EASIER - EwAS: quality control, meta-analysIs and EnRichment

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9 novembre 2020

Abstract

Description of your vignette

Package

EASIER 0.1.0

Contents

1 Prerequisites

The package requires other packages to be installed. These include: `ggplot2`, `VennDiagram`, `RColorBrewer`, `tibble`, `dplyr`, `stringr`, `rasterpdf`, `tidyverse`, `reshape`, `ggsignif`, `tools` and `meta` all available in CRAN. The package also requires other packages from Bioconductor to perform annotations and enrichment : `IlluminaHumanMethylation450kanno.ilmn12.hg19`, `IlluminaHumanMethylationEPICanno.ilm10b4.hg19`, `missMethyl`, `org.Hs.eg.db`, `GenomicRanges` and `rtracklayer`.

To perform meta-analyses we use GWAMA, a Software tool for meta analysis developed by Intitute of Genomics from University of Tartu, this software is available at https://genomics.ut.ee/en/tools/gwama-download, this software must be installed on the computer where we are running analysis (already installed in machines ws05 and ws06 from ISGlobal).

2 Overview

The EASIER package performs epigenetic wide-association study (EWAS) downstream analysis:

- Quality control of EWAS results
  - Folders: input and ouput
  - Configuration: array type, sample, ethnic, exclusion CpGs criteria
  - CpG filtering selection -> list of CpGs filtered and reason
  - QC with summaries -> summary SE, Beta, lambda, significatives...
  - QC with plots -> QQplot, Distribution plot, precision plot, ...
  - CpG annotation and adjustment -> QCed EWAS results file
- Meta-analysis of EWAS results (using GWAMA)
  - Folders: input and output
  - Link to GWAMA
  - Format QCed EWAS results file
  - Run GWAMA -> EWAS meta-analysis results file
  - Meta-analysis with summaries -> xxxxxxx
  - Meta-analysis with plots -> Heterogeneity plot, distribution plots, QQ-plots, Volcano plots, Manhattan plots andForest plots,
- Functional enrichment (pathway and molecular enrichments)

In this vignette we will show how to apply EASIER con the EWAS results from three cohorts and two distinct models for each cohort.

3 Getting started

First, we need to install and load the required packages

```
if (!require(rasterpdf, quietly = TRUE))
   install.packages('rasterpdf', repos = 'https://cran.rediris.es/' )
if (!require(meta, quietly = TRUE))
   install.packages('meta', repos = 'https://cran.rediris.es/' )
if (!require(tibble, quietly = TRUE))
   install.packages('tibble')
```

```
if (!require(dplyr, quietly = TRUE))
   install.packages('dplyr')
if (!require(tidyverse, quietly = TRUE))
   install.packages::install( "tidyverse" )
if (!require(stringr, quietly = TRUE))
   install.packages('stringr')
if (!require(meta, quietly = TRUE))
   install.packages('meta') # Forest Plot
if (!require(ggplot2, quietly = TRUE))
   install.packages('ggplot2')
if (!require(VennDiagram, quietly = TRUE))
   install.packages('VennDiagram')
if (!require(RColorBrewer, quietly = TRUE))
   install.packages('RColorBrewer')
if (!require(reshape, quietly = TRUE))
   install.packages('reshape')
if (!require(ggsignif, quietly = TRUE))
   install.packages('ggsignif')
if (!require(tools, quietly = TRUE))
   install.packages('tools')
```

```
# Required libraries from Bioconductor
if (!requireNamespace("BiocManager", quietly = TRUE))
install.packages("BiocManager")

BiocManager::install( c("missMethyl",
                        "org.Hs.eg.db",
                        "GenomicRanges",
                        "rtracklayer") )
```

```
# Load libraries from Bioconductor
library("missMethyl")
library("org.Hs.eg.db")
library("GenomicRanges")
library("rtracklayer")
```

We also need to install `devtools` package, this package allows us to install packages directly from github

```
if (!require(devtools, quietly = TRUE)) install.packages('devtools')
```

