R programming skills summary - Experimental Design and Data Management 2022

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Preface

This is a summary of the R programming skills sessions of the Experimental Design and Data Management (2223-MSB1005) course 2022. The course is part of the Master of Systems Biology at the Maastrich University. The course is taught by Prof. Michiel Adriaens and Prof. Aaron Isaacs.

1 Week3 - Introduction to Gene expression analysis part 1

Sebastian Ayala-Ruano
today

This R script serves as a scaffold for adding the code required to fulfill the assignments. It includes the assignments as well as a few hints.

Add the necessary code and type your answers in this document for your own record.

```r
library(ggplot2)
library(biomaRt)
library(pcaMethods)
library(readr)
library(dplyr)
library(tibble)
library(reshape2)
library(conflicted)
library(tidyr)
library(purrr)
```

1.1 Assignment 1: Importing the data and inspecting sample information

After unzipping the required transcriptomics (gene expression) file and sample information file, import both files as two separate objects. First set the active working directory to the folder containing the files.

```r
# Load data
gxData <- read_delim("Data/MAGNET_GeneExpressionData_CPM_19112020.txt", delim ="\t")
```

```
sampleInfo <- read_csv("Data/MAGNET_SampleData_18112022.csv")

# Add gene ID as the row names
gxData <- column_to_rownames(gxData, var = "EnsemblGeneID")

# Add sample name as the row names
sampleInfo <- column_to_rownames(sampleInfo, var = "sample_name")
```

1.1.1 Using the sampleInfo object, answer the following questions: What does DCM, HCM and PPCM stand for? (hint: Google)

- **DCM:** Dilated cardiomyopathy
- **HCM:** Hypertrophic cardiomyopathy
- **PPCM:** Postpartum cardiomyopathy

1.1.2 How many co-variates are there? What do they mean? What type of value does each contain? (e.g. binary, continuous, categorical)

```
# Number of covariates
n_co_variates = ncol(sampleInfo)

# Types of variables of covariates
str(sampleInfo)
```

```
'data.frame':   366 obs. of  19 variables:
 $ tissue_source: chr  "NF" "NF" "NF" "NF" ...
 $ etiology     : chr  "NF" "NF" "NF" "NF" ...
 $ gender       : chr  "Male" "Male" "Male" "Female" ...
 $ race         : chr  "AA" "AA" "AA" "AA" ...
 $ age          : num  18 26 17 59 59 50 15 53 62 16 ...
 $ weight       : num  70 85 68 66 87 87 70 91 56 67 ...
 $ height       : num  175 183 173 157 157 175 183 165 168 163 ...
 $ hw           : num  NA NA 400 380 NA 640 NA NA NA 256 ...
 $ lv_mass      : num  NA NA NA NA NA NA NA NA NA NA ...
 $ afib         : chr  "No" "No" "No" "No" ...
 $ VTVF         : chr  "No" "No" "No" "No" ...
 $ Diabetes     : chr  "No" "No" "No" "No" ...
 $ Hypertension : chr  "No" "No" "No" "Yes" ...
 $ LVEF         : num  0.37 NA 0.27 0.6 0.55 0.65 0.15 NA NA 0.51 ...
 $ RIN          : num  8.4 9.1 7.8 9.4 8.8 8.6 7.8 10 7.8 6.4 ...
 $ Library.Pool : chr  "Magnet_10" "Magnet_11" "Magnet_09" "Magnet_06" ...
 $ disease_race : chr  "AA_NF" "AA_NF" "AA_NF" "AA_NF" ...
 $ minexpr      : num  7.47 7.47 7.47 7.47 7.47 ...
 $ TIN.median.  : num  72.8 74.3 77.4 69.5 73.6 ...
```

The number of co-variates is **19**.

Covariates are variables known to affect disease susceptibility and are independent of tested genotypes at the population level. They are used to control for confounding factors in the analysis of the association between a disease and a genetic variant.

1.1.3 Are all variables measured in all individuals?

No, there are NA values in some columns, which means that these values were not measured.

```r
na_values <- sampleInfo %>%
  dplyr::select(everything()) %>%
  summarise_all(list(~ sum(is.na(.))))

na_values
```

```
  tissue_source etiology gender race age weight height hw lv_mass afib VTVF
1             0        0      0    0   0      0      1  7     207    5    2
  Diabetes Hypertension LVEF RIN Library.Pool disease_race minexpr TIN.median.
1        2            1   80   6            0            0       0           0
```

1.1.4 Using the sampleInfo object, create an overview of the sample sizes and characteristics in each disease category: How many individuals are healthy? How many suffer from DCM, HCM, PPCM?

Method 1

```r
etiology1 <- sampleInfo %>%
  dplyr::select(etiology) %>%
  table()
etiology1
```

```
etiology
 DCM  HCM   NF PPCM 
 166   28  166    6 
```

Method 2

```
etiology2 <- sampleInfo %>%
  count(etiology)

etiology2
```

```
  etiology   n
1      DCM 166
2      HCM  28
3       NF 166
4     PPCM   6
```

Method 3

```
etiology3 <- sampleInfo %>%
  group_by(etiology) %>%
  tally()

etiology3
```

```
# A tibble: 4 x 2
  etiology     n
  <chr>    <int>
1 DCM        166
2 HCM         28
3 NF         166
4 PPCM         6
```

1.1.5 What is the average age in each disease category?

```
avg_etiology_by_age <- sampleInfo %>%
  group_by(etiology) %>%
  summarise(
    n = n(),
    age = mean(age, na.rm = TRUE)
  )

avg_etiology_by_age
```

```
# A tibble: 4 x 3
  etiology      n   age
  <chr>    <int> <dbl>
1 DCM        166  52.1
2 HCM         28  48.7
3 NF         166  55.9
4 PPCM         6  34.7
```

1.1.6 How many male and female individuals are there in each disease category?

```
count_etiology_by_gender <- sampleInfo %>%
  group_by(etiology) %>%
  count(gender)

count_etiology_by_gender
```

```
# A tibble: 7 x 3
# Groups:   etiology [4]
  etiology gender     n
  <chr>    <chr>  <int>
1 DCM      Female    66
2 DCM      Male     100
3 HCM      Female    11
4 HCM      Male      17
5 NF       Female    89
6 NF       Male      77
7 PPCM     Female     6
```

