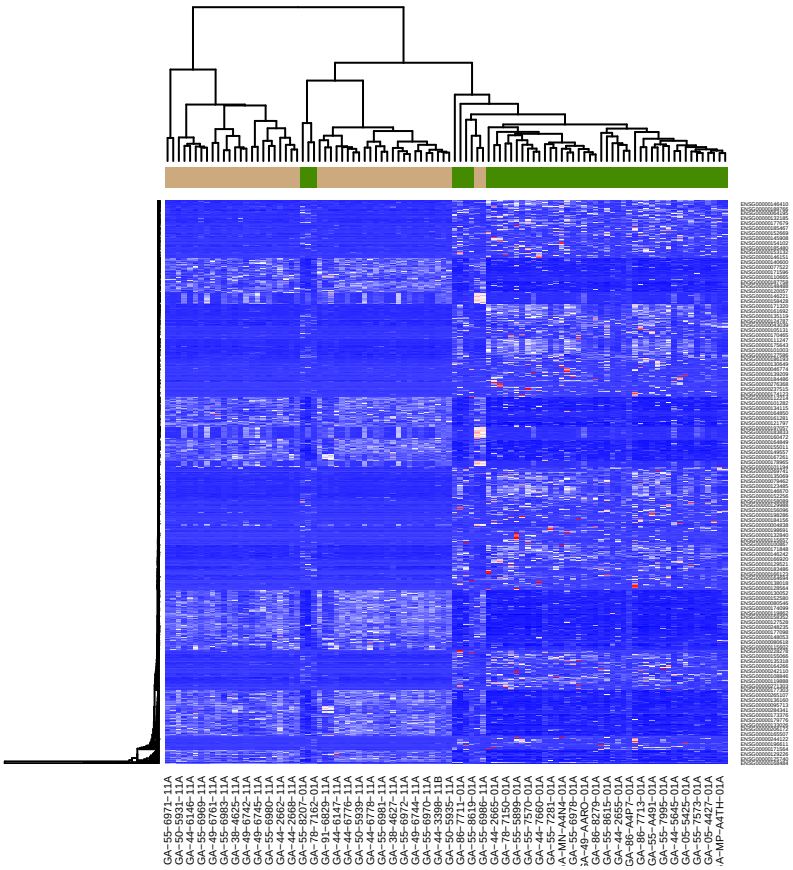


Figure 5: Heatmap up and down-regulated genes.

```
write.table(up_DEGs, file = "output/up_DEGs.txt", row.names = F, col.names = T, sep = "\t",
    quote = F)
write.table(down_DEGs, file = "output/down_DEGs.txt", row.names = F, col.names = T,
    sep = "\t", quote = F)
write.table(DEGs, file = "output/DEGs.txt", row.names = F, col.names = T, sep = "\t",
    quote = F)
```

Task 4: Gene set enrichment analysis

To perform the gene set enrichment analysis, we will use the `clusterProfiler` package.

```
DEGs <- read.table("output/DEGs.txt", header = T, sep = "\t", as.is = T)
table(DEGs$class)
```

```
##
##    -    +    =
## 1199  884 9258
```

We use the `biomaRt` package to retrieve the `entrezgene_id` for all the genes in the `DEGs` dataset.

```
ensembl <- useEnsembl(biomart = "ensembl", dataset = "hsapiens_gene_ensembl")
convert <- getBM(attributes = c("ensembl_gene_id", "entrezgene_id"), filters = c("ensembl_gene_id"),
    values = DEGs$ensembl_gene_id, mart = ensembl)

DEGs <- merge(DEGs, convert, by.x = "ensembl_gene_id", by.y = "ensembl_gene_id")  # include the new inf
DEGs <- DEGs[which(!is.na(DEGs$entrezgene_id)), ]
DEGs <- DEGs[-which(duplicated(DEGs$entrezgene_id)), ]
```

We removed all the NA and duplicates in the dataset DEGs.

We created new lists for the down and up-regulated genes.

```
# list of up-regulated genes
upDEGs <- DEGs %>%
    filter(class == "+")
# list of doum-regulated genes
downDEGs <- DEGs %>%
    filter(class == "-")
```

Performing enrichment analysis for GO biological process

```
# biological process up regulated genes
ego_BP_up <- enrichGO(gene = upDEGs$external_gene_name, OrgDb = org.Hs.eg.db, keyType = "SYMBOL",
    ont = "BP", pAdjustMethod = "BH", pvalueCutoff = 0.05, qvalueCutoff = 0.05)
```

```
# biological process doum regulated genes
ego_BP_down <- enrichGO(gene = downDEGs$external_gene_name, OrgDb = org.Hs.eg.db,
    keyType = "SYMBOL", ont = "BP", pAdjustMethod = "BH", pvalueCutoff = 0.05, qvalueCutoff = 0.05)
```

We report the top 10 enriched GO terms related to the Biological Process for both up and down regulated genes.
In the barplots we can see that the elements are ordered by adjusted p-value (where the most significant is placed on the top) and on the x-axis we have the gene counts, so the number of elements of our lists were found in the category.

```
barplot(ego_BP_up, showCategory = 10, main = "Up-regulated gene list: top 10 enriched BP terms")
```

