

## Bioinformatics Assignment 2

```
# Define file paths
data_dir <- file.path("../..data", "SRP164913")
data_file <- file.path(data_dir, "SRP164913_HUGO.tsv")
metadata_file <- file.path(data_dir, "metadata_SRP164913.tsv")
results_dir <- file.path("../..results")
plots_dir <- file.path("plots")

# Libraries
library(DESeq2)

## Loading required package: S4Vectors

## Loading required package: stats4

## Loading required package: BiocGenerics

##
## Attaching package: 'BiocGenerics'

## The following objects are masked from 'package:stats':
##
##   IQR, mad, sd, var, xtabs

## The following objects are masked from 'package:base':
##
##   anyDuplicated, aperm, append, as.data.frame, basename, cbind,
##   colnames, dirname, do.call, duplicated, eval, evalq, Filter, Find,
##   get, grep, grepl, intersect, is.unsorted, lapply, Map, mapply,
##   match, mget, order, paste, pmax, pmax.int, pmin, pmin.int,
##   Position, rank, rbind, Reduce, rownames, sapply, setdiff, table,
##   tapply, union, unique, unsplit, which.max, which.min

##
## Attaching package: 'S4Vectors'

## The following object is masked from 'package:utils':
##
##   findMatches

## The following objects are masked from 'package:base':
##
##   expand.grid, I, unname
```

```

## Loading required package: IRanges

## Loading required package: GenomicRanges

## Loading required package: GenomeInfoDb

## Loading required package: SummarizedExperiment

## Loading required package: MatrixGenerics

## Loading required package: matrixStats

##
## Attaching package: 'MatrixGenerics'

## The following objects are masked from 'package:matrixStats':
##
##   colAlls, colAnyNAs, colAnys, colAveragesPerRowSet, colCollapse,
##   colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
##   colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
##   colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
##   colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
##   colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
##   colWeightedMeans, colWeightedMedians, colWeightedSds,
##   colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAveragesPerColSet,
##   rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
##   rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
##   rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
##   rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
##   rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs, rowVars,
##   rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
##   rowWeightedSds, rowWeightedVars

## Loading required package: Biobase

## Welcome to Bioconductor
##
##   Vignettes contain introductory material; view with
##   'browseVignettes()'. To cite Bioconductor, see
##   'citation("Biobase)"', and for packages 'citation("pkgname)"'.

##
## Attaching package: 'Biobase'

## The following object is masked from 'package:MatrixGenerics':
##
##   rowMedians

## The following objects are masked from 'package:matrixStats':
##
##   anyMissing, rowMedians

```

```
library(ggplot2)
library(magrittr)
```

```
##
## Attaching package: 'magrittr'

## The following object is masked from 'package:GenomicRanges':
##
##      subtract
```

```
library(M3C)
library("umap")
```

```
##
## Attaching package: 'umap'

## The following object is masked from 'package:M3C':
##
##      umap
```

```
# Set seed for reproducible results
set.seed(12345)
```

**Group:** 5, **Date:** 09/25/2024 Order to run R scripts:

(task 1) preprocessing.R  
(task 2) PCA\_Plot.R  
(task 3) DifferentialAnalysis.R  
(task 4) heatmap.R  
(task 5-Hannah) gprofiler2.R  
(task 5-Luca) topGO.R

## Task 1 - Data analysis

Project report on the analysis of our RNA-seq data set on Neurological Immune-mediated Disorders.

The data set is composed of 88 total samples out of which 34 are Multiple Sclerosis, 14 are HAM/TSP and 20 are healthy controls.

URL: <https://www.refine.bio/experiments/SRP164913/comprehensive-analysis-of-tcr-s-repertoire-in-patients-with-neurological-immune-mediated-disorders>

### Preprocessing

At first we downloaded the quantile normalized version of our data set. After a discussion with our TA we switched to the non-normalized version, since some further analysis will use that Data. The following preprocessing steps have been taken.

1. Map Ensemble to HUGO Id's using the following tutorial: [https://alexlemonade.github.io/refinebio-examples/03-rnaseq/gene-id-annotation\\_rnaseq\\_01\\_ensembl.html](https://alexlemonade.github.io/refinebio-examples/03-rnaseq/gene-id-annotation_rnaseq_01_ensembl.html)

2. Some Ensemble ID's did not map to HUGO, these rows have been dropped.
3. Some Ensemble ID's mapped to multiple HUGO ID's we took the first match
4. A few Ensemble ID's mapped to the same HUGO ID's creating duplicate rows. These have been aggregated by the median into single rows.

Of 43'363 Genes in the original data set, 29'589 were used for the analysis. 13'774 rows from the original dataset were dropped or aggregated.

During the Preprocessing steps we also found out that even the non-quartile-normalized dataset from re-fine.bio was pre normalized. The consequences of this will be apparent in further chapters.

## Sample Size

```
data_analysis_df <- read.delim("../data/SRP164913/SRP164913_HUGO.tsv",header = TRUE, row.names = 1,
cat("Number of Genes in the expression matrix: ", dim(data_analysis_df)[1], "\n")
```

```
## Number of Genes in the expression matrix: 29589
```

```
cat("Number of Samples in the expression matrix: ", dim(data_analysis_df)[2], "\n")
```

```
## Number of Samples in the expression matrix: 88
```

There are 88 samples, however, there are three disease groups: healthy controls, MS subjects, and ham/tsp subjects. To make this a binary problem, from Task 2 onward, we removed the ham/tsp subjects to compare only MS vs healthy controls (hc). This reduces the number of samples to 62.