1.1.7 Are there other characteristics that are strikingly different between groups?

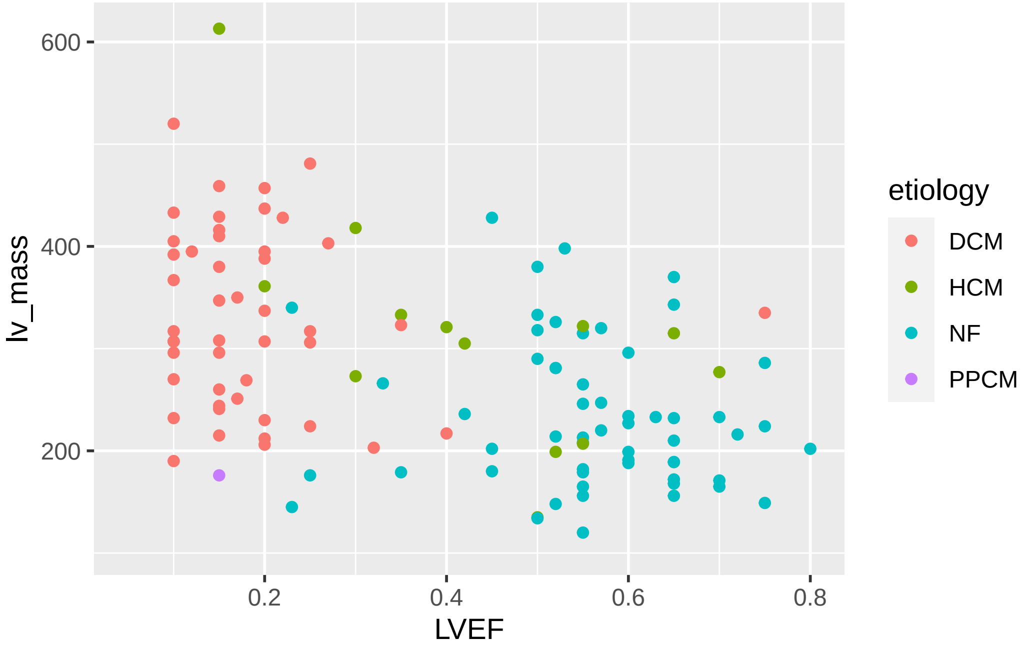
```
summary(sampleInfo)
```

```
tissue_source        etiology            gender              race
Length:366         Length:366         Length:366         Length:366
Class :character   Class :character   Class :character   Class :character
Mode  :character   Mode  :character   Mode  :character   Mode  :character
```

```
     age            weight           height            hw
Min.   :15.00   Min.   : 27.00   Min.   : 55.0   Min.   :151.0
1st Qu.:48.00   1st Qu.: 66.00   1st Qu.:163.0   1st Qu.:371.5
Median :55.00   Median : 78.00   Median :170.0   Median :448.0
Mean   :53.28   Mean   : 81.19   Mean   :168.7   Mean   :468.8
3rd Qu.:62.00   3rd Qu.: 91.00   3rd Qu.:178.0   3rd Qu.:556.5
Max.   :83.00   Max.   :267.00   Max.   :196.0   Max.   :923.0
                                 NA's   :1       NA's   :7
   lv_mass          afib               VTVF             Diabetes
Min.   :104.0   Length:366         Length:366         Length:366
1st Qu.:195.0   Class :character   Class :character   Class :character
Median :247.0   Mode  :character   Mode  :character   Mode  :character
Mean   :266.0
3rd Qu.:321.5
Max.   :613.0
NA's   :207
Hypertension            LVEF             RIN         Library.Pool
Length:366         Min.   :0.0500   Min.   : 5.800   Length:366
Class :character   1st Qu.:0.1500   1st Qu.: 8.100   Class :character
Mode  :character   Median :0.2000   Median : 8.500   Mode  :character
                   Mean   :0.3068   Mean   : 8.451
                   3rd Qu.:0.5200   3rd Qu.: 8.900
                   Max.   :0.8000   Max.   :10.000
                   NA's   :80       NA's   :6
disease_race          minexpr       TIN.median.
Length:366         Min.   :7.469   Min.   :23.38
Class :character   1st Qu.:7.469   1st Qu.:61.26
Mode  :character   Median :7.469   Median :70.12
                   Mean   :7.469   Mean   :65.03
                   3rd Qu.:7.469   3rd Qu.:73.32
                   Max.   :7.469   Max.   :81.52
```

An example of two features that are different between groups:

```r
ggplot(sampleInfo, aes(x= LVEF, y = lv_mass, color = etiology)) +
  geom_point()
```



1.2 Assignment 2: Data exploration on the sample level

1.2.1 The gene expression dataset contains so-called log2-transformed CPM (counts per million) values: How are CPM values created from raw RNA-sequencing count data? And why is this needed? (HINT: Google "what the fpkm")

FPKM stands for fragments per kilobase of exon per million mapped fragments. It is used specifically in paired-end RNA-seq experiments. The interpretation of FPKM is that if you sequence your RNA sample again, you expect to see for gene i, FPKMi reads divided by gene i length over a thousand and divided by the total number of reads mapped over a million.

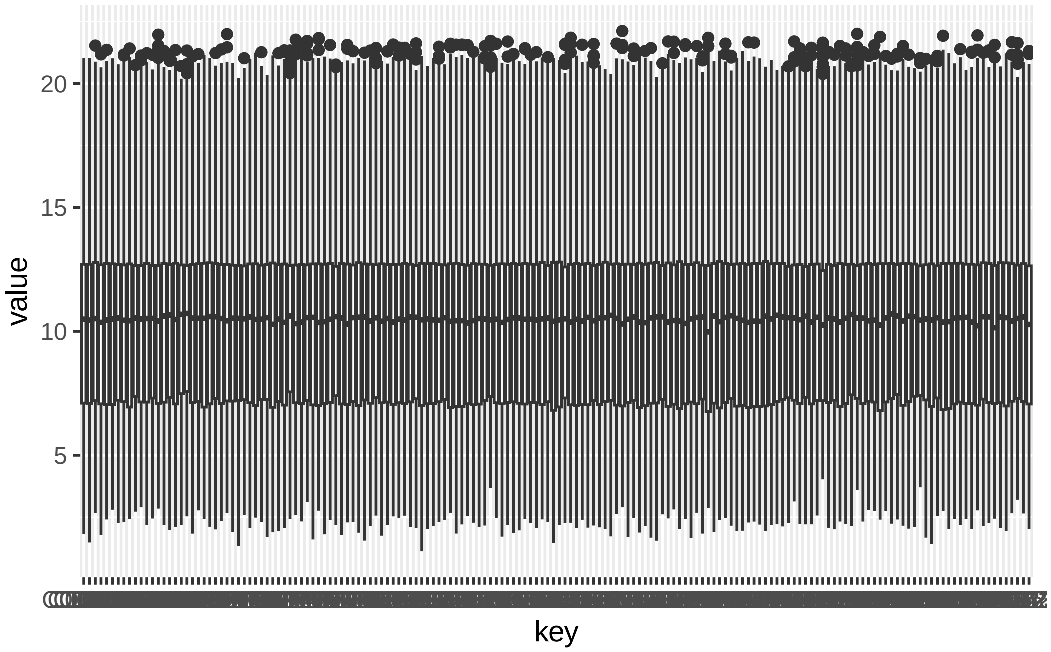
1.2.2 Create and interpret 4 figures containing boxplots for all samples in the dataset, one for DCM, one figure for HCM, one for PPCM and one for the healthy controls.

Create dataframes with information of the samples in each disease category.

```r
# Create a vector of the names of etiologies
names_et <- etiology2$etiology

# Get the names of the samples per etiology
for (i in names_et){
    # Define the name of the dataframes with the list of samples per etiology
    var_name <- paste(i,"columns",sep = "_")
    # Get the dataframes with the list of sample names per etiology
    df <- rownames_to_column(sampleInfo, var = "sample") %>%
              dplyr::filter(etiology == i) %>%
              dplyr::select(sample) %>%
              pull(sample)
    # Assign the names to the dataframes
    assign(var_name, df)
}
```

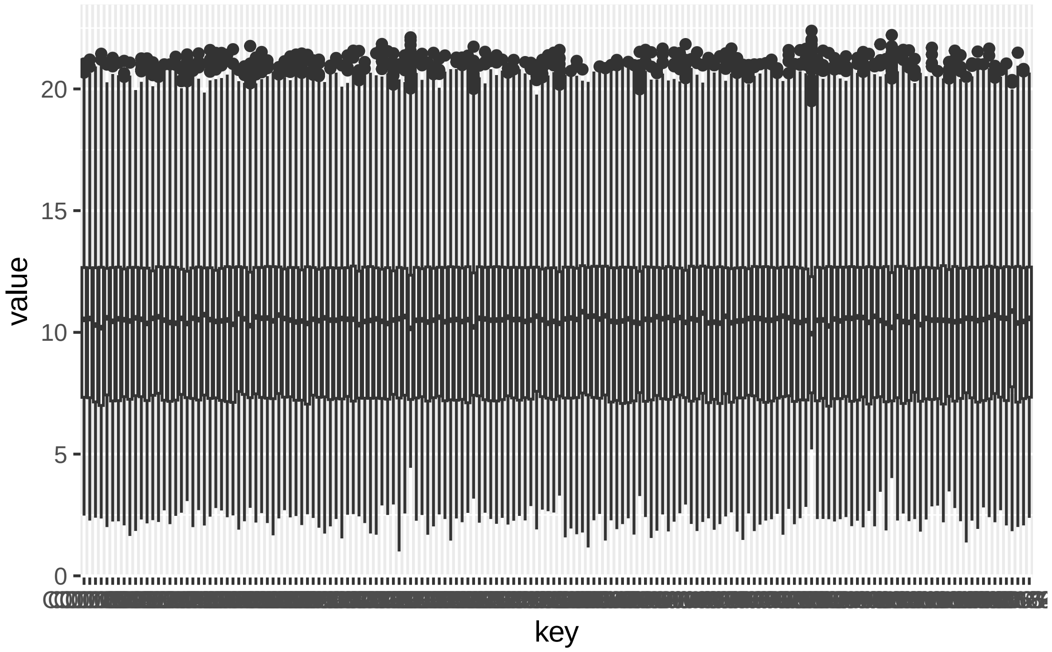
1.2.2.1 Boxplot of NF patients

```r
NF_data <- gxData %>%
           dplyr::select(NF_columns)

NF_data_group <- gather(NF_data) # melt also works, but it is deprecated

ggplot(NF_data_group, aes(x = key, y = value)) +
  geom_boxplot()
```



