PCA, UMAP and Hierarchical Clustering Boeva Neuroblastoma Cells

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Contents

1) Objectives of Analysis

This notebook uses RNA-Seq data from a published paper (Boeva et al. 2017, Nature Genetics, doi:10.1038/ng.3921) to compare Hierarchical Clustering, Principal Component and Uniform Manifold Approximation and Projection (UMAP) analysis for the classification of cells derived from pediatric cancer neuroblastoma, described by Boeva et al., 2017.

The article brings the concept of neuroblatoma as a heterogeneous tumor composed fundamentally of two distict cell populations: adrenergic and mesenchymal cells. Adrenergic cells represent a group of differentiated cells in the neuroblastoma tumor, which are more easily targeted by chemotherapy than mesenchymal cells.

On the other hand, chemotherapy may leave Minimal Residual Disease in the form of mesenchymal cells which may lead to disease progression after treatment, by allowning tumor to continue its malignant development. One possible therapeutic venue is the use of drugs that break the homestatic equilibrium between the

adrenergic and mesenchymal states of cells and make cells become more differentiated, or turn mesenchymal cells into adrenergic cells.

We are currenlty developing a study to quantify ADRN and MES phenotypes using wither RNA-Seq or epigenetic modification 5hmC profiles of cells, tumors and cfDNA. Our hypotheses is that MES scores can be measured from cfDNA 5hmC differentially expressed gene lists.

2) Creating Analysis folders

File organization is essential for clear and neat procedure steps. The chunk bellow creates folders for the data, plots and results in the same directory where the Rmd notebook is saved.

```
# Create the data folder if it doesn't exist
if (!dir.exists("data")) {
  dir.create("data")
}

# Define the file path to the plots directory
plots_dir <- "plots"

# Create the plots folder if it doesn't exist
if (!dir.exists(plots_dir)) {
  dir.create(plots_dir)
}

# Define the file path to the results directory
results_dir <- "results"

# Create the results folder if it doesn't exist
if (!dir.exists(results_dir)) {
  dir.create(results_dir)
}
```

2. 2) Check File Structure

The analysis folder, downloaded from GitHub, where the Rmd notebook is saved, should contain:

- The example analysis `.Rmd` downloaded
- A folder called "data" which contains:
  - The `Boeva` folder which contains:
    - The gene expression file GSE90683_Log2FPKMExpressionSummary.txt
    - The metadata file metadata_Boeva_Modified.txt
- A folder for `plots` (currently empty)
- A folder for `results` (currently empty)

Your example analysis folder should now look something like this.

In order for the example here to run without a hitch, we need these files to be in these locations. The next chunks are a test to check before we get started with the analysis. These chunks will declare your file paths and double check that your files are in the right place.

First we will declare our file paths to our data and metadata files, which should be in our data directory. This is handy to do because if we want to switch the dataset (see next section for more on this) we are using for this analysis, we will only have to change the file path here to get started.

```
# Define the file path to the data directory
# Replace with the path of the folder the files will be in
data_dir <- file.path("data", "Boeva")

# Declare the file path to the gene expression matrix file
# inside directory saved as `data_dir`
# Replace with the path to your dataset file
data_file <- file.path(data_dir, "GSE90683_Log2FPKMExpressionSummary.txt")

# Declare the file path to the metadata file
# inside the directory saved as `data_dir`
# Replace with the path to your metadata file
metadata_file <- file.path(data_dir, "metadata_SRP133573.tsv")
metadata_file <- file.path(data_dir, "metadata_Boeva_Modified.txt")
```

Now that our file paths are declared, we can use the `file.exists()` function to check that the files are where we specified above.

```
# Check if the gene expression matrix file is at the path stored in `data_file`
file.exists(data_file)
```

```
## [1] TRUE
```

```
# Check if the metadata file is at the file path stored in `metadata_file`
file.exists(metadata_file)
```

```
## [1] TRUE
```