## Density Plot of Gene Expressions

```
# Get the median of expressions by gene
gene_median <- apply(data_analysis_df, 1, median)
head(gene_median)
```

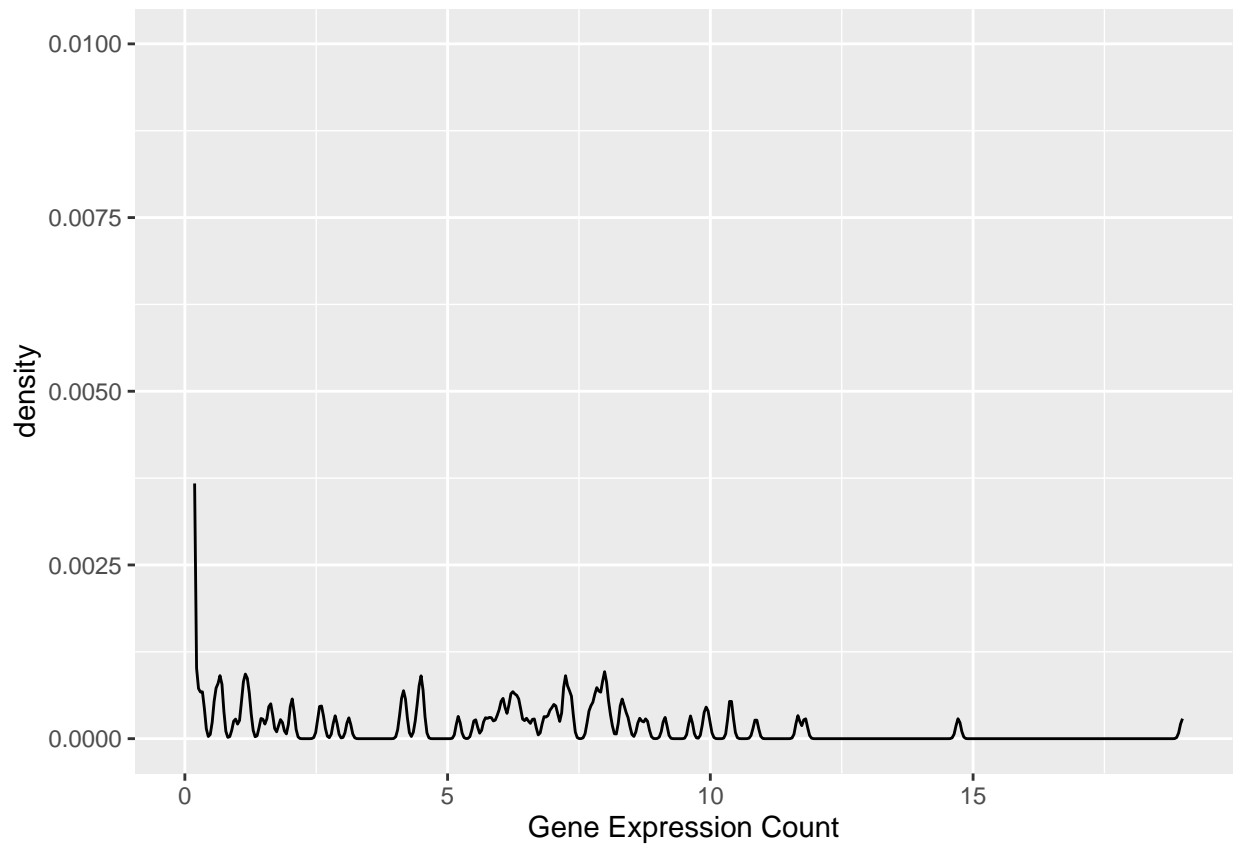
```
##      A1BG A1BG-AS1      A1CF      A2M A2M-AS1      A2ML1
##         0         0         0         0         0         0
```

```
gene_median = log2(gene_median +1)
cat("Variance between gene expression medians:", var(gene_median, na.rm = TRUE))
```

```
## Variance between gene expression medians: 0.1234497
```

```
# Create a Data frame from the numerical array
gene_median_df <- data.frame(Median = gene_median)
# Plot the values
ggplot(gene_median_df, aes(x = Median)) + geom_density() + xlab("Gene Expression Count") + ylim(0,1e-2)
```

```
## Warning: Removed 72 rows containing non-finite outside the scale range
## ('stat_density()').
```



The density plot shows a left skewed distribution of gene expression counts with most of the expressions being between 0 and 10 but with a few outliers between 10 and 15. We downloaded the un-normalized data, however when we opened the raw file, most count values were 0 or very close to 0. We suspect the data came pre-normalized, and this is causing some issues. This carries over through the entire assignment.

## Task 2 - Principal Component Analysis

We were able to get a PCA, tsne, and umap plot following the tutorials. All three show a clear difference between the healthy control (hs) and MS (ms) groups, with a few samples in the midst of the other cluster. The number of crossovers is different.