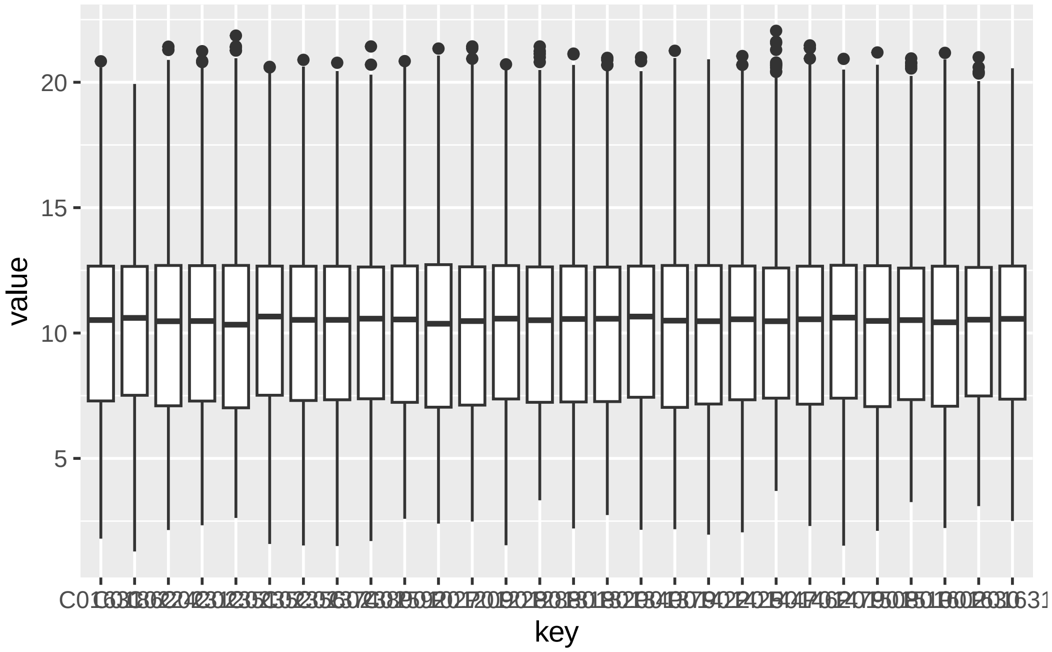
1.2.2.2 Boxplot of DCM patients

```
DCM_data <- gxData %>%
           dplyr::select(DCM_columns)

DCM_data_group <- gather(DCM_data) # melt also works, but it is deprecated

ggplot(DCM_data_group, aes(x = key, y = value)) +
  geom_boxplot()
```



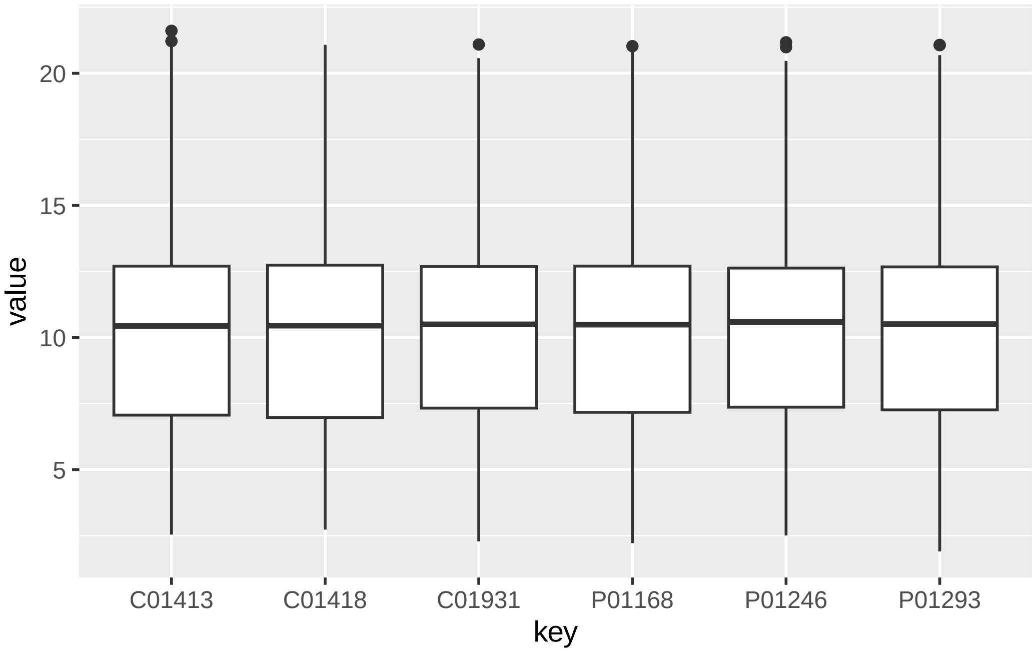
1.2.2.3 Boxplot of HCM patients

```
HCM_data <- gxData %>%
           dplyr::select(HCM_columns)

HCM_data_group <- gather(HCM_data) # melt also works, but it is deprecated

ggplot(HCM_data_group, aes(x = key, y = value)) +
  geom_boxplot()
```



1.2.2.4 Boxplot of PPCM patients

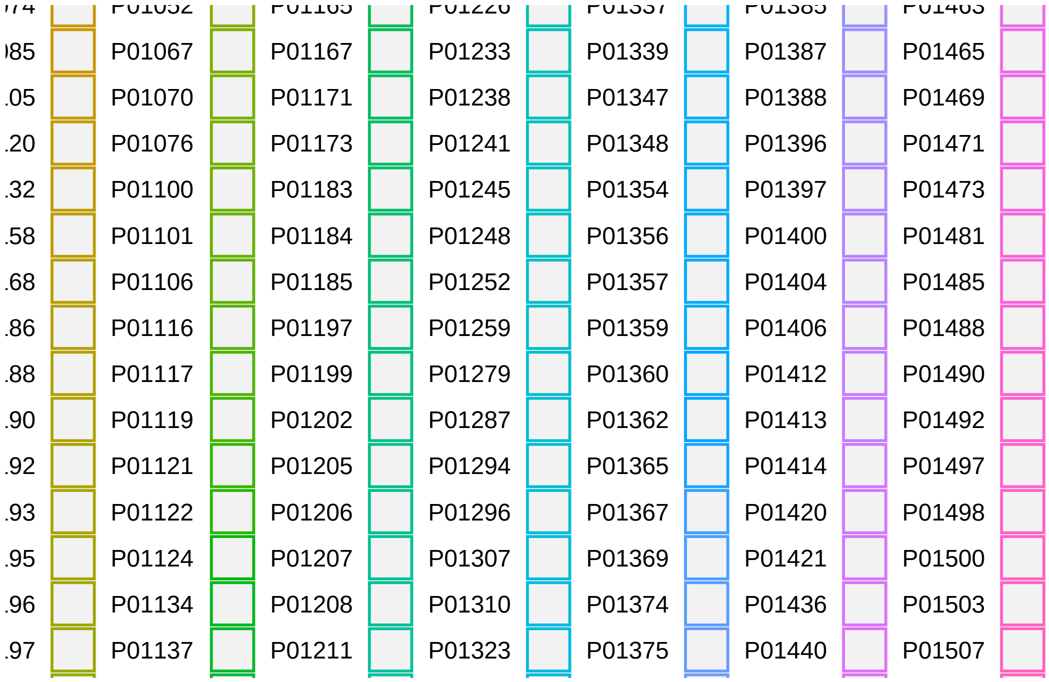
```
PPCM_data <- gxData %>%
           dplyr::select(PPCM_columns)

PPCM_data_group <- gather(PPCM_data) # melt also works, but it is deprecated

ggplot(PPCM_data_group, aes(x = key, y = value)) +
  geom_boxplot()
```



1.2.3 Create and interpret 4 figures containing density plots for all samples in the dataset, one figure for DCM, one for HCM, one for PPCM and one for the healthy controls.