3) UMAP Visualization - RNA-seq

Install libraries

We will use libraries DESeq2 umap, ggplot2 and magrittr. If you do not have the libraries installed, install them. If you have these libraries installed, you can skip this step.

```
if (!("DESeq2" %in% installed.packages())) {
  # Install DESeq2
  BiocManager::install("DESeq2", update = FALSE)
}

if (!("umap" %in% installed.packages())) {
  # Install umap package
  BiocManager::install("umap", update = FALSE)
}

if (!("ggfortify" %in% installed.packages())) {
  # Install ggfortify package
  BiocManager::install("ggfortify", update = FALSE)
}

if (!("pheatmap" %in% installed.packages())) {
```

```
  # Install pheatmap package
  BiocManager::install("pheatmap", update = FALSE)
}
```

Attach packages used in this analysis:

```
# Attach the `DESeq2` library
library(DESeq2)

# Attach the `umap` library
library(umap)

# Attach the `ggplot2` library for plotting
library(ggplot2)

# We will need this so we can use the pipe: %>%
library(magrittr)

# Attach the `ggfortify` library for PCA using prcomp()
library(ggfortify)

# Attach the `pheatmap` library for PCA using prcomp()
library(pheatmap)

# Set the seed so our results are reproducible:
set.seed(12345)
```

Import and set up data

In the chunk bellow, the gene expression table needs to be downloaded from the Gene Expression Omnibus or used directly from this repository. It was not possible to import the gene expression data-frame using the exact same code provided in the original ALSF Rmd notebook, because that was giving an error due to duplicated gene names. The

```
# Read in metadata TSV file and gene expression data-frame file.
metadata <- readr::read_tsv(metadata_file)
```

```
##
## -- Column specification --------------------------------------------------------
## cols(
##   .default = col_character(),
##   refinebio_age = col_logical(),
##   refinebio_cell_line = col_logical(),
##   refinebio_compound = col_logical(),
##   refinebio_disease_stage = col_logical(),
##   refinebio_genetic_information = col_logical(),
##   refinebio_processed = col_logical(),
##   refinebio_sex = col_logical(),
##   refinebio_source_archive_url = col_logical(),
##   refinebio_specimen_part = col_logical(),
##   refinebio_time = col_logical()
## )
## i Use `spec()` for the full column specifications.
```

```
data_file <- file.path(data_dir, "GSE90683_Log2FPKMExpressionSummary.txt") ## repeated from second chun

# Original code from ALSF:
# Read in data TSV file
# First time, there was this error: Error in `.rowNamesDF<-`(x, value = value) : duplicate 'row.names'
# expression_df <- readr::read_tsv(data_file, ) %>%
# Tuck away the gene ID  column as row names, leaving only numeric values
#  tibble::column_to_rownames("gene")

## Import gene expression data-frame using the entire path provided by downloading our repository.
expression_df <- read.table("data/Boeva/GSE90683_Log2FPKMExpressionSummary.txt", header = T)
## This way of gene expression data-frame upload needs to deal with the duplicated gene names problem.
## Need to remove repeated gene names with line of code bellow.
## Remove duplicated rows
expression_df <- expression_df[!duplicated(expression_df$gene), ]
## Name rows with gene names
rownames(expression_df) <- expression_df$gene
## Remove Gene Extra-column
expression_df <- subset(expression_df, select = -c(gene))
```

Check that metadata and data are in the same sample order.

```
# Make the data in the order of the metadata
expression_df <- expression_df %>%
  dplyr::select(metadata$refinebio_accession_code)

# Check if this is in the same order
all.equal(colnames(expression_df), metadata$refinebio_accession_code)
```

```
## [1] TRUE
```

Choose metadata annotation columns to be used in analysis.

```
# convert the columns we will be using for annotation into factors
metadata_metadata <- metadata %>%
  dplyr::select( # select only the columns that we will need for plotting
    refinebio_accession_code,
    refinebio_treatment,
    refinebio_disease
  )
```

Set minimum of counts to be used in analysis.

```
filtered_expression_df <- expression_df %>%
  dplyr::filter(rowSums(.) >= 100)
```