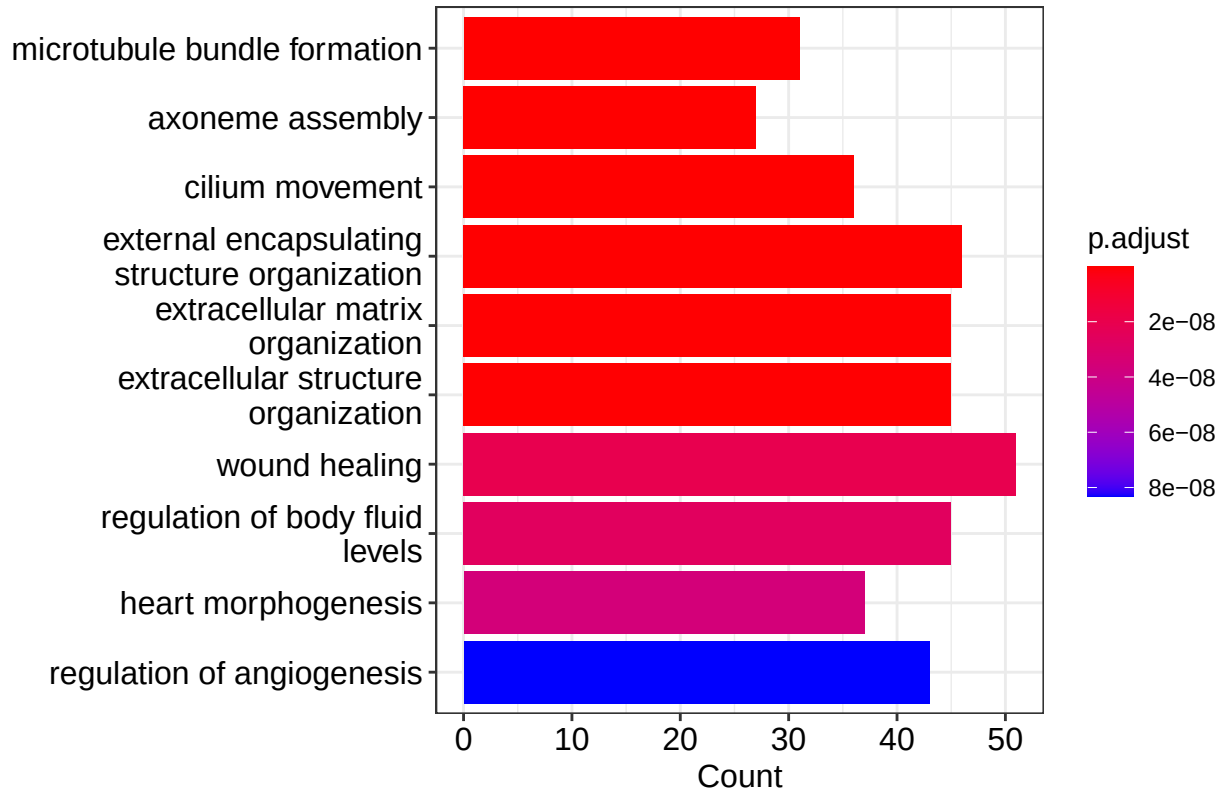


Figure 6: Biological process up regulated genes

Both the first two biological processes are related to the microtubules, which might be explained by the fact that tumour cells have a high growth rate thus the cytoskeleton is assembled over and over again. We also notice that angiogenesis is among the most enriched biological processes as we expected. In fact, angiogenesis is particularly important in tumorigenesis to allow the growth of the tumour tissue, providing nutrients to cells.

```
barplot(ego_BP_down, showCategory = 10, main = "Down-regulated gene list: top 10 enriched BP terms")
```

We would expect most of these biological processes to be enriched in the up-regulated genes since tumour cells tend to replicate more than normal cells. A possible explaination is that tumour cells genes accumulate numerous mutations usually ending up in loss of function or downregulation. These mutations might occur on transcription factors binding sites or RNA binding sites, reducing their expression.