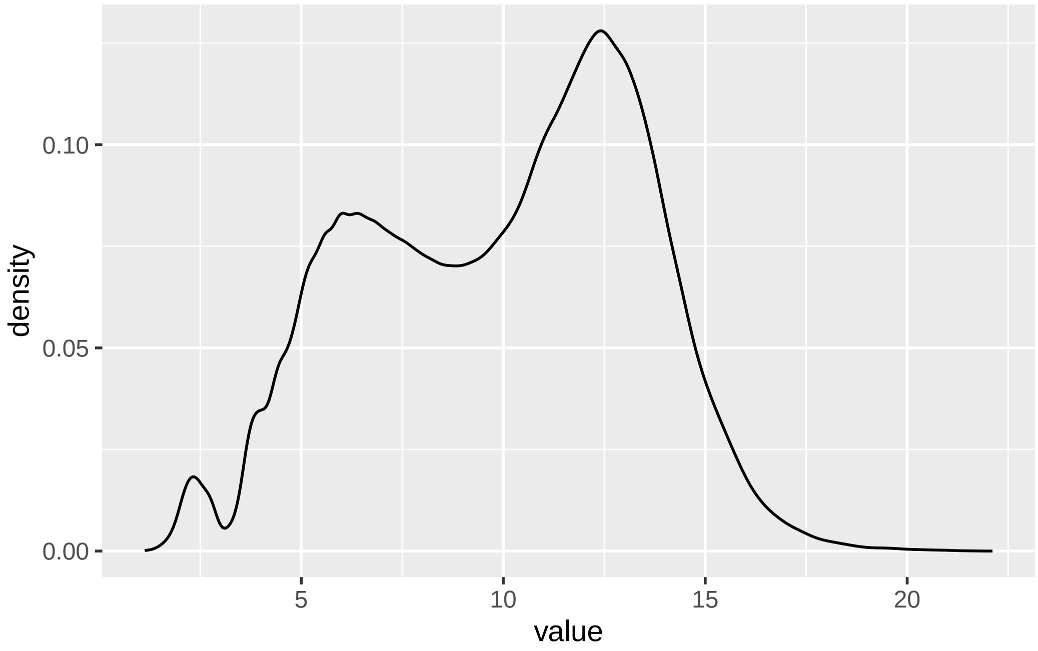
1.2.3.1 Density plot of all the values from the NF group colored by sample

```
ggplot(NF_data_group, aes(x = value, color = key)) +
  geom_density(alpha = 0.2)
```



1.2.3.2 Density plot of all the values from the NF group

```
ggplot(NF_data_group, aes(x = value)) +
  geom_density()
```



The same code applies for the pther groups

1.2.4 Assess the normality of all samples and summarize the results in a table or figure. (HINT: try ‘shapiro.test’)

```
# Get random sample for all the samples
norm_df <- sample_n(gxData, 5000) %>%
  #slice(gxData, 5000:8000) %>%
  sapply(., shapiro.test) %>%
  as_tibble() %>%
  slice(2) %>%
  gather(sample, p_value) %>%
  mutate(normality = p_value < 0.05)

norm_df %>%
  count(normality)
```

```
# A tibble: 1 x 2
  normality     n
  <lgl>     <int>
1 TRUE        366
```

1.2.5 Perform a principal component analysis (PCA), visualize the results and color by the disease category, sex and other variables of interest. Interpret the results: what patterns do you see?

HINT: use the functions ‘pca’ and ‘plotPcs’ from the package ‘pcaMethods’

1.2.5.1 Calculate the PCAs

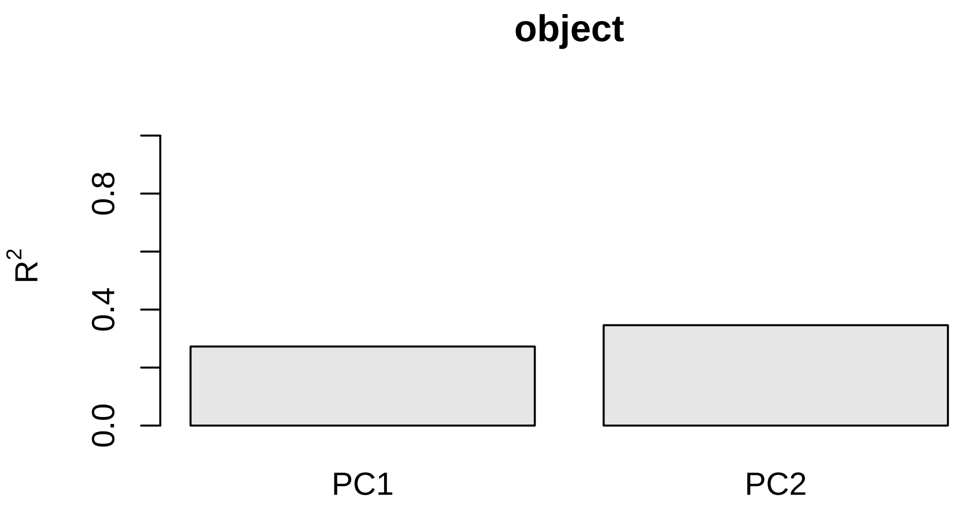
```
# Transpose dataframe to merge with the metadata
t_gxData <- t(gxData)

# Calculate pca
pca_hf <- pca(t_gxData, method = "svd")
```

1.2.5.2 Summary and barplot of the number of instances in the PCAs

```
# Get a short summary on the calculated model
summary(pca_hf)
```

```
svd calculated PCA
Importance of component(s):
                 PC1     PC2
R2            0.2726 0.07351
Cumulative R2 0.2726 0.34612
```

```
plot(pca_hf)
```

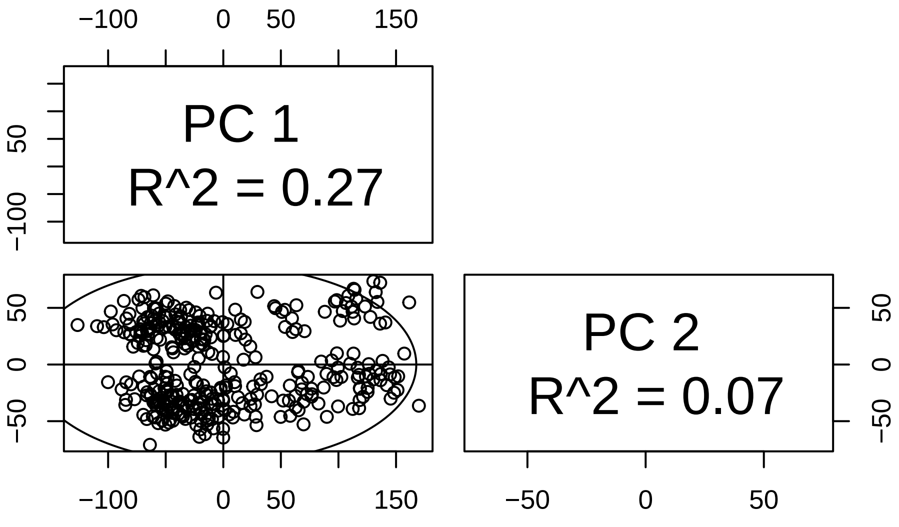


1.2.5.3 Merge pca scores with metadata for plotting

```
# Merge pca scores with metadata
df_hf <- merge(scores(pca_hf), sampleInfo, by = 0)
```

