# Day2_ReviewRNAseqFromDay1

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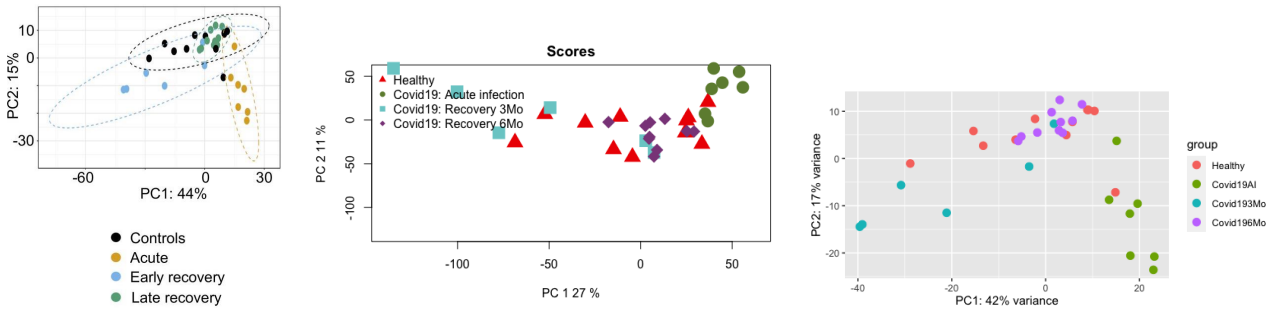
## Contents



### Comparing the results from Brauns et al

In the recent study titled "Functional reprogramming of monocytes in patients with acute and convalescent severe COVID-19" published by the team of Brauns et al (2022) (Ref. 1), they tested the TLR-induced reaction of blood cells from coming from patients presenting either mild to acute response and in their recovering phases.

A Principal Component Analysis (PCA) showed that the recovering patients from COVID clustered according to their stage, early stages separate from the healthy control (Fig. 1a). Late recovery stage patients clsutered together with the healthy controls. Acute infections samples formed their own sample (Fig. 1a). This pattern was recreated after analyzing the raw counts with our own pipelines (Fig. 1b and c) with both the NOISeq and the DESeq2 package. Please note, how the variance captured by the PC1 increases from 27% to 42% after the DESeq2 normalization.




a) b) c)

**Figure 1:** a) PCA of RNA seq samples taken from paper published by Brauns et al, b) PCA made with NOISeq, and c) PCA made with DESeq2.

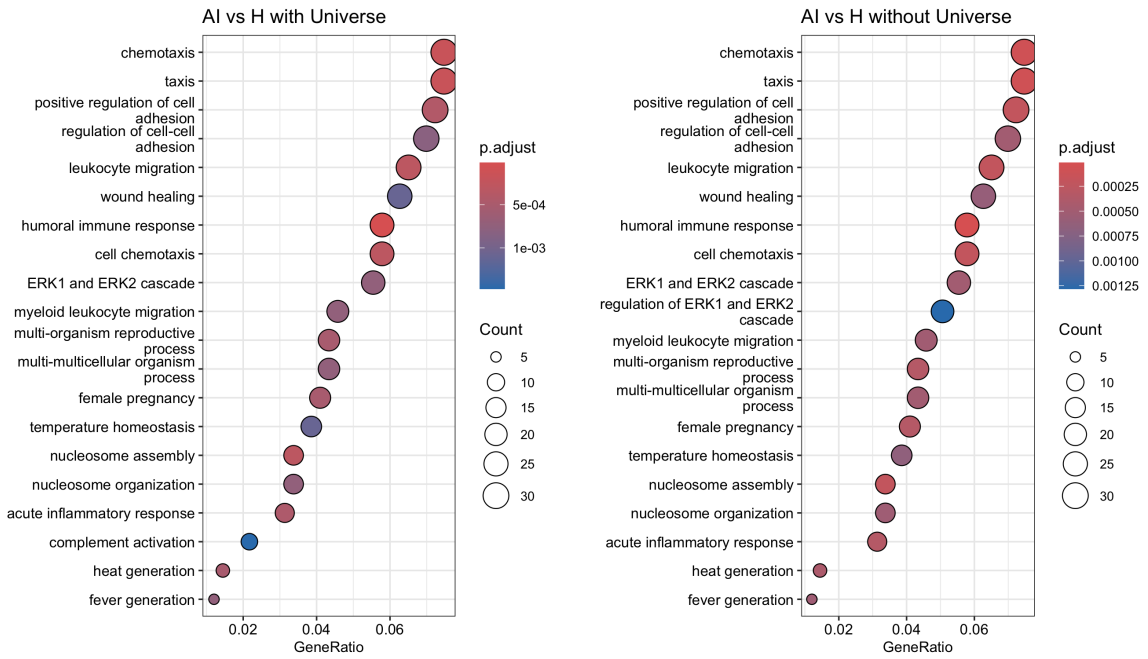
The research team used DESeq2 to perform the differential expression analysis. Nevertheless, different parameters were used when filtering out the lowly expressed genes. In the article, genes with counts lowered or equal to 20 in at least one sample were removed, meanwhile within our pipeline we filtered out genes which do not have at least 10 counts in at least 6 samples (size of each group). Afterwards, we called DESeq2 and performed DGEA for three comparisons: acute infection (AI) against healthy (H), and the two stages of

recovery against healthy three and six months, 3Mo and 6Mo, respectively. We kept the genes that passed the thresholds mentioned in the article, FDR $< 0.05$ and $|FC| > 2$.