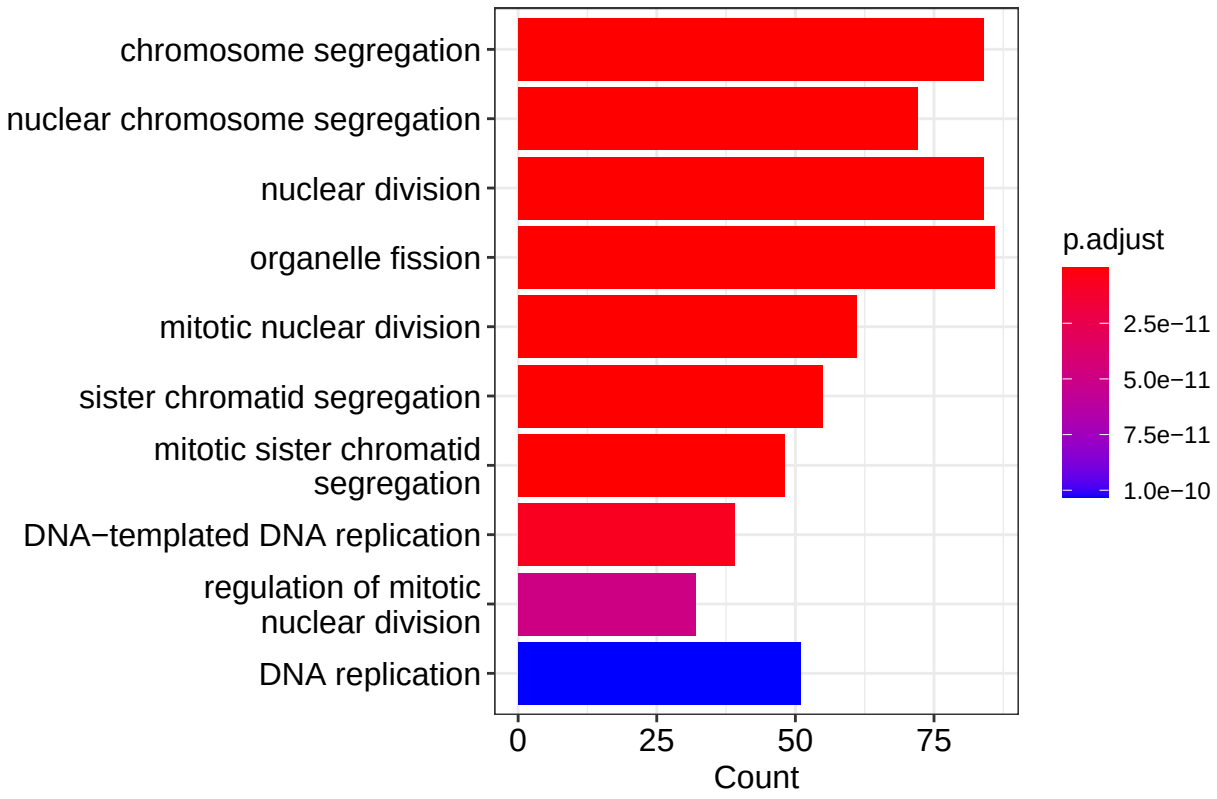


Figure 7: Biological process down regulated genes

Performing enrichment analysis for GO molecular function

The same analysis was done also for the molecular function.

```
# molecular function up regulated genes
ego_MF_up <- enrichGO(gene = upDEGs$external_gene_name, OrgDb = org.Hs.eg.db, keyType = "SYMBOL",
    ont = "MF", pAdjustMethod = "BH", pvalueCutoff = 0.05, qvalueCutoff = 0.05)
```

```
# molecular function down regulated genes
ego_MF_down <- enrichGO(gene = downDEGs$external_gene_name, OrgDb = org.Hs.eg.db,
    keyType = "SYMBOL", ont = "MF", pAdjustMethod = "BH", pvalueCutoff = 0.05, qvalueCutoff = 0.05)
```

```
barplot(ego_MF_up, showCategory = 10, main = "Up-regulated gene list: top 10 enriched MP terms")
```