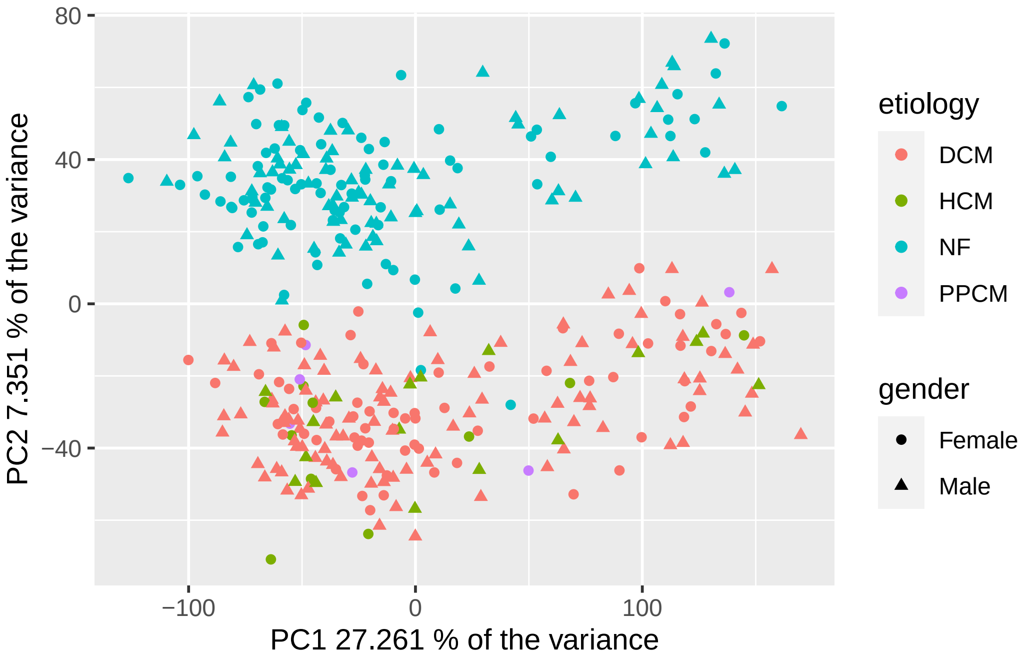
1.2.5.4 Plot the PCAs with the default them of plotsPcs

```
## Create scatteplot of the PCA with ggplot version 2
plotPcs(pca_hf)
```



1.2.5.5 Plot the PCAs with ggplot

```
## Create scatteplor of the PCA with ggplot version 1
ggplot(df_hf, aes(PC1, PC2, shape=gender, color=etiology)) +
  geom_point() +
  xlab(paste("PC1", pca_hf@R2[1] * 100, "% of the variance")) +
  ylab(paste("PC2", pca_hf@R2[2] * 100, "% of the variance"))
```



2 Week4 - Introduction to Gene expression analysis part 2

Sebastian Ayala Ruano
today

This R script serves as a scaffold for adding the code required to fulfill the assignments. It includes the assignments as well as a few hints.

Add the necessary code and type your answers in this document for your own record.

```r
library(ggplot2)
library(readr)
library(dplyr)
library(tibble)
library(reshape2)
library(conflicted)
library(tidyr)
library(purrr)
library(limma)
library(edgeR)
library(corrmorant)
```

2.1 Assignment 3: Data exploration on the gene level

2.1.1 Convert the CPM values to FPKM values. For some of the exercises below, we need to convert the CPM expression values to FPKM expression values.

```r
# Load data
geneTotExonLengths <- read_delim("Data/MAGNET_exonLengths.txt", delim = "\t")
gxData <- read_delim("Data/MAGNET_GeneExpressionData_CPM_19112020.txt", delim = "\t")
sampleInfo <- read_csv("Data/MAGNET_SampleData_18112022.csv")

# Add gene ID as the row names
gxData <- column_to_rownames(gxData, var = "EnsemblGeneID")
geneTotExonLengths <- column_to_rownames(geneTotExonLengths, var = "EnsemblGeneID")

# Check that row names are the same
all(rownames(geneTotExonLengths) == rownames(gxData)) # TRUE (just a check)
```

```
[1] TRUE
```

```r
# Add sample name as the row names
sampleInfo <- column_to_rownames(sampleInfo, var = "sample_name")

# Convert CPM expression values to FPKM
cpm2fpkm <- function(x) {
    t <- 2^(x) * 1E3 / geneTotExonLengths[, 1] # . before variable makes it a hidden varia
}
gxData_fpkm <- cpm2fpkm(gxData)
```

2.1.2 What does FPKM stand for? How does this measure differ from CPM? (Google)

These metrics attempt to normalize for sequencing depth and gene length. Normalized expression units are necessary to remove technical biases in sequenced data such as depth of sequencing and gene length, and make gene expressions directly comparable within and across samples. More sequencing depth produces more read count for a gene expressed at the same level and differences in gene length generate unequal reads count for genes expressed at the same level.

CPM is a basic gene expression unit that normalizes only for sequencing depth (depth-normalized counts). It is biased in some applications where the gene length influences gene expression, such as RNA-seq.

$$CPM = \frac{N\ reads\ mapped\ to\ gene \times 10^6}{Total\ N\ of\ mapped\ reads} \tag{2.1}$$

RPKM (reads per kilobase of transcript per million reads mapped) is a gene expression unit that measures the expression levels (mRNA abundance) of genes or transcripts. RPKM is a gene length normalized expression unit that is used for identifying the differentially expressed genes by comparing the RPKM values between different experimental conditions. Generally, the higher the RPKM of a gene, the higher the expression of that gene.

$$RPKM = \frac{N\ reads\ mapped\ to\ gene \times 10^3 \times 10^6}{Total\ N\ of\ mapped\ reads \times gene\ length\ in\ bp} \tag{2.2}$$

Here, 10^3 normalizes for gene length and 10^6 for sequencing depth factor.

FPKM (fragments per kilobase of exon per million mapped fragments) is a gene expression unit which is analogous to RPKM. FPKM is used especially for normalizing counts for paired-end RNA-seq data in which two (left and right) reads are sequenced from the same DNA fragment. Generally, the higher the FPKM of a gene, the higher the expression of that gene.

When we map paired-end data, both reads or only one read with high quality from a fragment can map to reference sequence. To avoid confusion or multiple counting, the fragments to which both or single read mapped are counted and represented for FPKM calculation.

2.1.3 In you own words, describe what the code above does.

The code takes the gene expression values in CPM and normalize them by the length of the genes, according to the formula expressed before.

2.1.4 Can we compare the FPKM value of gene A between two samples to state in which sample gene A is more highly expressed?

No, because FPKM values are normalized by the length of genes, which means that we cannot compare the values across different samples.

2.1.5 Can we compare the FPKM value of gene A to gene B in a single sample to state which gene is more highly expressed?

Yes, because FPKM values are normalized by the length of the genes.

2.1.6 Can we compare the CPM values of gene A between two samples to determine in which sample gene A is more highly expressed?

Yes, because CPM values are normalized by the sequencing depth (depth-normalized counts) and they do not take into account the length of genes for the normalization process.

2.1.7 Can we compare the CPM value of gene A to the value of gene B in a single sample to determine which gene is more highly expressed?

No, because CPM values are not normalized by the length of the genes.