Another popular package used to perform DGEA is edgeR. We used this package too to perform analysis and compare how much the results from both protocols overlap. As we can see, although we used the same cut-off values DESeq2 seems to be more conservative finding less genes consistenly. Surprisingly, EdgeR did not find any DEG in the comparison between healthy individuals and those whoe are 6 months into recovery. For the first comparison, AI vs H, they are reporting 339 differentially expressed genes (DEG), 184 up- and 155 down-regulated genes). Our analysis found 510 genes.


**Figure 2:** Venn diagrams for a) acute infection vs healthy subjects, b) 3 months recoveing individuals vs healthy subjects, and c) 6 months into recovery vs healthy subjects; the list of DEGs were intersected in order to compare how much both analysis overlapped.

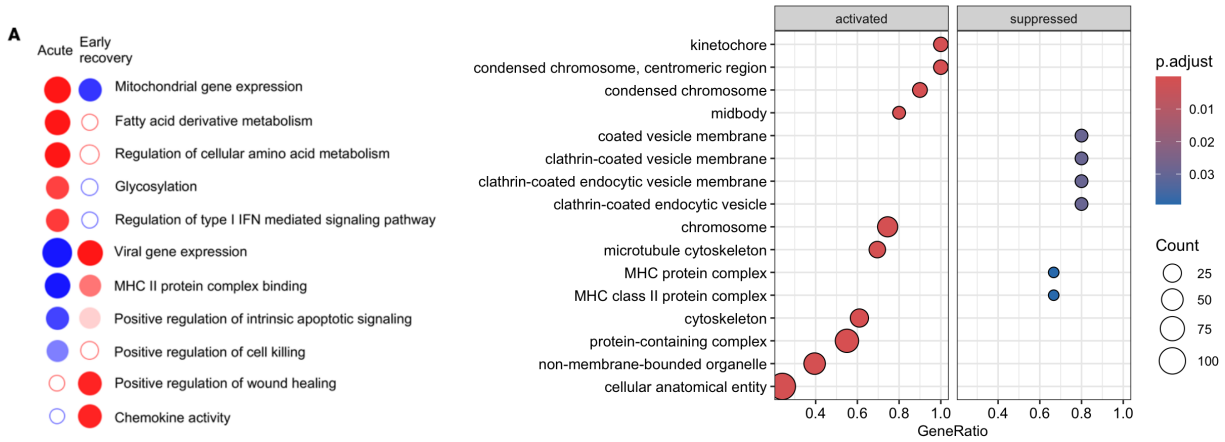
When comparing the GO analysis performed with and without the universe there is not much difference. This maybe due to the DEGs capture to the majority of the genes invlved genes in the captured processes by the enrichment.




a) b)

**Figure 3:** Comparison between a ORA performed with and without background or universe, panel a) and b) respectively.

Brauns et al, report upon gene set enrichment analysis (GSEA) comparing the AI to the healthy controls. For the GSEA the research team used BubbleGUM while we used the R package clusterProfiler. Although the results are not exactly the same, we would like to highlight the similarity between the two analysis, like finding downregulated the MHC complex in the acute group.

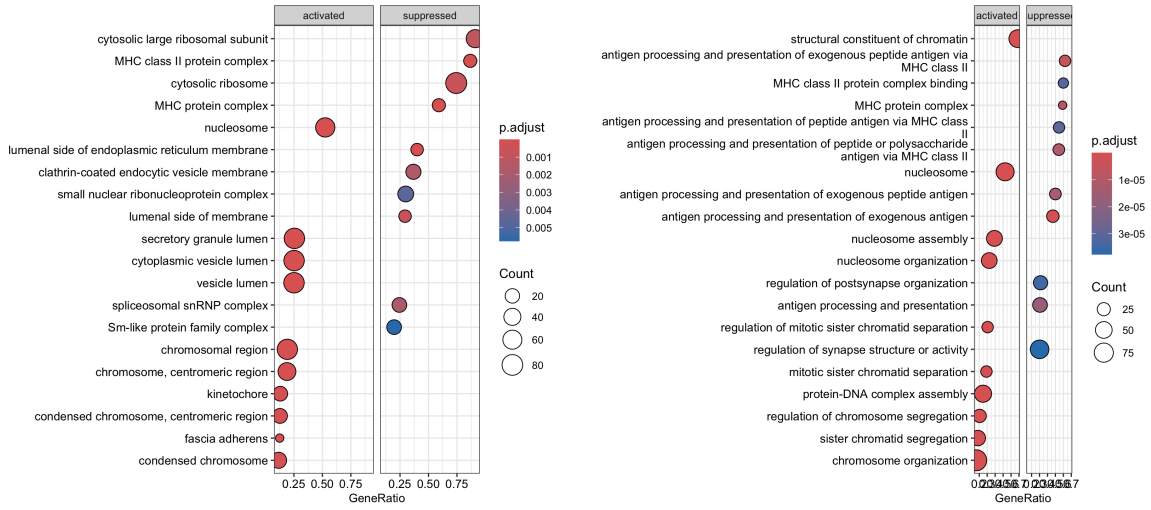



a) b)

**Figure 4:** a) GSEA study taken from Brauns et al, and b) GSEA generated in this study focusing in the Cellular Components terms

A mistake was made when doing that GSEA, only the FC of the DEGs were used to perform the analysis. If we compare the CC results for both approaches, we can see a huge change in the terms found by the analysis. When examining the CC terms, the MHC proteins are ranked higher than in the previous figure.