Counts need to be rounded before their values are passed to `DESeqDataSetFromMatrix()` function.

```
filtered_expression_df <- round(filtered_expression_df)
```

The chunck bellow creates `DESeqDataSet` object from gene expression data-frame as input. This highlights the DESeqDataSetFromMatrix function.

```
dds <- DESeqDataSetFromMatrix(
  countData = filtered_expression_df, # the counts values for all samples in our dataset
  colData = metadata, # annotation data for the samples in the counts data frame
  design = ~1 # Here we are not specifying a model
  # Replace with an appropriate design variable for your analysis
```

```
)
```

```
## converting counts to integer mode
```

4) PCA with DESeq

4.1) PCA with DESeq

```
combinedDNAHTSeq<- DESeq(dds)
```

```
## Warning in DESeq(dds): the design is ~ 1 (just an intercept). is this intended?
```

```
## estimating size factors
```

```
## estimating dispersions
```

```
## gene-wise dispersion estimates
```

```
## mean-dispersion relationship
```

```
## -- note: fitType='parametric', but the dispersion trend was not well captured by the
##    function: y = a/x + b, and a local regression fit was automatically substituted.
##    specify fitType='local' or 'mean' to avoid this message next time.
```

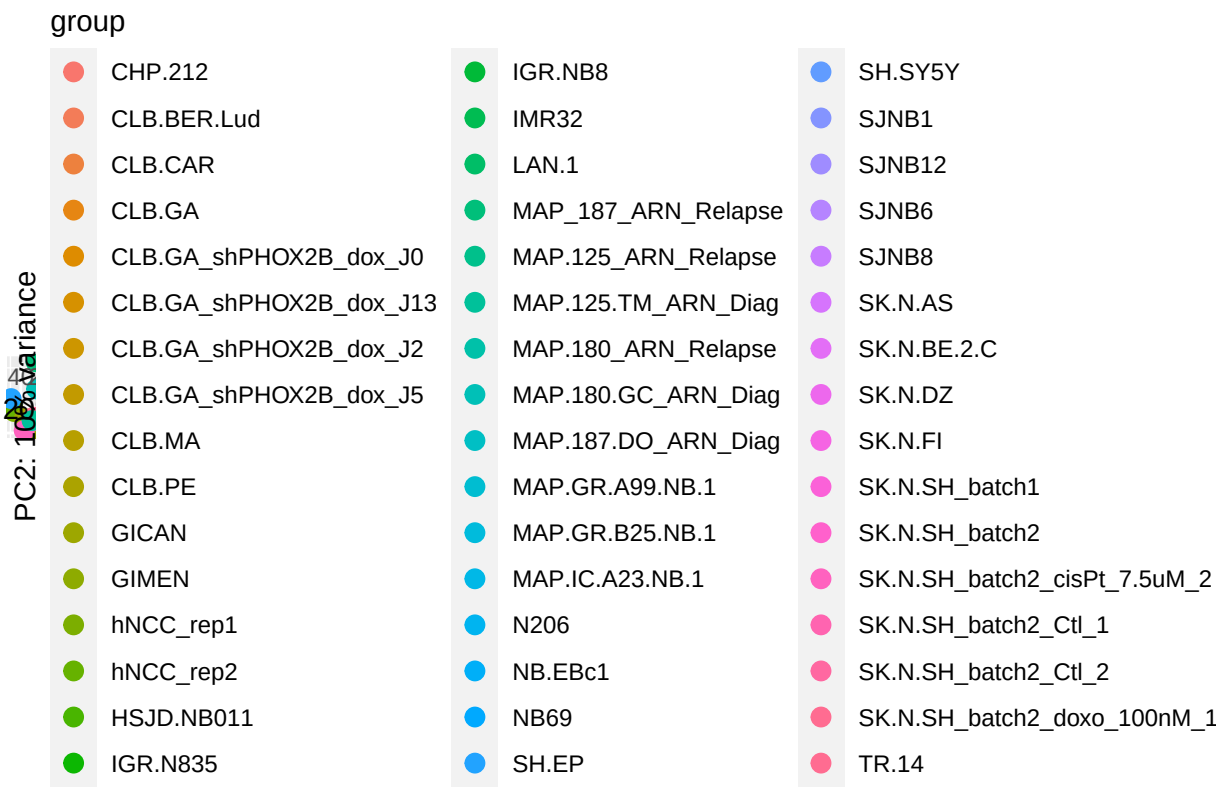
```
## final dispersion estimates
```

```
## fitting model and testing
```

```
summary(combinedDNAHTSeq)
```

```
## [1] "DESeqDataSet object of length 11298 with 19 metadata columns"
```

```
vst_Boeva <- vst(combinedDNAHTSeq, blind=FALSE)
## Plot PCA
plotPCA(vst_Boeva, intgroup = c("refinebio_accession_code"))
```




```
colData(dds)
```