The development version of `EASIER` package can be installed from BRGE GitHub repository:

```
devtools::install_github("isglobal-brge/EASIER@HEAD")
```

```
library(EASIER)
library(readtext)
```

4 Quality control

4.1 Quality Control Flowchart

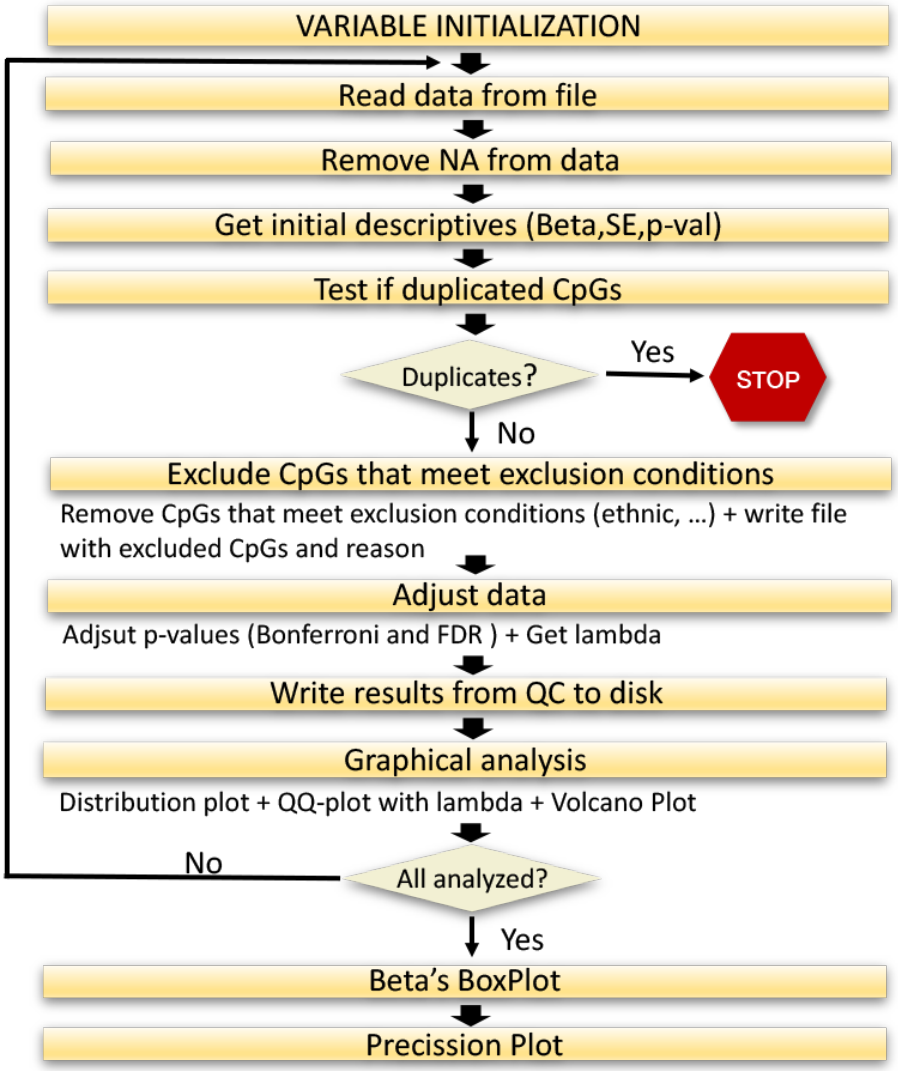


Figure 1: Quality control flowchart
This flowchart is used in the script under test folder to perform the quality control (QuqlityControl.R). The most important step in this workflow is the first step where we have to define the variables, if variables are well defined all the process is 'automatic'

We have programmed the script QualityControl.R using the library functions to carry out the *quality control process* automatically only by defining the previous variables. The script follows the **Figure**1 workflow. In this vignette we will explain how the script works to allow you to modify if necessary.

4.2 Initial Variables definition

We need to define the variables to work in all Quality control process, and the files containing the results of the EWAS to perform the downstream analysis.