This can show the power and presicion a correct use of the tools can bring. As well, if we analyze the top terms coming from the 3 GO categories, a lot of antigen-related terms become are found as regulated, which seems matches the nature of the data that is studying the immune reaction to a viral infection.




a) b)

**Figure 5:** a) GSEA for Cellular component terms when taken into account all genes, and b) GSEA for BP, CC and MF terms with the same correction

Regarding the comparisons between healthy and the two recovery stages, we found 470 and 9 DEGs for 3 and 6 months, respectively. In the article, the authors found 521 DEG for the 3 moths recovery stage. Genes highlighted in the text such as as ATF4, FOXO3, MAFF, MAFB, FOSL1, and PPARG, are up-regulated in our analysis as well. Regarding the late recovery stage, we only find 9 DEGs, this is in accordance with the authors which say they hardly find any regulated genes.

If we take a closer look into the overall expression regulation, we can see that the acute infection samples how a similar pattern of gene regulation as the one seen in the research article. Its worth noting that the dendogram group together the acute infection samples while the recovering and healthy are mixed. Also, the 3 months samples are clustering separately from the controls and the six months.

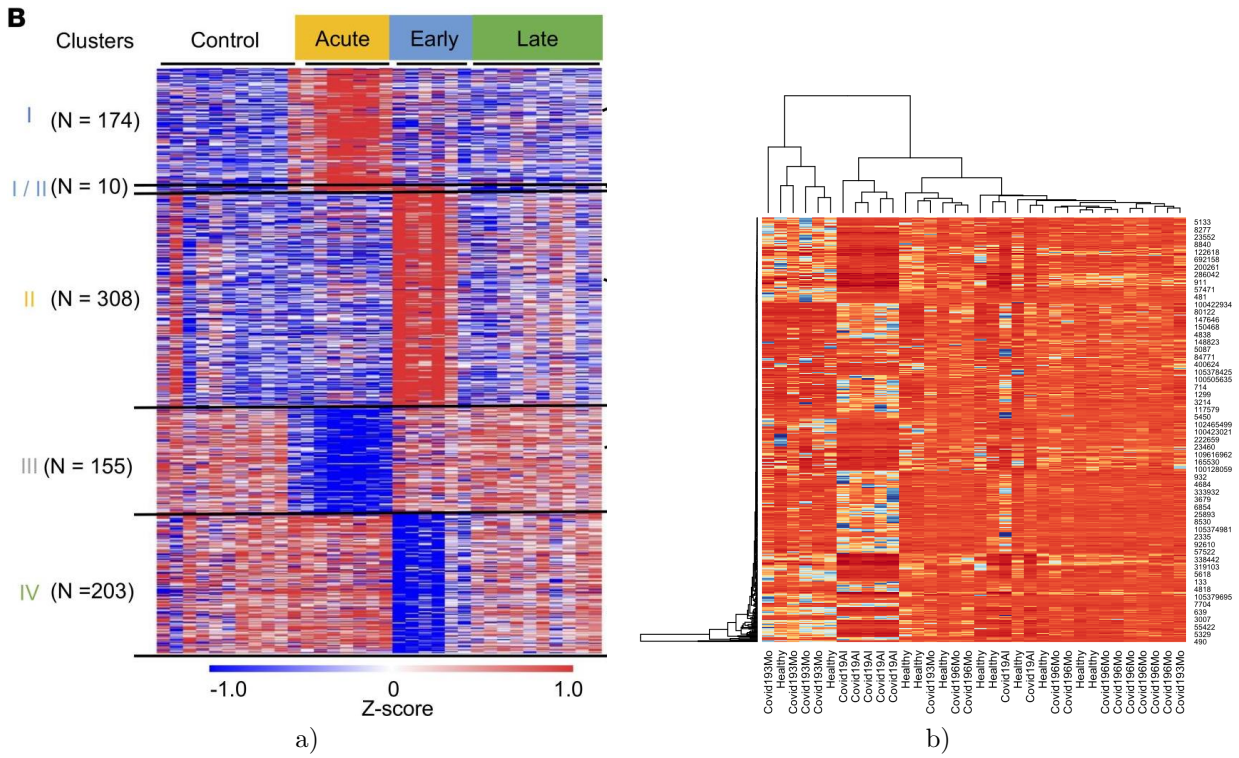



**Figure 6:** a) Heatmap taken from Brauns et al. and b) heatmap generated by our analysis.

##Code

Load the needed libraries:

```
library(GEOquery)
library(NOISeq)
library(DESeq2)
library(clusterProfiler)
library(org.Hs.eg.db)
library(RColorBrewer)
library(gplots)
```

Download the counts and metadata:

```
#load counts table from GEO2R
urld <- "https://www.ncbi.nlm.nih.gov/geo/download/?format=file&type=rnaseq_counts"
path <- paste(urld, "acc=GSE198256", "file=GSE198256_raw_counts_GRCh38.p13_NCBI.tsv.gz", sep="&");
tbl <- as.matrix(data.table::fread(path, header=T, colClasses="integer"), rownames=1)
entrez_ids <- write.csv(x = rownames(tbl), file = "Gene_EntrezID.txt", row.names = FALSE, col.names = FA

#geo
gset <- GEOquery::getGEO("GSE198256")[[1]]

#Get the sample metadata
metadata <- gset@phenoData@data
health_state <- metadata[,c("disease state:ch1")]
```