### PCA Plot

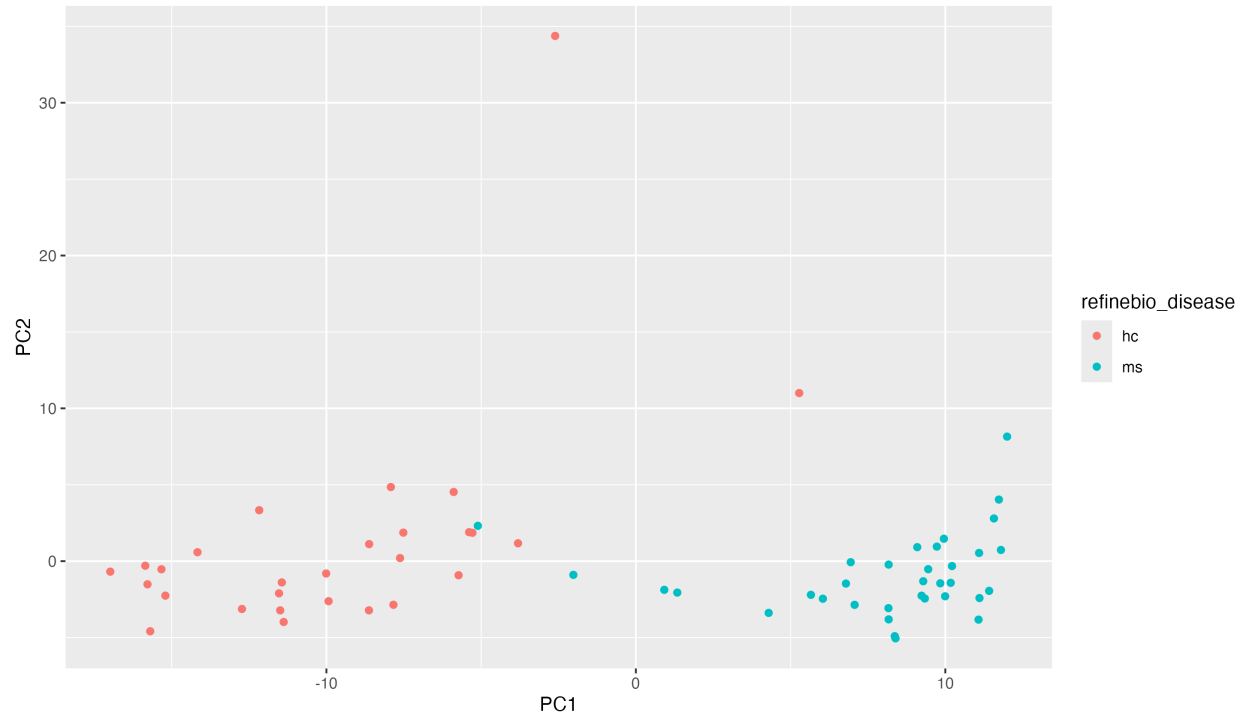
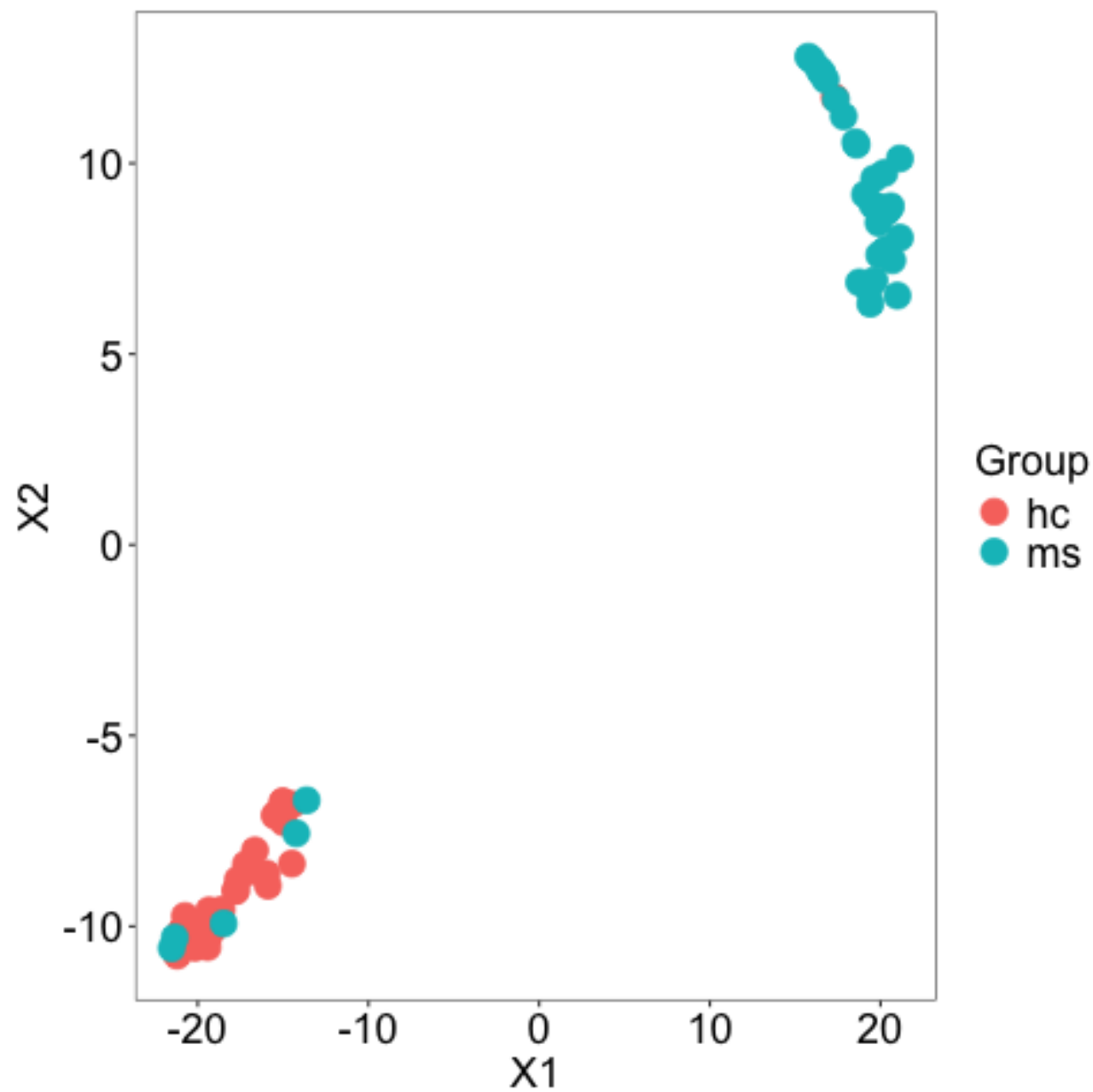