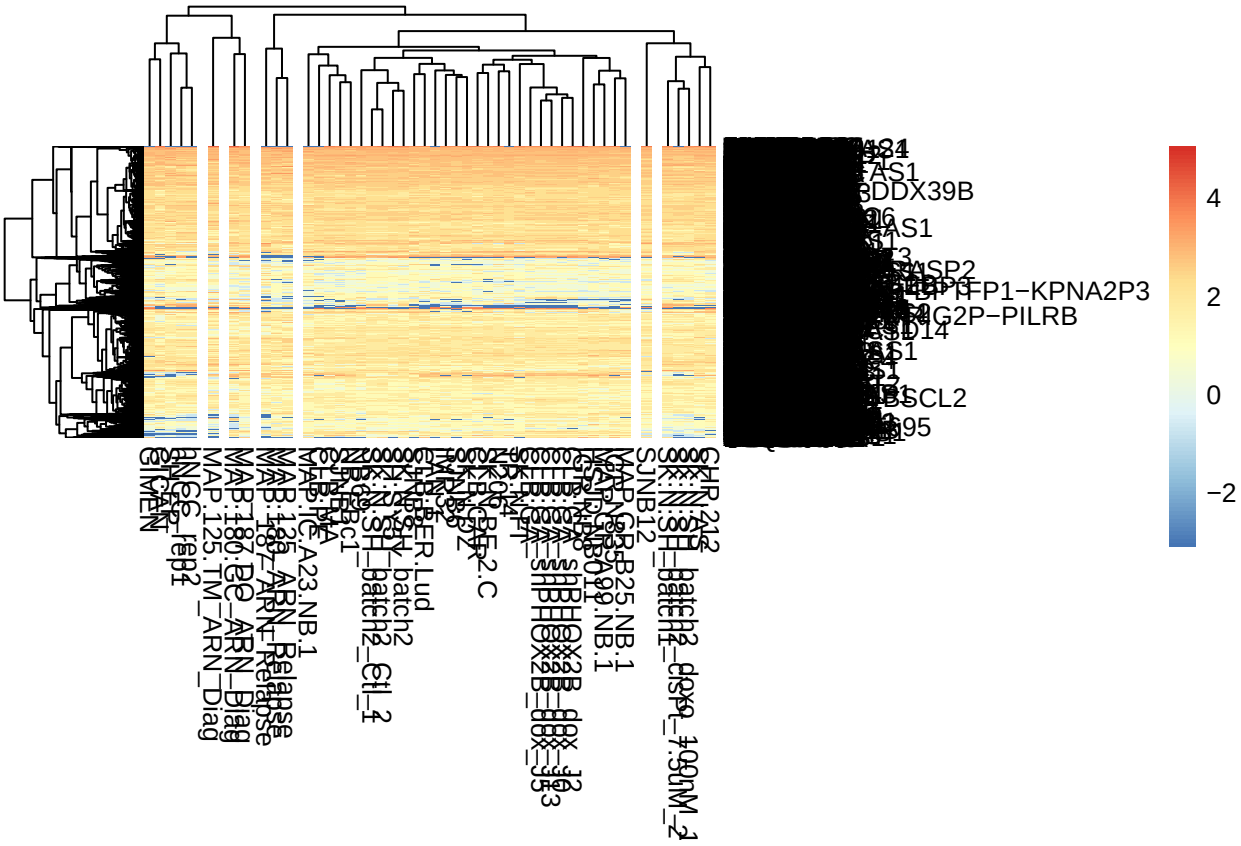
```
## DataFrame with 48 rows and 20 columns
##                     refinebio_accession_code experiment_accession refinebio_age
##                                  <character>          <character>     <logical>
## SJNB6                                SJNB6            SRP133573            NA
## SJNB8                                SJNB8            SRP133573            NA
## SK.N.AS                            SK.N.AS            SRP133573            NA
## CLB.CAR                            CLB.CAR            SRP133573            NA
## CLB.PE                              CLB.PE            SRP133573            NA
## ...                                    ...                  ...           ...
## MAP.180.GC_ARN_Diag      MAP.180.GC_ARN_Diag            SRP133573            NA
## MAP.187.DO_ARN_Diag      MAP.187.DO_ARN_Diag            SRP133573            NA
## MAP.180_ARN_Relapse      MAP.180_ARN_Relapse            SRP133573            NA
## MAP.125_ARN_Relapse      MAP.125_ARN_Relapse            SRP133573            NA
## MAP_187_ARN_Relapse      MAP_187_ARN_Relapse            SRP133573            NA
##                     refinebio_cell_line refinebio_compound
##                               <logical>          <logical>
## SJNB6                                NA                 NA
## SJNB8                                NA                 NA
## SK.N.AS                              NA                 NA
## CLB.CAR                              NA                 NA
## CLB.PE                               NA                 NA
## ...                                 ...                ...
## MAP.180.GC_ARN_Diag                  NA                 NA
## MAP.187.DO_ARN_Diag                  NA                 NA
## MAP.180_ARN_Relapse                  NA                 NA
## MAP.125_ARN_Relapse                  NA                 NA
## MAP_187_ARN_Relapse                  NA                 NA
```

```
##                         refinebio_disease refinebio_disease_stage
##                               <character>               <logical>
## SJNB6                radical prostatectomy                      NA
## SJNB8                radical prostatectomy                      NA
## SK.N.AS              radical prostatectomy                      NA
## CLB.CAR              radical prostatectomy                      NA
## CLB.PE                             biopsy                       NA
## ...                                   ...                      ...