4.2.1 Input data

As we commented before, we will perform an EWAS with three different cohorts and two distinct models for each cohort, so we need to define where the data is stored for each model and each cohort (six files). We do that in a character vector, and the variable is called files:

```
files <- c('data/PROJ1_Cohort3_Model1_date_v2.txt',
           'data/PROJ1_Cohort3_Model2_date_v2.txt',
           'data/PROJ1_Cohort2_Plate_ModelA1_20170309.txt',
           'data/PROJ1_Cohort2_Plate_ModelA2_20170309.txt',
           'data/Cohort1_Model1_20170713.txt',
           'data/Cohort1_Model2_20170713.txt')
```

files must contain at least the following fields :

probeID	BETA	SE	P_VAL
cg13869341	0.00143514362777834	0.00963411132344512	0.678945643213567
cg24669183	-0.0215342789035512	0.0150948404044624	0.013452341234512
cg15560884	0.00156725345562218	0.0063878467810596	0.845523221223523

4.2.2 Where to store output

We can also define the folder where we will save the results, for example in a variable called `result_folder`, in this case the results will be stored stored in a folder named *QC_Results*.

```
# Result folder
results_folder <- 'QC_Results'
```

4.2.3 Make results understandable

To make the analysis more understandable and do not have very complex file names we have to define an abbreviated form for each of the files defined above. For example, PROJ1_Cohort3_Model1_date_v2 will be treated as PROJ1_Cohort3_A1 or PROJ1_Cohort2_Plate_ModelA1_20170309 as PROJ1_Cohort2_A2 The length of the prefix vector must be equal to that of the files indicated above:

```
# Prefixes for each file
prefixes <- c('PROJ1_Cohort3_A1', 'PROJ1_Cohort3_A2',
              'PROJ1_Cohort2_A1','PROJ1_Cohort2_A2',
              'Cohort1_A1', 'Cohort1_A2')
```

4.2.4 Illumina Array type and filter conditions

The Illumina array type has to be indicated with one of these two possible values: 450K and EPIC. Filter CpGs is dependent on the Illumina array, thus this field has to be completed.

```
# Array type, used : EPIC or 450K
artype <- '450K'
```

In the quality control (QC) process, we exclude those CpGs that do not accomplish the defined parameters (based on *Zhou et al. 2017, Solomon et al. 2018, Fernandez-Jimenez et al. 2019*). These parameters are defined in a character vector and are the following:

4.2.4.1 Perform CpG exclusions –> non CpG probes and sexual CpGs:

- **Control probes ("control_probes")**: technical control probes that do not correspond to CpGs, such as bisulfite conversion I, bisulfite conversion II, extension, hybridization and negative. Classified as "rs" in the filtering variable named "probeType";
- **Non-cpg probes ("noncpg_probes")**: non-cpg probes classified as "ch" in the filtering variable named "probeType";
- **Sex chromosomes ("Sex")**: to avoid misleading results due to differences in sex-chromosome dosage on the human methylome. Filtering variable "Sex"; #

4.2.4.2 Perform CpG exclusions –> hybridizing problems:

- **Poor mapping probes ("MASK_mapping")**: Probes that have poor quality mapping to the target genomic location as indicated in the array's manifest file based on genome build GRCh37 and GRCh38 (for example due to the presence of INDELs (Insertion–deletion mutations present in the genome);
- **Cross-hybridising probes ("MASK_sub30")**: The sequence of the last 30bp at the 3' end of the probe is non-unique (problematic because the beta value of such probes is more likely to represent a combination of multiple sites and not the level of initially targeted CpG sites); Zhou et al. recommend 30bp, but in the code we prepared there is the possibility to adapt this to probes with non-unique 25bp, or 35bp, or 40bp, or 45bp 3'-subsequences (**"MASK_sub25", "MASK_sub40", "MASK_sub45"**).