Figure 1: PCA Plot

### TSNE Plot

The TSNE (t-distributed stochastic neighbor embedding) plot visualizes n-dimensional data in two dimensions. Using this method, our data is nicely split in two clusters, with a few outliers in the wrong cluster. This gives a first hint that there are some differentially expressed genes among the two groups, Multiple Sclerosis and Human Control.



## UMAP Plot

Similar to t-SNE UMAP also reduces n-dimensional data to two dimensions. In our case it yields similar results to the t-SNE plot with slightly less outliers.

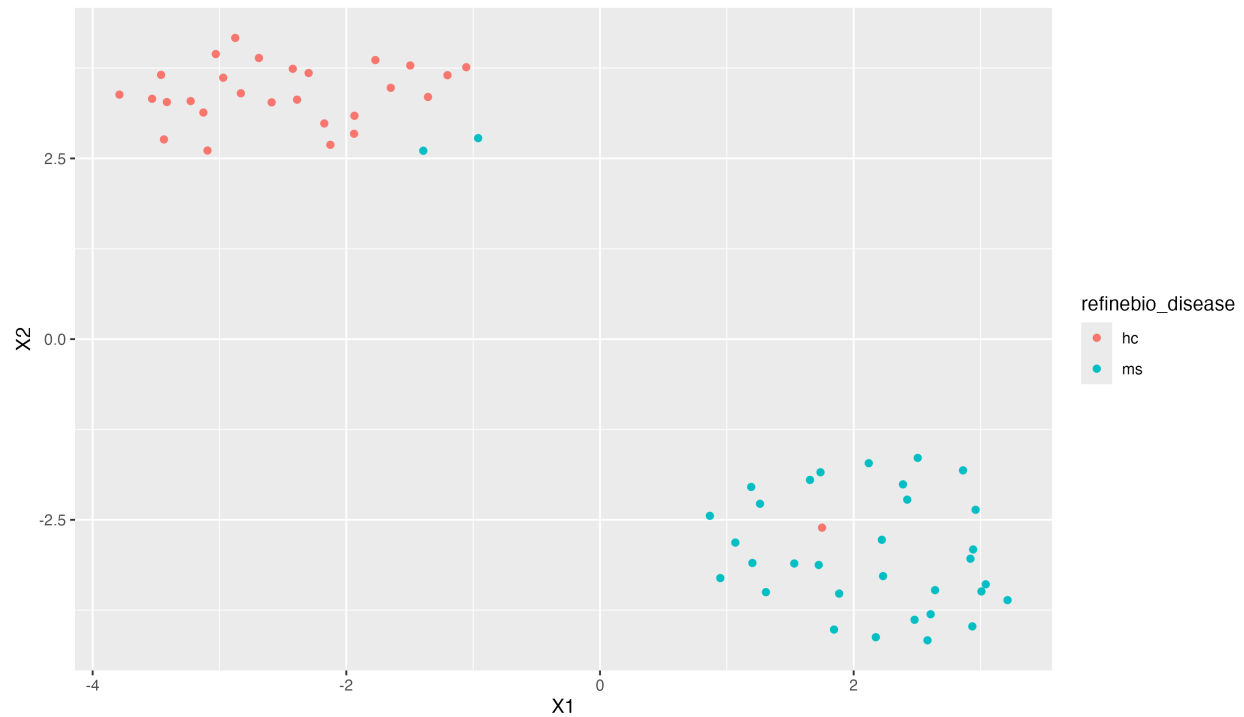


Figure 3: umap Plot

## Task 3 - Differential Analysis

When we did our differential analysis, because the data was so small and normalized, we had to adjust the filter value to 0.01 or else only ~1000 rows were making it to the `DESeqDataSetFromMatrix()` step. This was causing our volcano plot to look very sparse. We were able to get a list of the top 50 differentially expressed genes. The results, both the entire table and only the top 50, are stored in the results folder.

```
top_50_file <- file.path(results_dir, "SRP164913_diff_expr_top_50_results.tsv")
top_50 <- readr::read_tsv(top_50_file)

## Rows: 50 Columns: 7
## -- Column specification -----
## Delimiter: "\t"
## chr (1): Gene
## dbl (5): baseMean, log2FoldChange, lfcSE, pvalue, padj
## lgl (1): threshold
##
## i Use 'spec()' to retrieve the full column specification for this data.
## i Specify the column types or set 'show_col_types = FALSE' to quiet this message.
```