## MAP.180.GC_ARN_Diag  radical prostatectomy                      NA
## MAP.187.DO_ARN_Diag                biopsy                       NA
## MAP.180_ARN_Relapse                biopsy                       NA
## MAP.125_ARN_Relapse                biopsy                       NA
## MAP_187_ARN_Relapse                biopsy                       NA
##                      refinebio_genetic_information refinebio_organism
##                                          <logical>        <character>
## SJNB6                                           NA       HOMO_SAPIENS
## SJNB8                                           NA       HOMO_SAPIENS
## SK.N.AS                                         NA       HOMO_SAPIENS
## CLB.CAR                                         NA       HOMO_SAPIENS
## CLB.PE                                          NA       HOMO_SAPIENS
## ...                                            ...                ...
## MAP.180.GC_ARN_Diag                             NA       HOMO_SAPIENS
## MAP.187.DO_ARN_Diag                             NA       HOMO_SAPIENS
## MAP.180_ARN_Relapse                             NA       HOMO_SAPIENS
## MAP.125_ARN_Relapse                             NA       HOMO_SAPIENS
## MAP_187_ARN_Relapse                             NA       HOMO_SAPIENS
##                                    refinebio_platform refinebio_processed
##                                           <character>           <logical>
## SJNB6                Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## SJNB8                Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## SK.N.AS              Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## CLB.CAR              Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## CLB.PE               Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## ...                                                      ...                 ...
## MAP.180.GC_ARN_Diag  Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## MAP.187.DO_ARN_Diag  Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## MAP.180_ARN_Relapse  Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## MAP.125_ARN_Relapse  Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
## MAP_187_ARN_Relapse  Illumina HiSeq 2500 (IlluminaHiSeq2500)                TRUE
##                      refinebio_race refinebio_sex refinebio_source_archive_url
##                         <character>     <logical>                     <logical>
## SJNB6                         white            NA                            NA
## SJNB8                         white            NA                            NA
## SK.N.AS                       white            NA                            NA
## CLB.CAR                       white            NA                            NA
## CLB.PE                        white            NA                            NA
## ...                             ...           ...                           ...