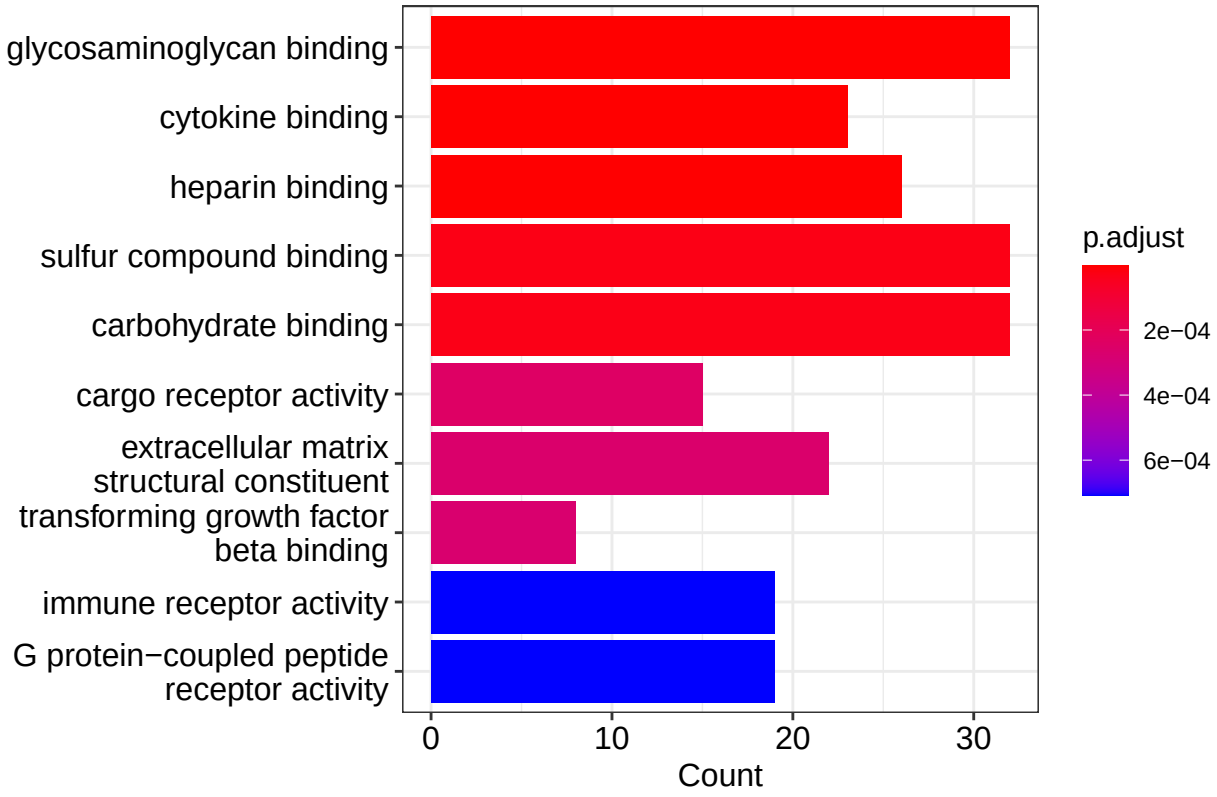


Figure 8: Molecular function up regulated genes

```
barplot(ego_MF_down, showCategory = 10, main = "Down-regulated gene list: top 10 enriched MP terms")
```

```
eWP_up <- enrichWP(gene = upDEGs$entrezgene_id, organism = "Homo sapiens", pvalueCutoff = 0.05,
    qvalueCutoff = 0.1)

head(eWP_up, n = 10)
```