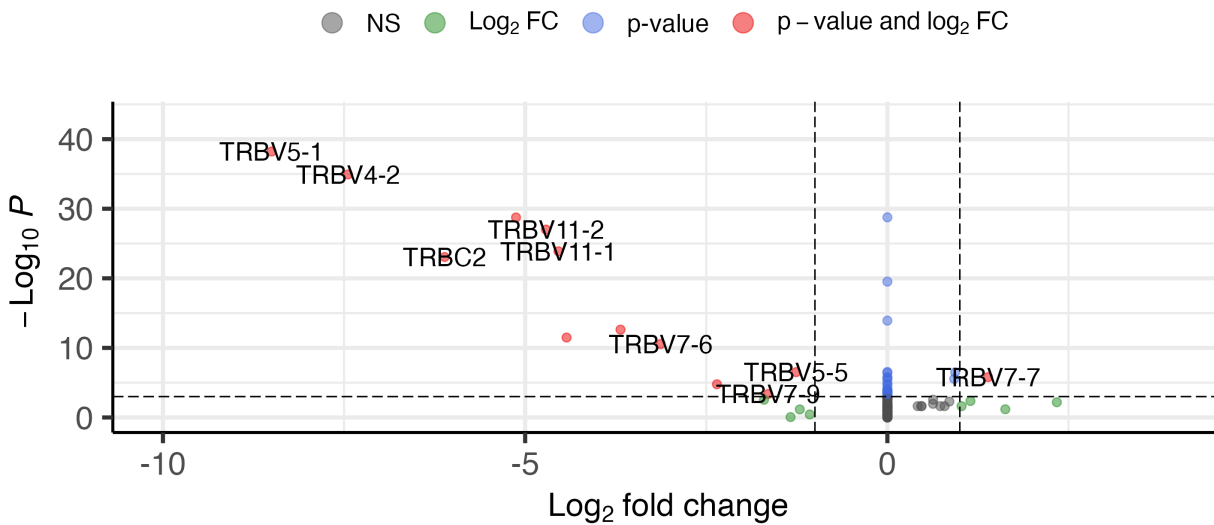


```
head(top_50)
```

```
## # A tibble: 6 x 7
##   Gene      baseMean log2FoldChange lfcSE      pvalue      padj threshold
##   <chr>      <dbl>          <dbl> <dbl>      <dbl>      <dbl> <lgl>
## 1 TRBV23-1    9.45            2.34  0.614 0.000168    0.00652    TRUE
## 2 TRBV16     18.9            1.63  0.424 0.0633     0.0663    FALSE
## 3 TRBV7-7    557.            1.39  0.269 0.0000000137 0.00000158 TRUE
## 4 TRBV7-4     31.7            1.15  0.362 0.000108    0.00435    TRUE
## 5 TRBV12-4    389.            1.02  0.509 0.000837    0.0234     TRUE
## 6 TRBV15     729.            0.936 0.172 0.00000000247 0.000000326 TRUE
```

## Volcano plot

*EnhancedVolcano*



The volcano plot, while still somewhat sparse, is far more populated than it was with the highly filtered data. Most significant results have a negative log fold change.

## Task 4 - Heatmap

A heatmap was generated for the top 50 differentially expressed genes. The counts for each gene in the top 50 were extracted and put into the heatmap plot. It was annotated with the two disease groups: ms subjects and healthy control subjects. Since many of our counts are 0 and therefore are not expressed differently, the map is largely blue.