## MAP.180.GC_ARN_Diag           white            NA                            NA
## MAP.187.DO_ARN_Diag           white            NA                            NA
## MAP.180_ARN_Relapse           white            NA                            NA
## MAP.125_ARN_Relapse           white            NA                            NA
## MAP_187_ARN_Relapse           white            NA                            NA
##                      refinebio_source_database refinebio_specimen_part
##                                    <character>               <logical>
```

```
## SJNB6                                  SRA                     NA
## SJNB8                                  SRA                     NA
## SK.N.AS                                SRA                     NA
## CLB.CAR                                SRA                     NA
## CLB.PE                                 SRA                     NA
## ...                                    ...                    ...
## MAP.180.GC_ARN_Diag                    SRA                     NA
## MAP.187.DO_ARN_Diag                    SRA                     NA
## MAP.180_ARN_Relapse                    SRA                     NA
## MAP.125_ARN_Relapse                    SRA                     NA
## MAP_187_ARN_Relapse                    SRA                     NA
##                         refinebio_subject refinebio_time refinebio_title
##                               <character>      <logical>     <character>
## SJNB6                 prostate tumor tissue             NA       CHU001.RP
## SJNB8                 prostate tumor tissue             NA       CHU001.RP
## SK.N.AS               prostate tumor tissue             NA       CHU001.RP
## CLB.CAR               prostate tumor tissue             NA       CHU001.RP
## CLB.PE                prostate tumor tissue             NA       CHU001.Bx
## ...                                     ...            ...            ...
## MAP.180.GC_ARN_Diag   prostate tumor tissue             NA       CHU009.RP
## MAP.187.DO_ARN_Diag   prostate tumor tissue             NA       CHU009.Bx
## MAP.180_ARN_Relapse   prostate tumor tissue             NA       CHU009.Bx
## MAP.125_ARN_Relapse   prostate tumor tissue             NA       CHU009.Bx
## MAP_187_ARN_Relapse   prostate tumor tissue             NA       CHU009.Bx
##                       refinebio_treatment
##                                 <character>
## SJNB6                             post.adt
## SJNB8                             post.adt
## SK.N.AS                           post.adt
## CLB.CAR                           post.adt
## CLB.PE                             pre.adt
## ...                                    ...
## MAP.180.GC_ARN_Diag               post.adt
## MAP.187.DO_ARN_Diag                pre.adt
## MAP.180_ARN_Relapse                pre.adt
## MAP.125_ARN_Relapse                pre.adt
## MAP_187_ARN_Relapse                pre.adt
```