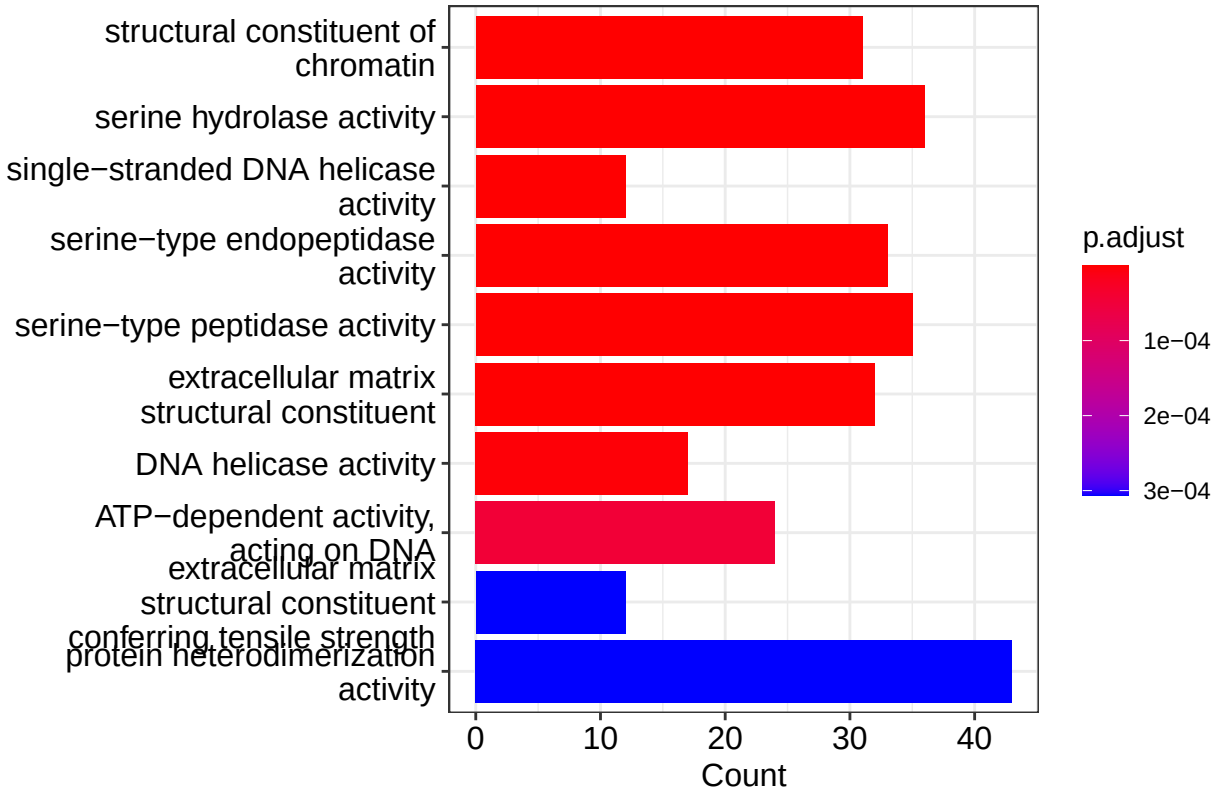


Figure 9: Molecular function down regulated genes

```
##            ID                                                Description
## WP2806 WP2806                                          Complement system
## WP545   WP545                                       Complement activation
## WP558   WP558                          Complement and coagulation cascades
## WP2431 WP2431                                        Spinal cord injury
## WP5094 WP5094                                     Orexin receptor pathway
## WP5090 WP5090 Complement system in neuronal development and plasticity
##        GeneRatio  BgRatio       pvalue     p.adjust       qvalue
## WP2806    19/436  97/8442 3.960197e-07 0.0001892974 0.0001725812
## WP545      8/436  23/8442 1.171095e-05 0.0027989174 0.0025517547
## WP558     12/436  58/8442 3.152788e-05 0.0050234421 0.0045798393
## WP2431    18/436 121/8442 4.427616e-05 0.0052910008 0.0048237708
## WP5094    24/436 201/8442 1.015995e-04 0.0087342052 0.0079629178
## WP5090    16/436 107/8442 1.096344e-04 0.0087342052 0.0079629178
##
## WP2806                         6401/729/2219/2160/7448/6404/730/653509/22918/5199/5648/11326/5806/235
## WP545                                                                                          732/729/
## WP558                                                                                729/1361/7450/730/5648/713/
## WP2431                                          2920/4842/57556/6347/1958/3164/7538/3569/7099/9353/2353/361/
## WP5094 6401/862/2920/4616/1839/8013/1958/1959/3164/2354/3949/5468/3569/9314/2890/1346/2353/5581/3400/
## WP5090                                                  732/729/2219/7448/730/5199/5648/8547/81035/1524/
##        Count
## WP2806    19
## WP545      8
## WP558     12
## WP2431    18
## WP5094    24
## WP5090    16
```

```
eWP_down <- enrichWP(gene = downDEGs$entrezgene_id, organism = "Homo sapiens", pvalueCutoff = 0.05,
    qvalueCutoff = 0.1)

head(eWP_down, n = 10)
```

```
##            ID
## WP2446 WP2446
## WP2361 WP2361
## WP179   WP179
## WP1604 WP1604
## WP45     WP45
## WP466   WP466
## WP4240 WP4240
## WP698   WP698
## WP4016 WP4016
## WP2525 WP2525
##                                                                  Description
## WP2446                                  Retinoblastoma gene in cancer
## WP2361                                       Gastric cancer network 1
## WP179                                                       Cell cycle
## WP1604                                 Codeine and morphine metabolism
## WP45                                       G1 to S cell cycle control
## WP466                                                  DNA replication
## WP4240 Regulation of sister chromatid separation at the metaphase-anaphase transition
## WP698                                                  Glucuronidation
```

```
## WP4016                                    DNA IR-damage and cellular response via ATR
## WP2525                  Trans-sulfuration, one-carbon metabolism and related pathways
##        GeneRatio  BgRatio       pvalue     p.adjust       qvalue
## WP2446    26/618  90/8442 5.976882e-10 3.107978e-07 2.850029e-07
## WP2361    13/618  28/8442 2.054960e-08 4.361461e-06 3.999477e-06
## WP179     28/618 120/8442 2.516227e-08 4.361461e-06 3.999477e-06
## WP1604     9/618  16/8442 4.091055e-07 4.391632e-05 4.027145e-05
## WP45      18/618  64/8442 4.222724e-07 4.391632e-05 4.027145e-05
## WP466     13/618  42/8442 5.366172e-06 4.650682e-04 4.264694e-04
## WP4240     7/618  15/8442 4.168136e-05 3.096330e-03 2.839347e-03
## WP698      9/618  26/8442 5.735318e-05 3.541417e-03 3.247494e-03
## WP4016    17/618  81/8442 6.129375e-05 3.541417e-03 3.247494e-03
## WP2525    15/618  68/8442 9.059661e-05 4.711024e-03 4.320028e-03
##
## WP2446    1870/54443/4175/4998/24137/8318/5427/1869/4173/898/1871/7272/7153/891/10733/890/1111/9133/5
## WP2361                                                                  6790/22974/4605/4173/1894/1063/715
## WP179  1870/4171/4175/4998/23594/8318/990/1869/4173/898/1871/7272/991/9088/891/9700/890/1029/1111/913
## WP1604                                                                                  1244/1565/87
## WP45                                                1870/4171/4175/4998/23594/8318/5427/1869/4173/898/1
## WP466                                                                55388/4171/4175/4998/23594/831
## WP4240
## WP698                                                                                   7358/7363/5457
## WP4016                                                 672/5888/4171/63967/8318/1869/2305/83990/55215/
## WP2525                                                   2729/2730/10797/1789/2571/23743/29968/570/55
##        Count
## WP2446    26
## WP2361    13
## WP179     28
## WP1604     9
## WP45      18
## WP466     13
## WP4240     7
## WP698      9
## WP4016    17
## WP2525    15
```

Task 5: Visualization of the enriched pathway

KEGG analysis was performed using the function `enrichKEGG`.

```
eWP_KEGG <- enrichKEGG(gene = upDEGs$entrezgene_id, organism = "human", pvalueCutoff = 0.05,
    qvalueCutoff = 0.1)
```