Download the genes information for NOISeq analysis:

```
#get biomart GC content, gene start, gene end, chr
biomart <- read.csv(file = "mart_export.txt", header = 1)
#filter out non cannonical chr, keep only the first annotation of each gene
biomart_filt <- biomart[biomart$Chromosome.scaffold.name %in% c(as.character(1:22) ,"X","Y"),]
biomart_filt <- biomart_filt[!duplicated(biomart_filt$NCBI.gene..formerly.Entrezgene..ID),]
row.names(biomart_filt) <- biomart_filt$NCBI.gene..formerly.Entrezgene..ID

#length
len <- biomart_filt$Gene.end..bp. - biomart_filt$Gene.start..bp.
names(len) <- biomart_filt$NCBI.gene..formerly.Entrezgene..ID

#GC%
gc <- biomart_filt$Gene...GC.content
names(gc) <- biomart_filt$NCBI.gene..formerly.Entrezgene..ID

#biotype
type <- biomart_filt$Gene.type
names(type) <- biomart_filt$NCBI.gene..formerly.Entrezgene..ID

#chr
chr <- data.frame(Chr=biomart_filt$NCBI.gene..formerly.Entrezgene..ID,
                  GeneStart=biomart_filt$Gene.start..bp.,
                  GeneEnd=biomart_filt$Gene.end..bp.,
                  row.names = biomart_filt$NCBI.gene..formerly.Entrezgene..ID)

#filter from tbl the not annotated genes
tbl_filt <- tbl[rownames(tbl) %in% rownames(biomart_filt),]
tbl_count_exc <- tbl[!(rownames(tbl) %in% rownames(biomart_filt)),]
annotgene_ord <- biomart_filt[rownames(tbl_filt ),]
#sum(rownames(annotgene_ord)==rownames(tbl_filt))

#df of exp design
myfactors <- data.frame(metadata [ colnames(tbl_filt),c("disease state:ch1")])
colnames(myfactors)[1]<- "Group"

#make data inot NOISeq object
NOISeq_obj <- readData(data = tbl, length = len, gc = gc,
                       chromosome = chr, factors = myfactors, biotype = type)

#PCA NOISeq
myPCA = dat(NOISeq_obj, type = "PCA")
par(mfrow = c(1, 1))
explo.plot(myPCA, factor = "Group")
```

Let's call DESeq2 and perform the DGEA:

```
#prepare matadata for deseq2
coldata <- pData(NOISeq_obj)
coldata[coldata=="Covid19: Acute infection"] <- "Covid19AI"
coldata[coldata=="Covid19: Recovery 3Mo"] <- "Covid193Mo"
coldata[coldata=="Covid19: Recovery 6Mo"] <- "Covid196Mo"
coldata$Group <- factor(coldata$Group, levels = unique(coldata$Group))
```

```
#create deseq2 obj
dds <- DESeqDataSetFromMatrix(countData = tbl_filt,
                              colData = coldata,
                              design = ~ Group)

#filter low counts genes
smallestGroupSize <- 6
keep <- rowSums(counts(dds) >= 10) >= smallestGroupSize
dss <- dds[keep,]

#Call DESeq2
dds <- DESeq(dds)

#PCA
vsd <- vst(dds, blind=FALSE)
plotPCA(vsd, intgroup=c("Group"), returnData=FALSE)

#retrieve results of desired comparisons
DEG_Covid19AI <- results(dds, name = "Group_Covid19AI_vs_Healthy")
DEG_Covid193Mo <- results(dds, name = "Group_Covid193Mo_vs_Healthy")
DEG_Covid196Mo <- results(dds, name = "Group_Covid196Mo_vs_Healthy")

#filter DEGs: |FC| > 2 and FDR < 0.05
DEG_Covid19AI_DF <- filter(as.data.frame(DEG_Covid19AI), padj <= 0.05 & abs(log2FoldChange)>2)
DEG_Covid193Mo_DF <- filter(as.data.frame(DEG_Covid193Mo), padj <= 0.05 & abs(log2FoldChange)>2)
DEG_Covid196Mo_DF <- filter(as.data.frame(DEG_Covid196Mo), padj <= 0.05 & abs(log2FoldChange)>2)
```

We can also compare the previous list of genes to the results edgeR can bring:

```
# set DGE class with edgeR and limma
dge <- DGEList(counts=GSE198256_count)

# Make sure on the metadata
rownames(Meta_GSE198256)==colnames(GSE198256_count)
Group[Group=="Covid19: Acute infection"] <- "Covid19AI"
Group[Group=="Covid19: Recovery 3Mo"] <- "Covid193Mo"
Group[Group=="Covid19: Recovery 6Mo"] <- "Covid196Mo"
design <- model.matrix(~ Group )
colnames(design) <- c("Intercept","Covid196Mo","Covid19AI","Healthy")

# Filter
keep <- filterByExpr(dge, design=design, min.count=10)
dge <- dge[keep,keep.lib.sizes=FALSE]

# Normalization
dge <- calcNormFactors(dge)

v <- voom(dge, design)

fit <- lmFit(v, design) #linear model for each gene

contrast.matrix <- makeContrasts(Covid19AI-Healthy, Healthy,
                                 Covid196Mo-Healthy,
```

```
                                levels=design)

fit2 <- contrasts.fit(fit, contrast.matrix)
fit2 <- eBayes(fit2) #dgea by empirical bayes moderation

# Store all of them
voom1 <- topTable(fit2,coef=1,sort="none",n=Inf, p.value = 0.05, fc = 2) #AI vs healthy
voom2 <- topTable(fit2,coef=2,sort="none",n=Inf, p.value = 0.05, fc = 2) #3Months vs healthy
voom3 <- topTable(fit2,coef=3,sort="none",n=Inf, p.value = 0.05, fc = 2) #6Months vs healthy
```