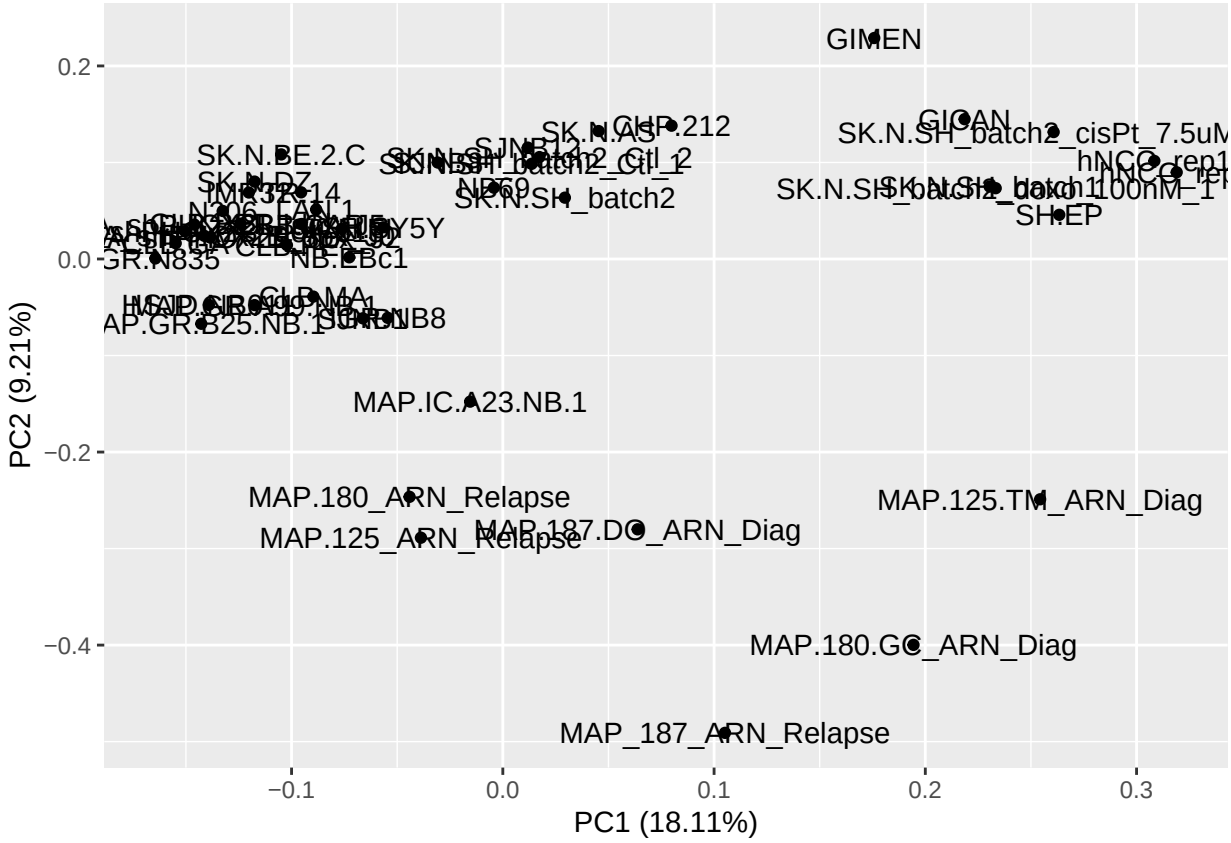
Hieracrchical Clustering Heatmap

```
## Set counts threshold to 100 for ease of plotting
library(pheatmap)
pheatmap(assay(vst_Boeva), cutree_cols = 7)
```



4.2) Plot PCA using expression_df object

```
pca_res <- prcomp(t(expression_df[, -1]))
autoplot(pca_res, label = TRUE, label.size = 4)
```



5) References

PCA plots

PCA GGfortify

https://cran.r-project.org/web/packages/ggfortify/vignettes/plot_pca.html

PCA datacarpentry

https://tavareshugo.github.io/data-carpentry-rnaseq/03_rnaseq_pca.html

https://tavareshugo.github.io/data-carpentry-rnaseq/

https://github.com/tavareshugo/data-carpentry-rnaseq/find/master

https://datacarpentry.org/R-ecology-lesson/

UMAP plots Alexe's Lemonade Stand Foundation

https://github.com/AlexsLemonade/refinebio-examples

https://alexslemonade.github.io/refinebio-examples/03-rnaseq/dimension-reduction_rnaseq_02_umap.html#analysis

UMAP Plots Tutorial with iris dataset, requires R function

https://cran.r-project.org/web/packages/umap/vignettes/umap.html

UMAP Package

https://github.com/lmcinnes/umap#installing

UMAPR Package

https://github.com/ropenscilabs/umapr