Here are reported the top 20 enriched KEGG pathways resulting from the up-regulated list of genes.

```
head(eWP_KEGG, n = 20)
```

```
##                ID                                                        Description
## hsa05144 hsa05144                                                           Malaria
## hsa04610 hsa04610                                  Complement and coagulation cascades
## hsa04270 hsa04270                                   Vascular smooth muscle contraction
## hsa04061 hsa04061 Viral protein interaction with cytokine and cytokine receptor
```

```
## hsa04614 hsa04614                                    Renin-angiotensin system
## hsa03320 hsa03320                                      PPAR signaling pathway
## hsa04380 hsa04380                                   Osteoclast differentiation
## hsa04514 hsa04514                                     Cell adhesion molecules
## hsa04060 hsa04060                       Cytokine-cytokine receptor interaction
## hsa04924 hsa04924                                              Renin secretion
## hsa04080 hsa04080                     Neuroactive ligand-receptor interaction
## hsa04925 hsa04925                        Aldosterone synthesis and secretion
## hsa04976 hsa04976                                               Bile secretion
##          GeneRatio  BgRatio       pvalue     p.adjust       qvalue
## hsa05144    13/382  50/8464 2.037683e-07 3.062438e-05 2.673245e-05
## hsa04610    17/382  86/8464 2.134103e-07 3.062438e-05 2.673245e-05
## hsa04270    17/382 134/8464 1.034508e-04 9.896797e-03 8.639052e-03
## hsa04061    14/382 100/8464 1.464287e-04 1.050626e-02 9.171062e-03
## hsa04614     6/382  23/8464 4.261842e-04 2.375352e-02 2.073478e-02
## hsa03320    11/382  75/8464 4.965893e-04 2.375352e-02 2.073478e-02
## hsa04380    15/382 128/8464 6.213148e-04 2.491966e-02 2.175272e-02
## hsa04514    17/382 157/8464 6.946247e-04 2.491966e-02 2.175272e-02
## hsa04060    26/382 297/8464 8.878939e-04 2.803929e-02 2.447589e-02
## hsa04924    10/382  69/8464 9.769787e-04 2.803929e-02 2.447589e-02
## hsa04080    30/382 367/8464 1.120368e-03 2.923141e-02 2.551651e-02
## hsa04925    12/382  98/8464 1.455772e-03 3.481721e-02 3.039243e-02
## hsa04976    11/382  89/8464 2.105813e-03 4.648988e-02 4.058167e-02
##
## hsa05144                                                                                                6401/14
## hsa04610                                                                                             732/729/1361/2160/74
## hsa04270                                                                                  10203/1906/50487/10268/196883/10266/
## hsa04061                                                                                                53832/2920/6369/
## hsa04614
## hsa03320
## hsa04380                                                                                             7305/6688/10326/54/110
## hsa04514                                                                                          6401/51208/8516/64115/6404/90952/584
## hsa04060                                  53832/2920/3568/6369/1440/6347/2690/3977/9173/8809/650/51554/3569/90865/9
## hsa04924
## hsa04080 153/10203/1906/1511/5023/2690/7433/1268/5737/8698/2922/187/1910/6751/185/2900/2890/5745/112
## hsa04925                                                                                                        4
## hsa04976
##          Count
## hsa05144    13
## hsa04610    17
## hsa04270    17
## hsa04061    14
## hsa04614     6
## hsa03320    11
## hsa04380    15
## hsa04514    17
## hsa04060    26
## hsa04924    10
## hsa04080    30
## hsa04925    12
## hsa04976    11
```

The more enriched pathway (using the list of up-regulated genes), with ID **AGGIUNGERE**, was then visualized using the package `pathview`:

```
logFC <- upDEGs$logFC
names(logFC) <- upDEGs$entrezgene_id
pathview(gene.data = logFC, pathway.id = "WP2446", species = "human")
```

Task 6: Enrichment score transcription factors

First of all, we retrieve the promoter sequence for all the genes in the list of those down-regulated using ensembl gene id as identifier.

```
promoter_regions <- getSequence(id = downDEGs$ensembl_gene_id, type = "ensembl_gene_id",
    seqType = "gene_flank", upstream = 500, mart = ensembl)
```

Then we load the background for MotifDb human PWMs and convert the obtained sequence into a DNAString object.

```
data(PWMLogn.hg19.MotifDb.Hsap)
seq <- lapply(promoter_regions$gene_flank, function(x) DNAString(x))
```

`motifEnrichment` function performs enrichment analysis on the promoter sequences we obtained a few chunks above.

```
enriched_TFs = motifEnrichment(seq, PWMLogn.hg19.MotifDb.Hsap, score = "affinity")
```

```
## Calculating motif enrichment scores ...
```

```
report = groupReport(enriched_TFs)
report
```

```
## An object of class 'MotifEnrichmentReport':
##        rank target              id        raw.score              p.value
## 1         1  PGAM2           PGAM2 7.47058707343782 2.41159784654048e-86
## 2         2  TFAP4      M2944_1.02 3.42391273544219 3.36836197404242e-84
## 3         3    SP2             SP2  119.54842162525 6.25197615973972e-84
## 4         4  CEBPB      M4556_1.02 2.30767167521828  1.7054813305699e-83
## 5         5   JUND      M4506_1.02  6.8634137704217 1.50332672791467e-81
## 6         6   MAFK      M4573_1.02 2.41935358645508 4.83938302985492e-81
## 7         7  ZMAT2           ZMAT2 2.16420212862343 9.19698805317131e-81
## 8         8    JUN      M4591_1.02 2.76520576103839 2.36897498529839e-80
## 9         9   KLF5            KLF5 30.1023912345585 2.91297830264159e-79
## 10       10    NNT             NNT 1.87741003326598 6.17885793377267e-79
## ...     ...    ...             ...              ...                    ...
## 2287 2001.5  Oct-1 NBT06/Oct-1.pwm 1.72351938773035                      1
##          top.motif.prop
## 1     0.177364864864865
## 2     0.178209459459459
## 3     0.185810810810811
## 4     0.174831081081081
## 5     0.185810810810811
## 6     0.171452702702703
## 7     0.163851351351351
```

```
## 8     0.168074324324324
## 9     0.158783783783784
## 10    0.173986486486486
## ...                 ...
## 2287 0.0278716216216216
```

Task 7

We select one among the top enriched TFs, compute the empirical distributions of scores for all PWMs that you find in MotifDB for the selected TF and determine for all of them the distribution (log2) threshold cutoff at 99.75%.

```
tfs <- report$target[1]
tfs_motifs = subset(MotifDb, organism == "Hsapiens" & geneSymbol == tfs)

# transformation to a PWM matrix
PWM = toPWM(as.list(tfs_motifs))

ecdf = motifEcdf(PWM, organism = "hg19", quick = TRUE)
# calculate for each motif of my gene the empirical distribution scores

thresholds = lapply(ecdf, function(x) log2(quantile(x, 0.9975)))
# for each of the distribution, take the quantile as reference
```