2.1.8 Using the FPKM values, answer the following questions:

2.1.8.1 What are the IDs of the 5 highest expressed genes? What is their function according to the GeneCards website?

```
# Create a column with the mean of expression values of all samples
exp_mean_df <- gxData_fpkm %>%
  rownames_to_column(var = "geneID") %>%
  rowwise() %>%
  mutate(exp_mean = mean(c_across(C00039:P01640))) %>%
  column_to_rownames(var = "geneID") %>%
  select(exp_mean)
```

```
# Select the 5 most expressed genes
max5_genes_mean <- exp_mean_df %>%
  slice_max(n= 5, exp_mean)
```

Gene ID	Name	Function
ENSG00000198804	MT-CO1	Contributes to cytochrome-c oxidase activity
ENSG00000198899	MT-ATP6	Contributes to proton-transporting ATP synthase activity
ENSG00000198938	MT-CO3	Involved in respiratory chain complex IV assembly
ENSG00000198712	MT-CO2	Contributes to cytochrome-c oxidase activity

Gene ID	Name	Function
ENSG00000198886	MT-ND4	Enables NADH dehydrogenase (ubiquinone) activity

All of the genes are involved in processes related to mitochondria activity, which make sense because the dataset has muscle samples.

2.1.8.2 What are the IDs of the 5 lowest expressed genes? What is their function according to the GeneCards website?

```
# Select the 5 lowest expressed genes
min5_genes_mean <- exp_mean_df %>%
  slice_min(n = 5, exp_mean)
```

Gene ID	Name	Function
ENSG00000015568	RGPD5	RAN is a small GTP-binding protein of the RAS superfamily that is associated with the nuclear membrane
ENSG00000162105	SHANK2	This gene encodes a protein that is a member of the Shank family of synaptic proteins that may function as molecular scaffolds in the postsynaptic density of excitatory synapses
ENSG00000267586	LINC00907	RNA Gene, and is affiliated with the lncRNA class
ENSG00000215126	ZNG1F	Predicted to enable ATP binding activity
ENSG00000183914	DNAH2	Dyneins are microtubule-associated motor protein complexes

There are pseudogenes, RNA genes, and others related to different processes (i.e. synaptic genes).

2.1.8.3 What are the IDs of the 5 most variable genes? What is their function according to the GeneCards website?

```
# Create a column with the mean of expression values of all samples
exp_var_df <- gxData %>%
  rownames_to_column(var = "geneID") %>%
  rowwise() %>%
  mutate(exp_var = var(c_across(C00039:P01640))) %>%
  column_to_rownames(var = "geneID") %>%
  select(exp_var)
```

```
# Select the 5 most variable genes
max5_genes_var <- exp_var_df %>%
  slice_max(n= 5, exp_var)
```

Gene ID	Name	Function
ENSG00000198692	EIF1AY	Eukaryotic Translation Initiation Factor 1A Y-Linked
ENSG00000129824	RPS4Y1	Ribosomal Protein S4 Y-Linked 1
ENSG00000114374	USP9Y	Ubiquitin Specific Peptidase 9 Y-Linked
ENSG00000067048	DDX3Y	DEAD-Box Helicase 3 Y-Linked
ENSG00000012817	KDM5D	Lysine Demethylase 5D - encodes a protein containing zinc finger domains

4 of these genes are related to Y chromosome, which are absent in the female samples.

Note: By using the FPKM dataset, we got the same genes as the 5 mots highly expressed.

2.1.8.3.1 What are the IDs of the 5 least variable (= stable!) genes? What is their function according to the GeneCards website?

```
# Select the 5 least variable genes
min5_genes_var <- exp_var_df %>%
  slice_min(n= 5, exp_var)
```

Gene ID	Name	Function
ENSG00000136709	WD Repeat Domain 33	WD repeats are conserved regions, which may facilitate formation of heterotrimeric or multiprotein complexes
ENSG00000089053	ANAPC5	Anaphase Promoting Complex Subunit 5
ENSG00000111361	EIF2B1	Eukaryotic Translation Initiation Factor 2B Subunit Alpha
ENSG00000086475	SEPHS1	Selenophosphate Synthetase 1
ENSG00000106609	TMEM248	Transmembrane Protein 248

All of the genes are related to conserved cellular functions - house keeping genes.

2.1.9 Using the CPM values, answer the following questions:

2.1.9.1 Which 5 genes show the strongest correlation to age in the control group?

```
# Get the dataframe with the list of sample names with NF
NF_columns <- rownames_to_column(sampleInfo, var = "sample") %>%
          dplyr::filter(etiology == "NF") %>%
          dplyr::select(sample) %>%
          pull(sample)

# Get the gene expression data from the NF patients
NF_data <- gxData %>%
           dplyr::select(NF_columns)

# Transpose the dataframe to have genes as columns
NF_data <- as.data.frame(t(NF_data))

# Get the metadata from NF patients
t_sampleInfo <- as.data.frame(t(sampleInfo))

NF_metadata <- t_sampleInfo %>%
               rownames_to_column(var = "covariate") %>%
               dplyr::select(c(NF_columns, covariate)) %>%
               column_to_rownames(var = "covariate")
```

```
# Transpose the dataframe to have age as column
NF_metadata <- as.data.frame(t(NF_metadata))

# Add age column into the gene expression dataframe
NF_data <- NF_data %>%
            mutate(age = NF_metadata$age)
```

```
# Calculate correlation values
data_cor <- cor(NF_data[ , colnames(NF_data) != "age"],
                as.numeric(NF_data$age))

data_cor <- as.data.frame(data_cor)

# Select the 5 most correlated genes with age in the control group
corr5_genes_age <- data_cor %>%
  arrange(desc(abs(V1))) %>%
  slice_head(n= 5)

# Get the gene expression data
corr5_gene_data <- NF_data %>%
           dplyr::select(row.names(corr5_genes_age))

# Calculate significance (p value) of the 5 most correlated genes with age
corr5_genes_age[1,2] <- cor.test(corr5_gene_data$ENSG00000244681, as.numeric(NF_data$age))
corr5_genes_age[2,2] <- cor.test(corr5_gene_data$ENSG00000244694, as.numeric(NF_data$age))
corr5_genes_age[3,2] <- cor.test(corr5_gene_data$ENSG00000182264, as.numeric(NF_data$age))
corr5_genes_age[4,2] <- cor.test(corr5_gene_data$ENSG00000154080, as.numeric(NF_data$age))
corr5_genes_age[5,2] <- cor.test(corr5_gene_data$ENSG00000250337, as.numeric(NF_data$age))

colnames(corr5_genes_age) <- c("estimate", "p_value")
```

- **Is the correlation positive or negative?**

4 of the values are positive and one is negative

- **Is the correlation significant?**

Yes, all of the correlations were significant

2.1.9.2 What is their function according to the GeneCards website? Are they genes of which the expression is known to change with age (use Pubmed)?

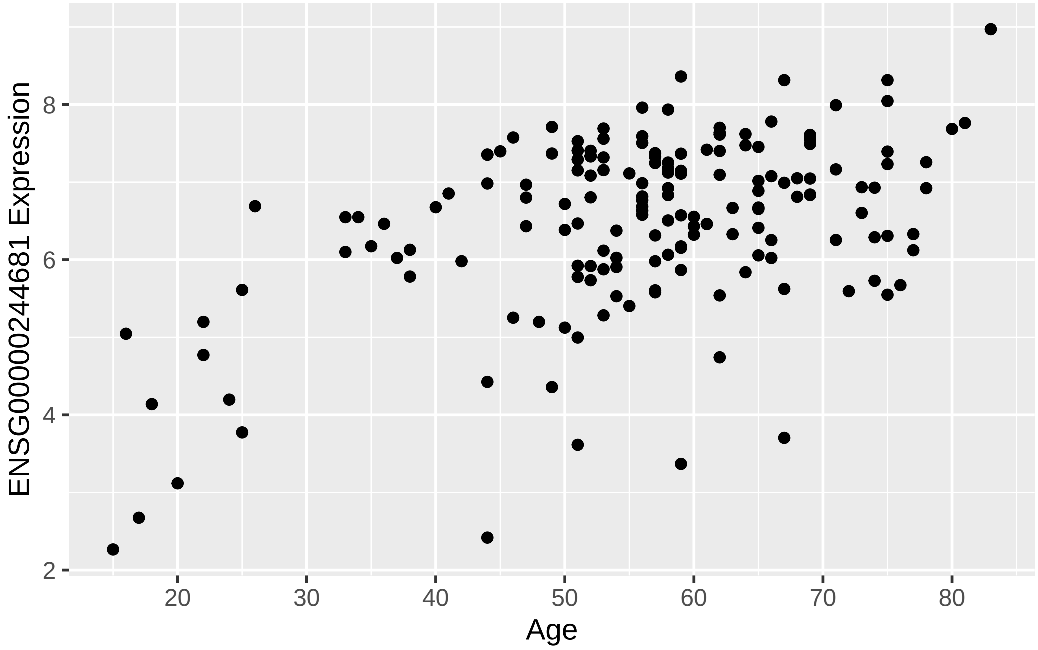
Gene ID	Name	Function
ENSG00000244681	MTHFD2P1	Pseudogene
ENSG00000244694	PTCHD4	Predicted to be integral component of membrane
ENSG00000182264	IZUMO1	The sperm-specific protein Izumo is essential for sperm-egg plasma membrane binding and fusion
ENSG00000154080	CHST9	Catalyzes the transfer of sulfate to position 4 of non-reducing N-acetylgalactosamine (GalNAc) residues in both N-glycans and O-glycans
ENSG00000250337	PURPL	RNA Gene, and is affiliated with the lncRNA class. Diseases associated with colorectal cancer and myasthenic syndrome