We can intersect the list of DEGs from both appproaches:

```
require(VennDiagram)

venn.diagram(x = list(rownames(voom1), rownames(DEG_Covid19AI_DF)),
        category.names = c("EdgeR" , "DESeq2"),
        filename = "VennCoef1.png",
        main = "AIvsHealthy",  imagetype = "png")

venn.diagram(x = list(rownames(voom2), rownames(DEG_Covid193Mo_DF)),
        category.names = c("EdgeR" , "DESeq2"),
        filename = "VennCoef2.png",
        main = "3MonthsvsHealthy", imagetype = "png")

venn.diagram(x = list(rownames(voom3), rownames(DEG_Covid196Mo_DF)),
        category.names = c("EdgeR" , "DESeq2"),
        filename = "VennCoef3.png",
        main = "6MonthsvsHealthy", imagetype = "png")
```

Get information on the DEGs:

```
#get annotations on DEGs
DEG_Covid19AI_DF$symbol <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid19AI_DF),
                            column="SYMBOL", keytype="ENTREZID", multiVals="first")
DEG_Covid19AI_DF$entrez <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid19AI_DF),
                            column="ENTREZID", keytype="ENTREZID", multiVals="first")
DEG_Covid19AI_DF$name <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid19AI_DF),
                           column="GENENAME", keytype="ENTREZID", multiVals="first")
DEG_Covid19AI_DF$refseq <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid19AI_DF),
                            column="REFSEQ", keytype="ENTREZID", multiVals="first")
DEG_Covid19AI_DF$ensembl <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid19AI_DF),
                             column="ENSEMBL", keytype="ENTREZID", multiVals="first")
#get annotations on DEGs
DEG_Covid193Mo_DF$symbol <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid193Mo_DF),
                            column="SYMBOL", keytype="ENTREZID", multiVals="first")
DEG_Covid193Mo_DF$entrez <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid193Mo_DF),
                            column="ENTREZID", keytype="ENTREZID", multiVals="first")
DEG_Covid193Mo_DF$name <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid193Mo_DF),
                           column="GENENAME", keytype="ENTREZID", multiVals="first")
DEG_Covid193Mo_DF$refseq <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid193Mo_DF),
                            column="REFSEQ", keytype="ENTREZID", multiVals="first")
DEG_Covid193Mo_DF$ensembl <- mapIds(org.Hs.eg.db, keys=rownames(DEG_Covid193Mo_DF),
                             column="ENSEMBL", keytype="ENTREZID", multiVals="first")
```

Heatmap of DEGs across groups:

```
#make matrix of FC for all groups
DEGs_FC <- matrix(0, nrow = 510, ncol = 3)
rownames(DEGs_FC) <- rownames(DEG_Covid19AI_DF)
colnames(DEGs_FC) <- c("AI", "3Mo", "6Mo")
idx1 <- rownames(DEG_Covid19AI) %in% rownames(DEGs_FC)
DEGs_FC[,1] <- as.data.frame(DEG_Covid19AI)[idx1,2]

idx2 <- rownames(DEG_Covid193Mo) %in% rownames(DEGs_FC)
DEGs_FC[,2] <- as.data.frame(DEG_Covid193Mo)[idx2,2]

idx3 <- rownames(DEG_Covid196Mo) %in% rownames(DEGs_FC)
DEGs_FC[,3] <- as.data.frame(DEG_Covid196Mo)[idx3,2]

DEGs_FC1 <- DEGs_FC[!(row.names(DEGs_FC)  == "1832"),]
pheatmap(DEGs_FC1,
         cluster_rows = TRUE,
         cluster_cols = TRUE,
         show_rownames = FALSE,
         show_colnames = TRUE)

col <- colorRampPalette(brewer.pal(10, "RdYlBu"))(256)
norm_counts <- counts(dds, normalized=TRUE)
norm_counts <- norm_counts[(row.names(norm_counts)  %in% rownames(DEGs_FC)),]
colnames(norm_counts) <- coldata$Group
heatmap <- heatmap(norm_counts, col=col)
```

Perform GO analysis:

In the previous version of this work, I didnt use a background list of genes.

```
#GO cmd
egoAIH.noU <- enrichGO(gene        = rownames(DEG_Covid19AI_DF),
                 OrgDb         = org.Hs.eg.db,
                 ont           = "ALL",
                 pAdjustMethod = "BH",
                 pvalueCutoff  = 0.05,
                 qvalueCutoff  = 0.1,
                 readable      = TRUE)
head(egoAIH.noU)

#dotplot
dotplot(egoAIH.noU, showCategory = 20,
        font.size = 10,
        title = "AI vs H without Universe")

egoAIH <- enrichGO(gene        = rownames(DEG_Covid19AI_DF),
                 OrgDb         = org.Hs.eg.db,
                 universe      = rownames(DEG_Covid19AI),
                 ont           = "ALL",
                 pAdjustMethod = "BH",
                 pvalueCutoff  = 0.05,
                 qvalueCutoff  = 0.1,
```

```
                readable      = TRUE)
head(egoAIH)

#dotplot
dotplot(egoAIH, showCategory = 20,
        font.size = 10,
        title = "AI vs H with Universe")
```

GSEA analysis for acute and early recovery:

In the previous version I didnt use as input all the genes with their corresponding fold change, instead I only used as input genes I selected as differentially expressed.

```
#sort list of genes according to LF value
DEG_Covid19AI_sg <- DEG_Covid19AI$log2FoldChange
names(DEG_Covid19AI_sg) <- rownames(DEG_Covid19AI)
DEG_Covid19AI_sg <- na.omit(DEG_Covid19AI_sg)
DEG_Covid19AI_sg <- sort(DEG_Covid19AI_sg, decreasing = TRUE)

gse_AI_All <- gseGO(geneList=DEG_Covid19AI_sg,
             ont ="ALL",
             keyType = "ENTREZID",
             minGSSize = 3,
             maxGSSize = 800,
             pvalueCutoff = 0.05,
             verbose = TRUE,
             OrgDb = org.Hs.eg.db,
             pAdjustMethod = "none")
gse_AI_BP <- gseGO(geneList=DEG_Covid19AI_sg,
             ont ="BP",
             keyType = "ENTREZID",
             minGSSize = 3,
             maxGSSize = 800,
             pvalueCutoff = 0.05,
             verbose = TRUE,
             OrgDb = org.Hs.eg.db,
             pAdjustMethod = "none")
gse_AI_CC <- gseGO(geneList=DEG_Covid19AI_sg,
             ont ="CC",
             keyType = "ENTREZID",
             minGSSize = 3,
             maxGSSize = 800,
             pvalueCutoff = 0.05,
             verbose = TRUE,
             OrgDb = org.Hs.eg.db,
             pAdjustMethod = "none")

dotplot(gse_AI_All, showCategory=10, split=".sign", font.size = 10) +
  facet_grid(.~.sign)  +
  scale_y_discrete(labels=function(egoBP) str_wrap(egoBP, width=70))

dotplot(gse_AI_BP, showCategory=10, split=".sign", font.size = 10) +
  facet_grid(.~.sign)  +
  scale_y_discrete(labels=function(egoBP) str_wrap(egoBP, width=70))
```

```
dotplot(gse_AI_CC, showCategory=10, split=".sign", font.size = 10) +
  facet_grid(.~.sign)  +
  scale_y_discrete(labels=function(egoBP) str_wrap(egoBP, width=70))
```

```
#sort list of genes according to LF value
DEG_Covid193Mo_sg <- DEG_Covid193Mo_DF$log2FoldChange
names(DEG_Covid193Mo_sg) <- DEG_Covid193Mo_DF$symbol
DEG_Covid193Mo_sg <- na.omit(DEG_Covid193Mo_sg)
DEG_Covid193Mo_sg <- sort(DEG_Covid193Mo_sg, decreasing = TRUE)

gse_3Mo_All <- gseGO(geneList=DEG_Covid193Mo_sg,
              ont ="ALL",
              keyType = "SYMBOL",
              minGSSize = 3,
              maxGSSize = 800,
              pvalueCutoff = 0.05,
              verbose = TRUE,
              OrgDb = org.Hs.eg.db,
              pAdjustMethod = "none")
gse_3Mo_BP <- gseGO(geneList=DEG_Covid193Mo_sg,
              ont ="BP",
              keyType = "SYMBOL",
              minGSSize = 3,
              maxGSSize = 800,
              pvalueCutoff = 0.05,
              verbose = TRUE,
              OrgDb = org.Hs.eg.db,
              pAdjustMethod = "none")
gse_3Mo_CC <- gseGO(geneList=DEG_Covid193Mo_sg,
              ont ="CC",
              keyType = "SYMBOL",
              minGSSize = 3,
              maxGSSize = 800,
              pvalueCutoff = 0.05,
              verbose = TRUE,
              OrgDb = org.Hs.eg.db,
              pAdjustMethod = "none")

dotplot(gse_3Mo_All, showCategory=10, split=".sign", font.size = 10) +
  facet_grid(.~.sign)  +
  scale_y_discrete(labels=function(egoBP) str_wrap(egoBP, width=70))

dotplot(gse_3Mo_BP, showCategory=10, split=".sign", font.size = 10) +
  facet_grid(.~.sign)  +
  scale_y_discrete(labels=function(egoBP) str_wrap(egoBP, width=70))

dotplot(gse_3Mo_CC, showCategory=10, split=".sign", font.size = 10) +
  facet_grid(.~.sign)  +
  scale_y_discrete(labels=function(egoBP) str_wrap(egoBP, width=70))
```

Reference:

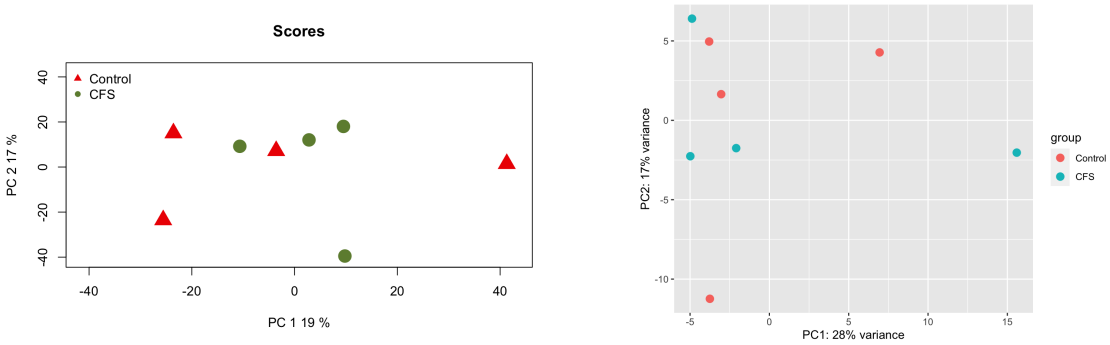

1. Brauns, E., Azouz, A., Grimaldi, D., Xiao, H., Thomas, S., Nguyen, M., ... & Goriely, S. (2022).

Functional reprogramming of monocytes in patients with acute and convalescent severe COVID-19. JCI insight, 7(9). JCI Insight. 2022;7(9):e154183. https://doi.org/10.1172/jci.insight.154183.