Task 8: Pattern matching

We identify which down-regulated genes have a region in their promoter (defined as previously) with binding scores above the computed thresholds for any of the previously selected PWMs.

```
scores = motifScores(seq, PWM, raw.scores = FALSE, cutoff = unlist(thresholds))  # in my promoters, i w

highscore_seq <- which(apply(scores, 1, sum) > 0)
genes_id <- promoter_regions$ensembl_gene_id[highscore_seq]
# down-regulated genes that have a region in their promoter with binding scores
# above the defined threshold for any PWMs
write(genes_id, "output/enriched_genes_id.txt")
```

Task 9: PPI interactions

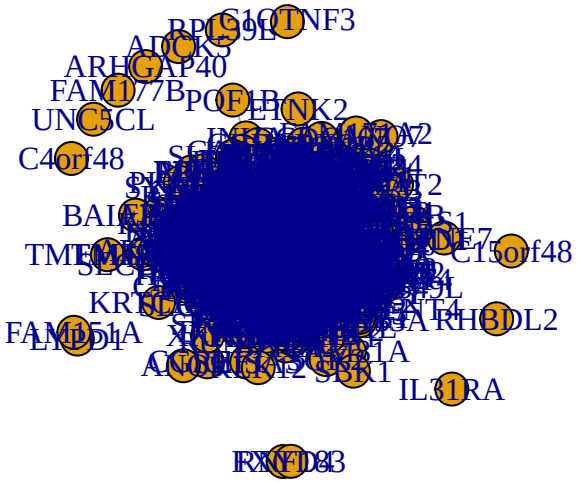
We use STRING database to ind PPI interactions among differentially expressed genes. The network is exported in TSV format.

```
dat <- read.table("output/down_DEGs.txt", sep = "\t", header = TRUE)

write.table(dat$ensembl_gene_id, file = "output/ens_gene_id_down.txt", row.names = FALSE,
    col.names = FALSE)
```

Task 10: visualize the network

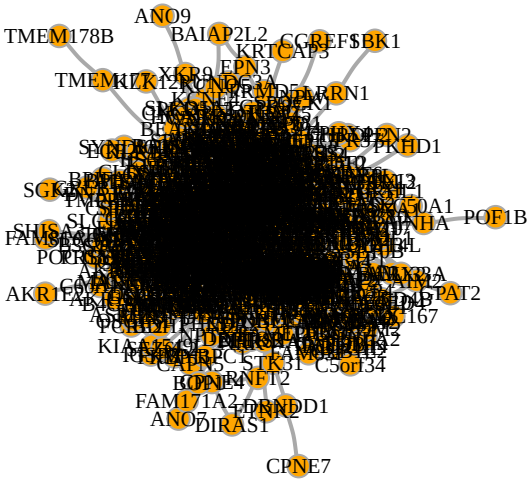
We import the network in R and, using `igraph` package, we identify and plot the largest connected component.

```
links_high_conf <- read.delim("string_interactions_short_high_conf.tsv")
links <- read.delim("string_interactions_short.tsv")
downDEGs <- read.table("output/down_DEGs.txt", sep = "\t", header = TRUE)

ensembl <- useMart(biomart = "ensembl", dataset = "hsapiens_gene_ensembl")
nodes <- getBM(attributes = c("external_gene_name", "ensembl_gene_id", "description",
    "gene_biotype", "start_position", "end_position", "chromosome_name", "strand"),
    filters = c("ensembl_gene_id"), values = downDEGs[, 1], mart = ensembl)  # search info about nodes
nodes <- unique(nodes[, c(1, 3:6)])

nodes <- nodes[nodes$external_gene_name %in% links$X.node1 & nodes$external_gene_name %in%
    links$X.node1, ]

links <- links[links$X.node1 %in% nodes$external_gene_name & links$node2 %in% nodes$external_gene_name,
    ]

# create network
net <- graph_from_data_frame(d = links, vertices = nodes, directed = FALSE)

plot(net)
```

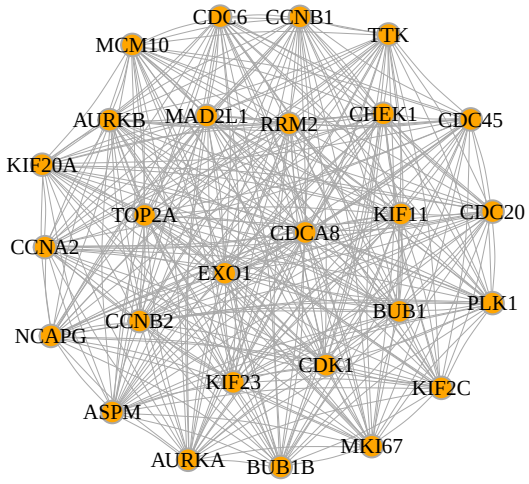



```
c <- components(net, mode = c("weak", "strong"))
net.c <- induced_subgraph(net, V(net)[which(c$membership == 1)])
```