2.1.9.3 Visualize the result for at least 1 gene (HINT: CPM values on the y-axis, age in years on the x-axis)

Scatter plot of one gene vs age

```r
# Add age column into the 5 most correlated genes dataframe
corr5_gene_data <- corr5_gene_data %>%
            mutate(Age = as.numeric(NF_metadata$age), .before = 1)

corr1gene_age <- corr5_gene_data %>%
        select(Age, ENSG00000244681)

corr1gene_age_plot <- ggplot(corr1gene_age, aes(x = Age, y = ENSG00000244681)) +
  geom_point() +
  labs(x = "Age", y = "ENSG00000244681 Expression") +
  scale_x_continuous(n.breaks = 10.0)
```

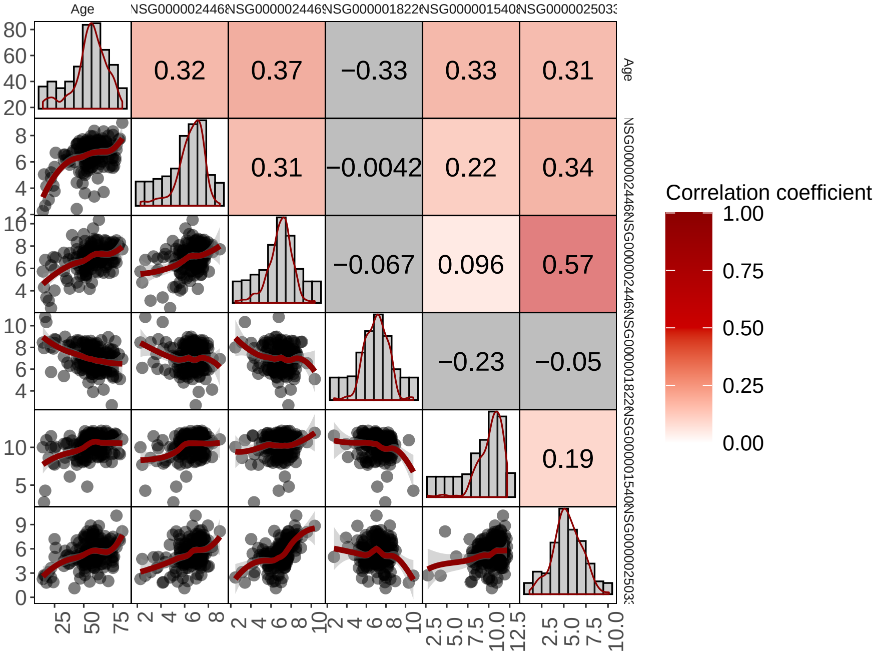
```
corr1gene_age_plot
```



Scatter plots, correlation values, and distributions of all genes and age

```
corr_plot_allgenes <- ggcorrm(data = corr5_gene_data) +
  theme_corrm(base_size = 6) +
  theme(axis.text.x = element_text(angle = 90, size = 8),
        axis.text.y = element_text(size = 8),
        strip.text.x = element_text(size = 5),
        strip.text.y = element_text(size = 5),
        legend.text = element_text(size = 8),
        legend.title = element_text(size = 8)) +
  lotri(geom_point(alpha = 0.5)) +
  lotri(geom_smooth(colour = "red4")) +
  utri_heatmap(alpha = 0.5, corr_method = "spearman") +
  utri_corrtext(corr_method = "spearman", size = 3.5) +
  dia_histogram(lower = 0.1, fill = "grey80", color = 1) +
  dia_density(lower = 0.1, alpha = .1, colour = "red4") +
  scale_fill_gradient2(low = "white", mid = "red3", high = "red4",
                       midpoint = 0.5, space = "rgb",
                       guide = guide_colorbar(title = "Correlation coefficient"),
                       limits = c(0, 1))
```

```
corr_plot_allgenes
```



2.2 Assignment 4: Differential gene expression analysis.

Now that we have explored the gene expression data, it is time to perform a differential gene expression analysis.

2.2.1 What is differential gene expression analysis (DGEA)? What are some of the most common packages in R for DGEA?

Differential expression analysis means taking the normalised read count data and performing statistical analysis to discover quantitative changes in
expression levels between experimental groups.

R packages:

- DESeq2
- limma

We are going to use the limma package to perform a DGEA. We need to use the CPM normalized values. Have a look at the limma guide section 15.4: (https://www.bioconductor.org/packages/devel/bioc/v

2.2.2 Implement the steps noted in the limma guide for the MAGNET dataset. Start with a DGEA between DCM patients and healthy controls.

2.2.2.1 Limma-trend

```r
# Convert counts to logCPM values
logCPM <- cpm(gxData, log = TRUE, prior.count = 3)

# Create design matrix
design = model.matrix(~0 + sampleInfo$etiology)

# Apply limma pipeline
fit <- lmFit(logCPM, design)
fit <- eBayes(fit, trend = TRUE)
topTable(fit, coef = ncol(design))
```

```
                   logFC  AveExpr        t P.Value adj.P.Val        B
ENSG00000089053 6.381604 6.384943 961.3848       0         0 1415.207
ENSG00000129351 6.429434 6.416465 955.8661       0         0 1413.270
ENSG00000105323 6.380228 6.365844 944.4249       0         0 1409.213
ENSG00000136709 6.329745 6.324768 937.9541       0         0 1406.892
ENSG00000106609 6.348231 6.340574 921.1237       0         0 1400.769
ENSG00000176915 6.281051 6.282988 907.5412       0         0 1395.732
ENSG00000075785 6.481443 6.478184 898.0510       0         0 1392.161
ENSG00000100711 6.284988 6.278731 891.0022       0         0 1389.480
ENSG00000182944 6.459927 6.446393 890.8330       0         0 1389.416
ENSG00000113648 6.404155 6.400461 890.3165       0         0 1389.218
```

```r
# Give more weight to fold-changes in the gene ranking
fit <- lmFit(logCPM, design)
fit <- treat(fit, lfc = log2(1.2), trend = TRUE)
topTreat(fit, coef = ncol(design))
```

```
                   logFC  AveExpr        t P.Value adj.P.Val
ENSG00000000003 6.117085 6.111910 388.5360       0         0
ENSG00000000419 6.294569 6.292874 480.5645       0         0
ENSG00000000457 6.100006 6.111824 433.3020       0         0
ENSG00000000460 5.886348 5.887347 308.1765       0         0
ENSG00000000938 5.992674 6.059126 206.5199       0         0
```

```
ENSG00000000971 6.391421 6.392179 255.8019       0         0
ENSG00000001036 6.299640 6.308049 581.1499       0         0
ENSG00000001084 6.273295 6.255830 284.7585       0         0
ENSG00000001167 6.134147 6.122626 201.0493       0         0
ENSG00000001460 5.943226 5.954836 370.8629       0         0
```

Results show the p value of 0 in some genes, which means that something is wrong.