## GSE214282

*Transcriptome profiling of classical monocytes in ME/CFS post exercise challenge*

Monocytes were collected 24 hrs after the participants performed a cardiopulmonary exercise test (CPET). Patients belonging to two different groups were included in this study: 4 myalgic encephalomyelitis, also called chronic fatigue syndrome or ME/CFS, and 4 healthy controls. The study has not been published yet. The raw counts were downloaded from the GEO database. A PCA showed poor separation betwwen the two groups of this study (Fig. 1).



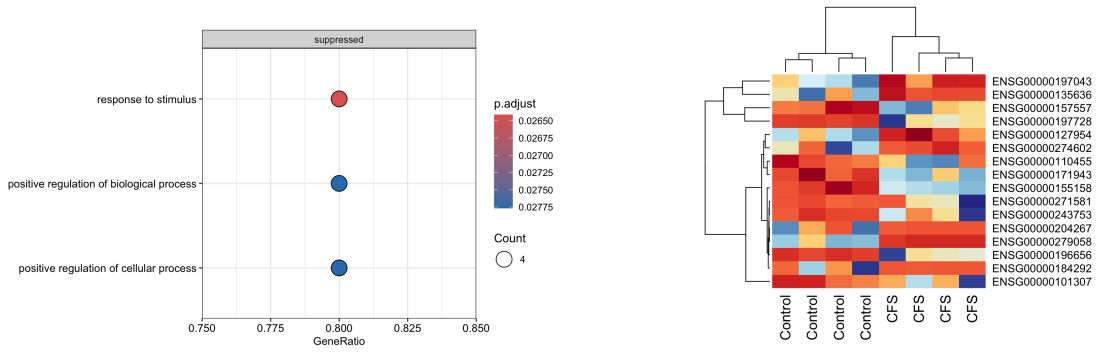

a) b)

**Figure 7:** a) PCA of RNA seq samples done with NOISeq and b) PCA made with DESeq2.

As expected not many DEGs were found: 16 genes which became 11 after LF shrinkage. This set of genes were found with not conservative parameters: padj $< 0.01$ and $|FC| > 1$. When performed GO analysis no relevant pathways came to light. For GSEA 3 pathways were found to be significantly down-regulated.

When we plot the FC of the DEGs as a heatmap, we can see genes do not show a clear different pattern of expression between the conditions.

The response this dataset shows is very noisy. This could be because the ME/CFS does not have a huge impact on the biology of these cells. Another explanation could be that effort the CPET is not a good proxy to examine how this syndrome affects the white blood cells.




a) b)

**Figure 8:** a) GSEA and b) Heatmap done only with the DEGs.

‘ ## Code

Load the needed libraries:

```
library(GEOquery)
library(NOISeq)
library(DESeq2)
library(clusterProfiler)
library(org.Hs.eg.db)
library(RColorBrewer)
library(gplots)
```

Download the counts and metadata:

```
#read countys in
counts <- read.table(file = "GSE214282_rawCounts.txt", sep = "\t", header = TRUE)
head(counts)

#geo
gset <- GEOquery::getGEO("GSE214282")[[1]]

#Get the sample metadata
metadata <- gset@phenoData@data
group <- metadata[,c("characteristics_ch1.1","geo_accession")]
group[group=="disease state: control",1] <- "Control"
group[group=="disease state: case",1] <- "CFS"
colnames(group) <- c("Group","SampleID")

#wirte gene names for biomart
#write.csv(counts$Id, file = "GSE214282_genes.txt",quote = FALSE,row.names = FALSE)

rownames(counts) <- counts$Id
counts <- counts[,2:9]
```

Download the genes information for NOISeq analysis:

```
#get biomart GC content, gene start, gene end, chr
biomart2 <- read.delim(file = "mart_export2.txt", header = 1, sep = "\t")
#filter out non cannonical chr, keep only the first annotation of each gene
biomart_filt2 <- biomart2[biomart2$Chromosome.scaffold.name %in% c(as.character(1:22) ,"X","Y"),]
biomart_filt2 <- biomart_filt2[!duplicated(biomart_filt2$NCBI.gene..formerly.Entrezgene..ID),]
biomart_filt2 <- na.omit(biomart_filt2)
row.names(biomart_filt2) <- biomart_filt2$NCBI.gene..formerly.Entrezgene..ID

#length
len <- biomart_filt2$Gene.end..bp. - biomart_filt2$Gene.start..bp.
names(len) <- biomart_filt2$NCBI.gene..formerly.Entrezgene..ID

#GC%
gc <- biomart_filt2$Gene...GC.content
names(gc) <- biomart_filt2$NCBI.gene..formerly.Entrezgene..ID

#biotype
type <- biomart_filt2$Gene.type
```

```
names(type) <- biomart_filt2$NCBI.gene..formerly.Entrezgene..ID

#chr
chr <- data.frame(Chr=biomart_filt2$NCBI.gene..formerly.Entrezgene..ID,
                  GeneStart=biomart_filt2$Gene.start..bp.,
                  GeneEnd=biomart_filt2$Gene.end..bp.,
                  row.names = biomart_filt2$NCBI.gene..formerly.Entrezgene..ID)

#filter from tbl the not annotated genes
counts_filt <- counts[rownames(counts) %in% rownames(biomart_filt2),]
counts_exc <- counts[!(rownames(counts) %in% rownames(biomart_filt2)),]
annotgene_ord <- biomart_filt2[rownames(counts_filt),]
#sum(rownames(annotgene_ord)==rownames(tbl_filt))

#df of exp design
myfactors <- data.frame(group$Group)
colnames(myfactors)[1]<- "Group"

#make data inot NOISeq object
NOISeq_obj <- readData(data = counts, length = len, gc = gc,
                       chromosome = chr, factors = myfactors, biotype = type)

#PCA NOISeq
myPCA = dat(NOISeq_obj, type = "PCA")
par(mfrow = c(1, 1))
explo.plot(myPCA, factor = "Group")

#counts
mycountsbio = dat(NOISeq_obj, factor = NULL, type = "countsbio")
par(mfrow = c(1, 1))
explo.plot(mycountsbio, toplot = 1, samples = 1, plottype = "boxplot")
```