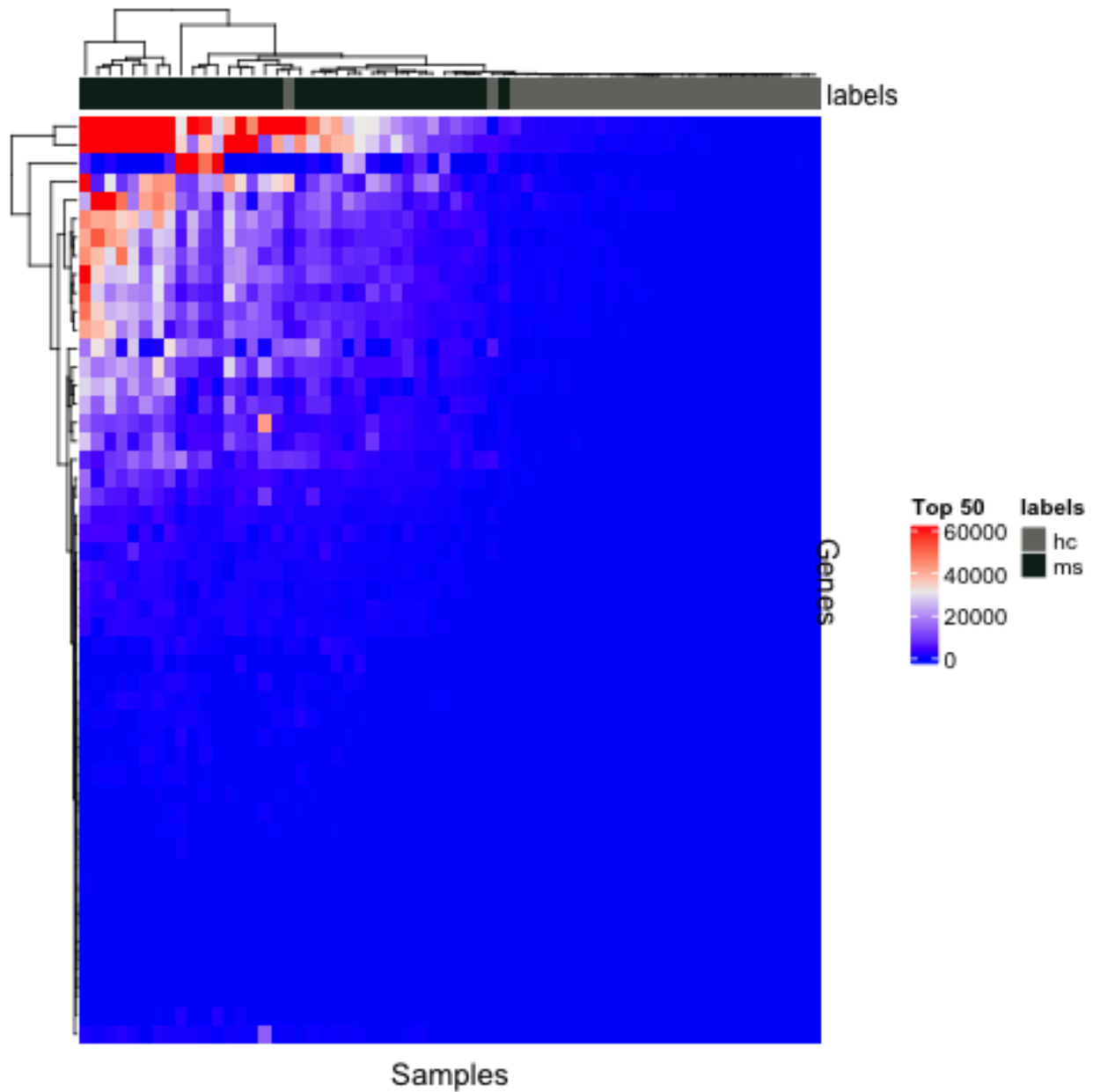


Figure 4: Heatmap

## Task 5 - Enrichment Analysis

### Hannah- gprofiler2

The function `gost` was used from `gprofiler2` to perform enrichment analysis on the differentially expressed data. Disease ontology was used, as the data was differentially expressed between the MS subjects and healthy controls. Only genes with adjusted p-values below 0.05 were put into the function. Two `gost` results were generated: one that contains all results, significant or not, and one that contained only significant results. The plot (created with `gostplot`) for the significant results is shown below. It seems there are a few points above the significance threshold.