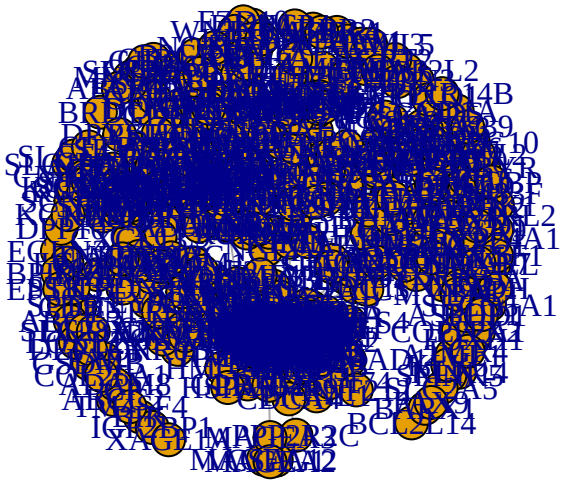
```
plot(net.c, edge.width = 2, vertex.color = "orange", vertex.size = 10, vertex.frame.color = "darkgray",
    vertex.label.color = "black", vertex.label.cex = 0.7, edge.curved = 0.1)
```




```
deg <- degree(net.c, mode = "all")
names_nodes <- names(deg[which(deg > 150)])

nodes.deg <- nodes[nodes$external_gene_name %in% names_nodes, ]

nodes.deg <- nodes.deg[nodes.deg$external_gene_name %in% links$X.node1 & nodes.deg$external_gene_name %
    links$node2, ]
links.deg <- links[links$X.node1 %in% nodes.deg$external_gene_name & links$node2 %in%
    nodes.deg$external_gene_name, ]

net.deg <- graph_from_data_frame(d = links.deg, vertices = nodes.deg, directed = FALSE)

plot(net.deg, edge.width = 0.5, vertex.color = "orange", vertex.size = 10, vertex.frame.color = "darkgra
    vertex.label.color = "black", vertex.label.cex = 0.7, edge.curved = 0.1)
```

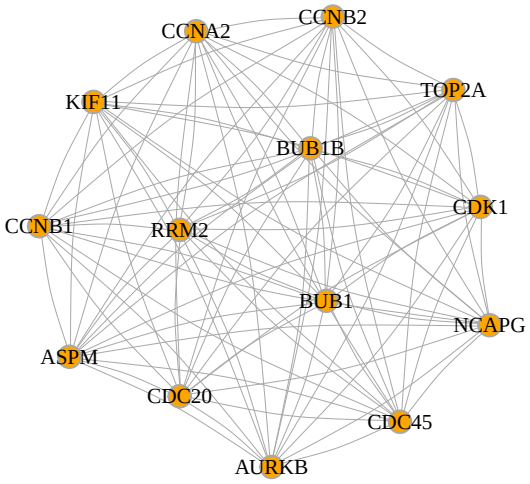



```
links_high_conf <- read.delim("string_interactions_short_high_conf.tsv")
downDEGs <- read.table("output/down_DEGs.txt", sep = "\t", header = TRUE)

ensembl <- useMart(biomart = "ensembl", dataset = "hsapiens_gene_ensembl")
nodes <- getBM(attributes = c("external_gene_name", "ensembl_gene_id", "description",
    "gene_biotype", "start_position", "end_position", "chromosome_name", "strand"),
    filters = c("ensembl_gene_id"), values = downDEGs[, 1], mart = ensembl)  # search info about nodes
nodes <- unique(nodes[, c(1, 3:6)])

nodes <- nodes[nodes$external_gene_name %in% links_high_conf$X.node1 & nodes$external_gene_name %in%
    links_high_conf$X.node1, ]

links_high_conf <- links_high_conf[links_high_conf$X.node1 %in% nodes$external_gene_name &
    links_high_conf$node2 %in% nodes$external_gene_name, ]

# create network
net <- graph_from_data_frame(d = links_high_conf, vertices = nodes, directed = FALSE)

plot(net)
```



```
c <- components(net, mode = "strong")
net.c <- induced_subgraph(net, V(net)[which(c$membership == 1)])

plot(net.c, edge.width = 2, vertex.color = "orange", vertex.size = 10, vertex.frame.color = "darkgray",
    vertex.label.color = "black", vertex.label.cex = 0.7, edge.curved = 0.1)
```


```
deg <- degree(net.c, mode = "all")
names_nodes <- names(deg[which(deg > 110)])

nodes.deg <- nodes[nodes$external_gene_name %in% names_nodes, ]

nodes.deg <- nodes.deg[nodes.deg$external_gene_name %in% links_high_conf$X.node1 &
    nodes.deg$external_gene_name %in% links_high_conf$node2, ]
links.deg.high <- links_high_conf[links_high_conf$X.node1 %in% nodes.deg$external_gene_name &
    links_high_conf$node2 %in% nodes.deg$external_gene_name, ]

net.deg <- graph_from_data_frame(d = links.deg.high, vertices = nodes.deg, directed = FALSE)

plot(net.deg, edge.width = 0.5, vertex.color = "orange", vertex.size = 10, vertex.frame.color = "darkgra
    vertex.label.color = "black", vertex.label.cex = 0.7, edge.curved = 0.1)
```


CCNB2
CCNA2
TOP2A
KIF11
BUB1B
CDK1
CCNB1
RRM2
BUB1
NCAPG
ASPM
CDC20
CDC45
AURKB