Let's call DESeq2 and perform the DGEA:

```
#prepare matadata for deseq2
coldata2 <- pData(NOISeq_obj)
coldata2$Group <- factor(coldata2$Group, levels = unique(coldata2$Group))

#create deseq2 obj
dds2 <- DESeqDataSetFromMatrix(countData = counts,
                               colData = coldata2,
                               design = ~ Group)

#filter low counts genes
smallestGroupSize <- 2
keep <- rowSums(counts(dds2) >= 1) >= smallestGroupSize
dss2 <- dds2[keep,]

#Call DESeq2
dds2 <- DESeq(dds2)
resultsNames(dds2)

#PCA
```

```
vsd2 <- vst(dds2, blind=FALSE)
plotPCA(vsd2, intgroup=c("Group"), returnData=FALSE)

#counts dist
boxplot(log10(counts), pch=".",
        horizontal=TRUE, cex.axis=0.5,
        las=1, ylab="Samples", xlab="log2(Counts +1)")

#library(reshape2)
count_melt <- reshape2::melt(log2(counts + 1))
ggplot(data=count_melt, mapping=aes(x=value, color=variable)) + geom_density()

plotCounts(dds2, gene=which.min(CFSvsCTL$padj), intgroup="Group")

sizeFactors(dds2)

#retrieve results of desired comparisons
CFSvsCTL <- results(dds2, name = "Group_CFS_vs_Control")

CFSvsCTL_LFC <- lfcShrink(dds2, coef="Group_CFS_vs_Control", type="apeglm")

plotMA(CFSvsCTL, ylim=c(-2,2))
plotMA(CFSvsCTL_LFC, ylim=c(-2,2))

#filter DEGs: |FC| > 2 and FDR < 0.05
CFSvsCTL_DF <- filter(as.data.frame(CFSvsCTL), padj <= 0.1 & abs(log2FoldChange)>0.5)
CFSvsCTL_LFC_DF <- filter(as.data.frame(CFSvsCTL_LFC), padj <= 0.1 & abs(log2FoldChange)>0.5)
```

Get information on the DEGs:

```
#get annotations on DEGs
CFSvsCTL_DF$symbol <- mapIds(org.Hs.eg.db, keys=rownames(CFSvsCTL_DF),
                             column="SYMBOL", keytype="ENSEMBL", multiVals="first")
CFSvsCTL_DF$entrez <- mapIds(org.Hs.eg.db, keys=rownames(CFSvsCTL_DF),
                             column="ENTREZID", keytype="ENSEMBL", multiVals="first")
CFSvsCTL_DF$name <- mapIds(org.Hs.eg.db, keys=rownames(CFSvsCTL_DF),
                           column="GENENAME", keytype="ENSEMBL", multiVals="first")
```

Heatmap of DEGs across groups:

```
#make matrix of FC for all groups
col <- colorRampPalette(brewer.pal(10, "RdYlBu"))(256)
norm_counts <- counts(dds2, normalized=TRUE)
norm_counts <- norm_counts[(row.names(norm_counts)  %in% rownames(CFSvsCTL_DF)),]
colnames(norm_counts) <- coldata2$Group
heatmap <- heatmap(norm_counts, col=col)
```

Perform GO analysis:

```
#GO cmd
egoCFS <- enrichGO(gene         = rownames(CFSvsCTL_DF),
                   OrgDb        = org.Hs.eg.db,
```

```
                ont           = "ALL",
                pAdjustMethod = "BH",
                pvalueCutoff  = 0.05,
                qvalueCutoff  = 0.1,
                readable      = TRUE)
head(egoCFS)

#dotplot
dotplot(egoCFS, showCategory = 20,
        font.size = 10,
        title = "AI vs H")
```

GSEA analysis for acute and early recovery:

```
#sort list of genes according to LF value
CFSvsCTL_sg <- CFSvsCTL_DF$log2FoldChange
names(CFSvsCTL_sg) <- rownames(CFSvsCTL_DF)
CFSvsCTL_sg <- na.omit(CFSvsCTL_sg)
CFSvsCTL_sg <- sort(CFSvsCTL_sg, decreasing = TRUE)

gse_CFS_All <- gseGO(geneList=CFSvsCTL_sg,
             ont ="ALL",
             keyType = "ENSEMBL",
             minGSSize = 3,
             maxGSSize = 800,
             pvalueCutoff = 0.05,
             verbose = TRUE,
             OrgDb = org.Hs.eg.db,
             pAdjustMethod = "none")

head(gse_CFS_All)

dotplot(gse_CFS_All, showCategory=10, split=".sign", font.size = 10) +
  facet_grid(.~.sign)  +
  scale_y_discrete(labels=function(egoBP) str_wrap(egoBP, width=70))
```