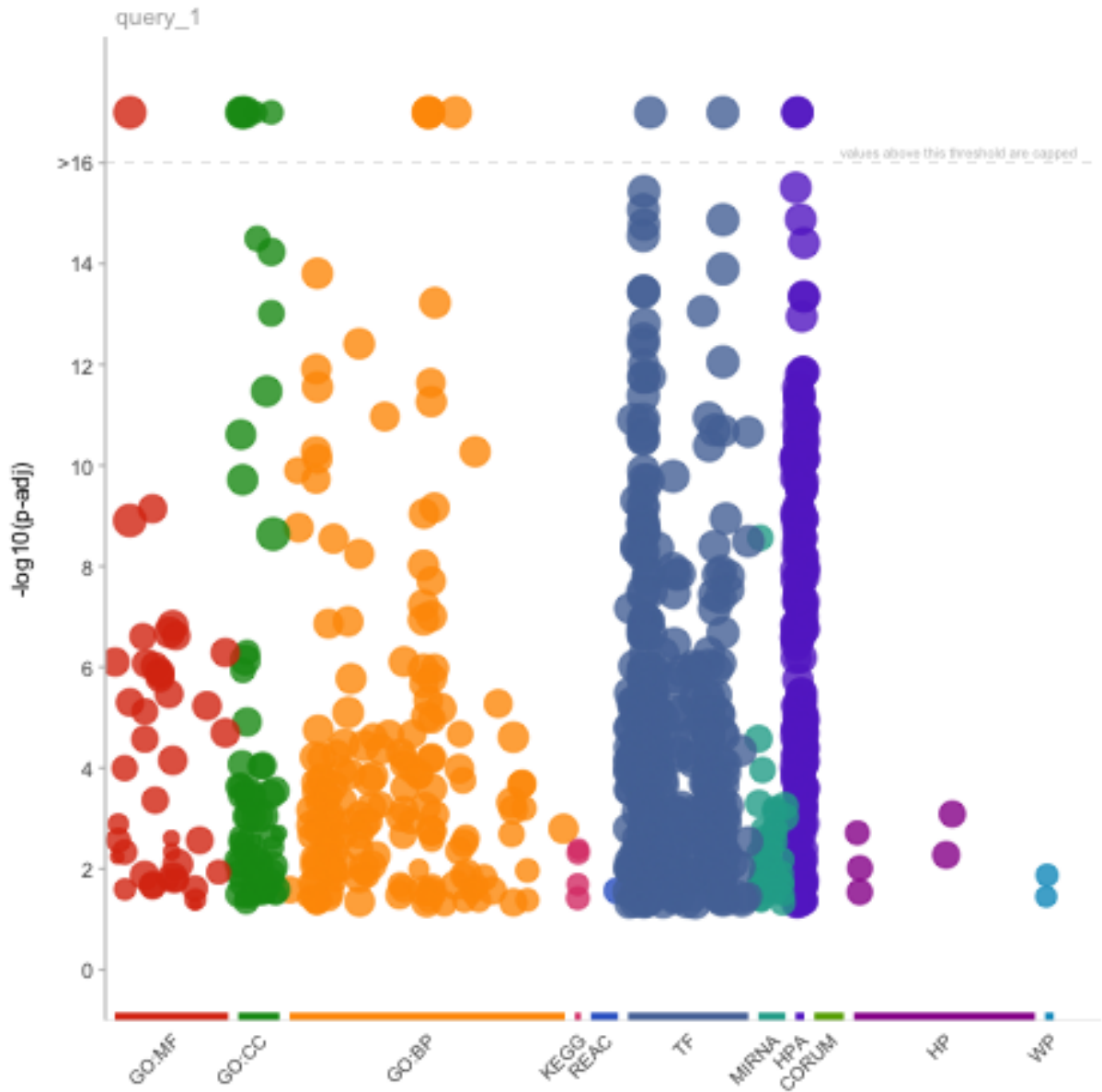


Figure 5: gprofiler2 Plot

However, when `publish_gosttable(gost_res3)` was run, the function would not complete. It would continue

to run for over 15 minutes and never produce results. I was unable to generate a table from the results, and when I tried to print the results, it would return NULL for both significant and non-significant gost results. The NULL result is shown below along with the gost function that was run. It is possible that no truly significant results were generated and so the results were empty.

```
results_dir <- file.path(".././results")
diff_expr_file <- file.path(results_dir, "SRP164913_diff_expr_results.tsv")
library(gprofiler2)
diff_expr_df <- readr::read_tsv(diff_expr_file)

## Rows: 5115 Columns: 7
## -- Column specification -----
## Delimiter: "\t"
## chr (1): Gene
## dbl (5): baseMean, log2FoldChange, lfcSE, pvalue, padj
## lgl (1): threshold
##
## i Use 'spec()' to retrieve the full column specification for this data.
## i Specify the column types or set 'show_col_types = FALSE' to quiet this message.

gost_res3 <- gost(
  query = unlist(diff_expr_df[diff_expr_df$padj<0.05, 'Gene']),
  significant=TRUE,
  organism = "hsapiens"
)
head(gost_res3$results)

## NULL
```

When I had initially tried to run gost with every gene name, no p-value factor, it would produce a result that could be printed, but that is not as relevant to our dataset. This does prove that something is off with specifically the differential data, and the function does work.

Unfortunately, due to the circumstances described above, no table was generated using the gprofiler2 method.

## Luca - topGO

Given a set of genes and a gene differential expression analysis, topGo enriches the most relevant Genes with Gene Ontology (GO) terms. This helps to find out what function the differential expressed genes perform in the Human Body.

**Methodology** The Methodology described is analogous to the Quick Start Guide found here “<https://bioconductor.org/packages/release/bioc/vignettes/topGO/inst/doc/topGO.pdf>”. The Output of Task 1-4 was a list of differential expressed genes, with their probability, only trivial transformations were required to input the data into the topGO function which returns an object containing all gene identifiers and their scores, GO annotations and further information. The cutoff for expressed gene selection has been set to the default of  $p < 0.05$ .

Using the Fisher exact test a enrichment analysis is performed, testing for the over-representation of GO terms within the differentially expressed genes. Due to time constraints only the classic method, testing each GO category independently was used.