2.2.3 Which co-variates should be taken along for correction? (confounding; see the “alcohol causes lung cancer” example from the lecture)

```r
# Convert counts to logCPM values
logCPM <- cpm(gxData, log = TRUE, prior.count = 3)

# Create design matrix considering confounding variables
design = model.matrix(~0 + etiology + gender + age, data = sampleInfo)

# Apply limma pipeline with confounding variables
fit <- lmFit(logCPM, design)

cont.matrix <- makeContrasts(DCMvsControl = etiologyDCM - etiologyNF,
                             levels = design)

fit <- contrasts.fit(fit, cont.matrix)

efit <- eBayes(fit, trend = TRUE)

dgeRes <- topTable(efit, coef = 'DCMvsControl', number = nrow(gxData))

glimpse(dgeRes)
```

```
Rows: 20,781
Columns: 6
$ logFC     <dbl> 0.15831103, 0.33966098, 0.21263013, -0.44141913, 0.36628640,~
$ AveExpr   <dbl> 6.286287, 5.952977, 6.101884, 6.022681, 6.049712, 6.371697, ~
$ t         <dbl> 29.01545, 27.11832, 25.18996, -25.06301, 24.77563, -24.26241~
$ P.Value   <dbl> 7.851116e-97, 1.689807e-89, 7.264246e-82, 2.342191e-81, 3.33~
$ adj.P.Val <dbl> 1.631540e-92, 1.755794e-85, 5.031943e-78, 1.216827e-77, 1.38~
$ B         <dbl> 210.1073, 193.3613, 175.9173, 174.7550, 172.1180, 167.3887, ~
```

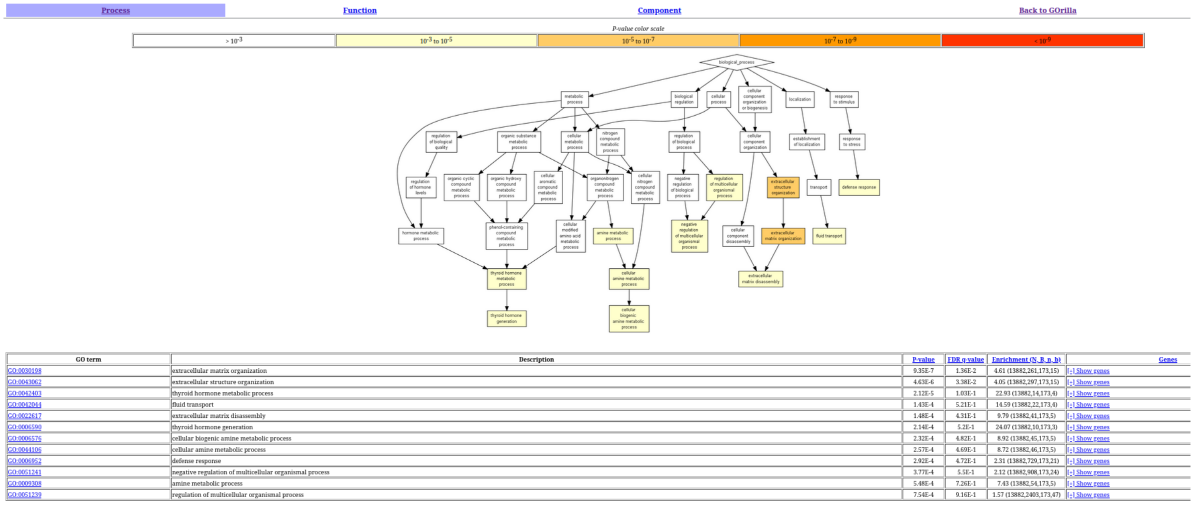
After including the cofounding variables, the p values are not 0, which means that the cofounding variables are important for the analysis.

2.2.4 Copy the top 200 differentially expressed genes to for a quick GO enrichment analysis. Which processes are changed between DCM and controls? Do these processes make biological sense? (quick literature check!)

```
# Select the 200 most correlated genes with age in the control group
to200_corr_genes <- dgeRes %>%
  slice_head(n = 200) %>%
  rownames_to_column(var = "Gene_ID")  %>%
  select(Gene_ID)

# Select the names of all genes in the control group
all_corr_genes <- dgeRes %>%
  rownames_to_column(var = "Gene_ID")  %>%
  select(Gene_ID)

# Export target list to csv file
write_csv(to200_corr_genes, "Outputs/to200_corr_genes.csv", col_names = FALSE)

# Export background list to csv file
write_csv(all_corr_genes, "Outputs/all_corr_genes.csv", col_names = FALSE)
```

In the GOrilla server, the inputs are the target and background tables exported in the previous step. The results are shown in the following figure:

The results show that the most enriched GO terms are related to inflammation and structural processed, and the immune system, which is consistent with the literature.



Process | Function | Component | Back to GOrilla

P-value color scale

> 10^{-3}	10^{-3} to 10^{-5}	10^{-5} to 10^{-7}	10^{-7} to 10^{-9}	< 10^{-9}

biological_process
metabolic process
biological regulation
cellular process
cellular component organization or biogenesis
localization
response to stimulus
regulation of biological quality
organic substance metabolic process
cellular metabolic process
nitrogen compound metabolic process
regulation of biological process
cellular component organization
establishment of localization
response to stress
regulation of hormone levels
organic cyclic compound metabolic process
organic hydroxy compound metabolic process
cellular aromatic compound metabolic process
organonitrogen compound metabolic process
cellular nitrogen compound metabolic process
negative regulation of biological process
regulation of multicellular organismal process
extracellular structure organization
transport
defense response
hormone metabolic process
phenol-containing compound metabolic process
cellular modified amino acid metabolic process
amine metabolic process
negative regulation of multicellular organismal process
cellular component disassembly
extracellular matrix organization
fluid transport
thyroid hormone metabolic process
cellular amine metabolic process
extracellular matrix disassembly
thyroid hormone generation
cellular biogenic amine metabolic process

GO term	Description	P-value	FDR q-value	Enrichment (N, B, n, b)	Genes
GO:0030198	extracellular matrix organization	9.35E-7	1.36E-2	4.61 (13882,261,173,15)	[+] Show genes
GO:0043062	extracellular structure organization	4.63E-6	3.38E-2	4.05 (13882,297,173,15)	[+] Show genes
GO:0042403	thyroid hormone metabolic process	2.12E-5	1.03E-1	22.93 (13882,14,173,4)	[+] Show genes
GO:0042044	fluid transport	1.43E-4	5.21E-1	14.59 (13882,22,173,4)	[+] Show genes
GO:0022617	extracellular matrix disassembly	1.48E-4	4.31E-1	9.79 (13882,41,173,5)	[+] Show genes
GO:0006590	thyroid hormone generation	2.14E-4	5.2E-1	24.07 (13882,10,173,3)	[+] Show genes
GO:0006576	cellular biogenic amine metabolic process	2.32E-4	4.82E-1	8.92 (13882,45,173,5)	[+] Show genes
GO:0044106	cellular amine metabolic process	2.57E-4	4.69E-1	8.72 (13882,46,173,5)	[+] Show genes
GO:0006952	defense response	2.92E-4	4.72E-1	2.31 (13882,729,173,21)	[+] Show genes
GO:0051241	negative regulation of multicellular organismal process	3.77E-4	5.5E-1	2.12 (13882,908,173,24)	[+] Show genes
GO:0009308	amine metabolic process	5.48E-4	7.26E-1	7.43 (13882,54,173,5)	[+] Show genes
GO:0051239	regulation of multicellular organismal process	7.54E-4	9.16E-1	1.57 (13882,2403,173,47)	[+] Show genes

Figure 2.1: GOrilla results