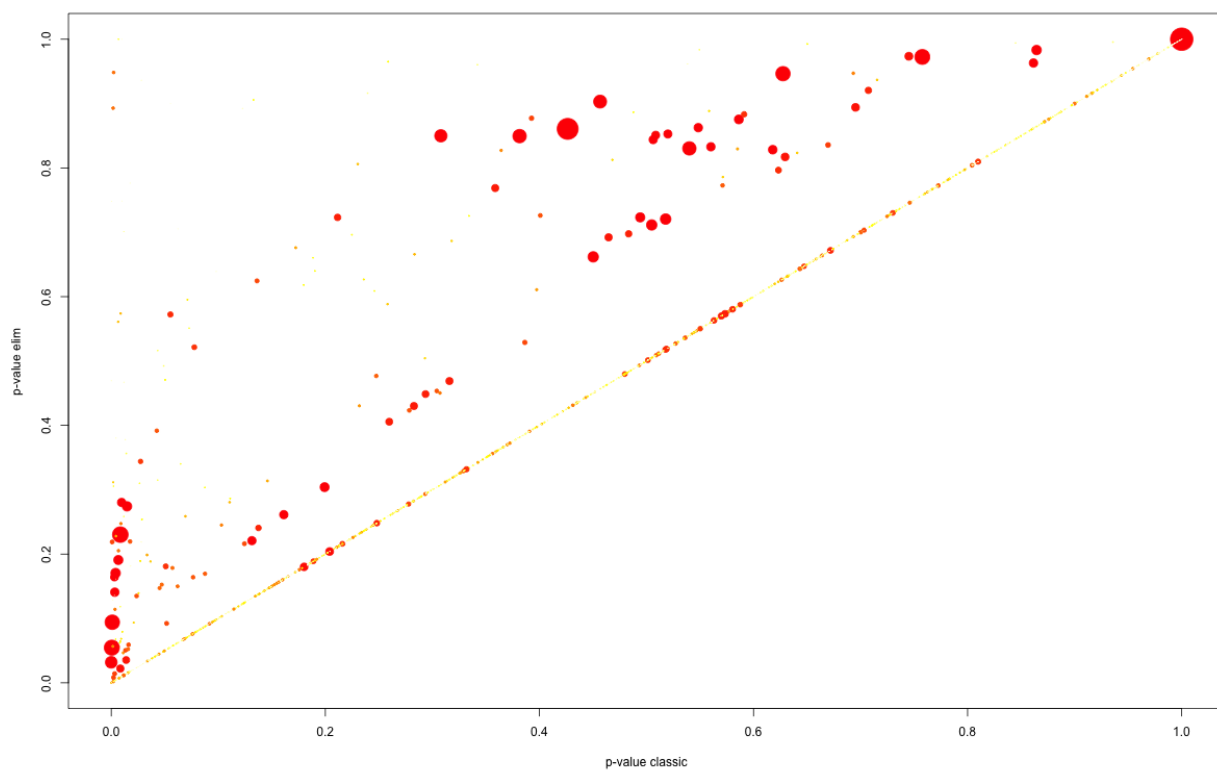


Figure 8: Fisher vs Elim

## Peter- ClusterProfiler

Cluster Profiler is designed for the analysis and visualization of functional profiles of genes and gene clusters. It helps researchers identify biological themes, functions, and pathways that are overrepresented in gene sets. In this specific example, it was used to identify the enriched biological processes, and molecular functions associated with the gene set.

Here is the plot I was able to generate after using the enrichGO function to find overrepresentation.

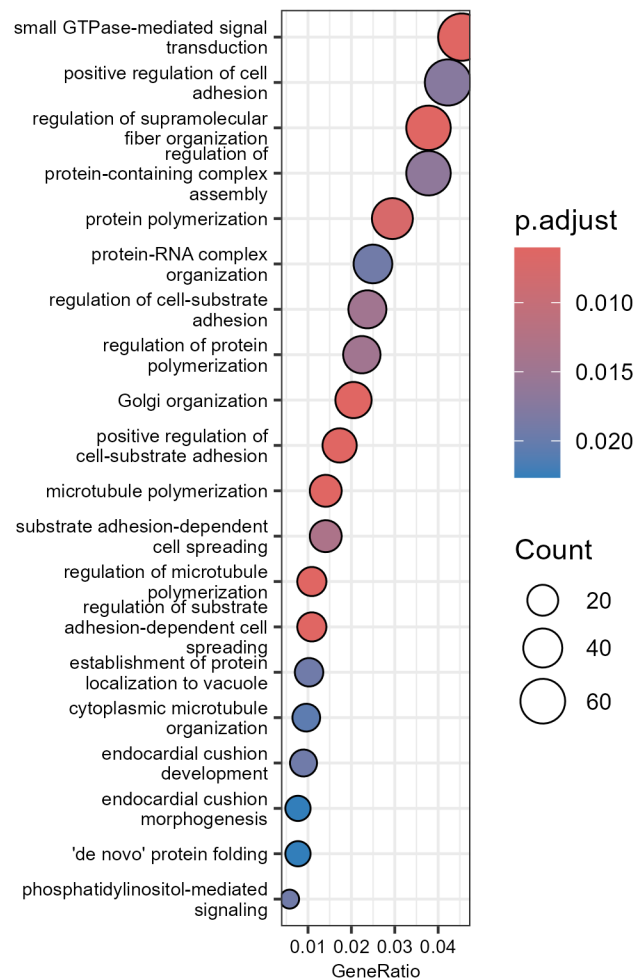


Figure 9: ClusterProfiler Plot

As shown, the top result was small GTPase-mediated signal transduction, which I found to play a significant role in signal transduction pathways that regulate various cellular functions, including cell growth and immune responses. Small GTPases like Rho, Rac, and Cdc42 are vital for activating and migrating immune cells. In MS, these cells lead to neuroinflammation and tissue damage. They also regulate cytokine production in immune and glial cells, with dysregulation potentially worsening MS-related inflammation.

## Justin - Genomic Super Signature

Justin was unable to complete his portion of the assignment.

## Task 6/7 - Joint Enrichment Analysis