4.2.4.3 Perform CpG exclusions -> presence of SNPs:

- **"MASK_extBase"**: Probes with a SNP altering the CpG dinucleotide sequence context and hence the ability of target cytosines to be methylated (regardless of the MAF);
- **"MASK_typeINextBaseSwitch"**: Probes with a SNP in the extension base that causes a color channel switch from the official annotation (regardless of the MAF);
- **"MASK_snp5.GMAF1p"**: probes with SNPs at the last 5bp of the 3' end of the probe, with an average minor allele frequency (MAF) >1%, by ethnic group;
- **"MASK_snp5.common"**: probes with SNPs at the last 5bp of the 3' end of the probe, with any average minor allele frequency (MAF) (can be <1%), by ethnic group;

4.2.4.4 Perform CpG exclusions -> array consistency:

- **"Unrel_450_EPIC_blood"**: These are probes that are known to yield different results for the 450K and EPIC array in BLOOD, suggesting that results are unreliable for at least one of these arrays. CpGs based on Solomon et al. (2018).

- **"Unrel_450_EPIC_pla_restrict"** or **"Unrel_450_EPIC_pla"**: These are probes that are known to yield different results for the 450K and EPIC array in PLACENTA, suggesting that results are unreliable for at least one of these arrays. CpGs based on Fernandez-Gutierrez et al. (2019).

In this example we filter CpGs that meet the following conditions: MASK_sub35_copy, MASK_typeINextBaseSwitch, noncpg_probes, control_probes, Unreliable_450_EPIC and Sex.

```
# Parameters to exclude CpGs
exclude <- c( 'MASK_sub35_copy',
              'MASK_typeINextBaseSwitch',
              'noncpg_probes',
              'control_probes',
              'Unrel_450_EPIC_blood',
              'Sex')
```

We also need to define the ethnic origin of the study population. Ethnic origins can be one of the table or *GMAF1p* if population is very diverse.

Population Code	Population Description	Super Population Code
AFR	*African*	AFR
AMR	*Ad Mixed Americn*	AMR
EAS	*East Asian*	EAS
EUR	*European*	EUR
SAS	*South Asian*	SAS
CHBB	Han Chinese in Beijing, China	EAS
JPT	Japanese in Tokyo, Japan	EAS
CHS	Southern Han Chinese	EAS
CDX	Chinese Dai in Xishuangbanna, China	EAS
KHV	Kinh in Ho Chi Minh City, Vietnam	EAS
CEU	Utah Residents (CEPH) with Northern and Western European Ancestry	EUR
TSI	Toscani in Italia	EUR
FIN	Finnish in Finland	EUR
GBR	British in England and Scotland	EUR
IBS	Iberian Population in Spain	EUR
YRI	Yoruba in Ibadan, Nigeria	AFR
LWK	Luhya in Webuye, Kenya	AFR
GWD	Gambian in Western Divisions in the Gambia	AFR
MSL	Mende in Sierra Leone	AFR
ESN	Esan in Nigeria	AFR
ASW	Americans of African Ancestry in SW USA	AFR
ACBB	African Caribbeans in Barbados	AFR
MXL	Mexican Ancestry from Los Angeles USA	AMR
PUR	Puerto Ricans from Puerto Rico	AMR
CLM	Colombians from Medellin, Colombia	AMR
PEL	Peruvians from Lima, Peru	AMR
GIH	Gujarati Indian from Houston, Texas	SAS
PJL	Punjabi from Lahore, Pakistan	SAS
BEBB	Bengali from Bangladesh	SAS
STU	Sri Lankan Tamil from the UK	SAS
ITU	Indian Telugu from the UK	SAS

Population Code	Population Description	Super Population Code
GMAF1p	If population is very diverse	

```
ethnic <- 'EUR'
```

4.2.5 Other variables :

To obtain the precision plot and to perform the GWAMA meta-analysis we need to know the number of samples in the EWAS results, so we store this information in "N"for each of the files. In addition, for case-control EWAS, we need to know the sample size of exposed or diseased individuals. This informaiotn is storaed as "n" for each of the files

```
N <- c(100, 100, 166, 166, 240, 240 )
n <- c(NA)
```

4.3 Quality Control - general code

As we show in the quality control flowchart, this code can be executed for each file defined in previous variable `files` but in this example we only show the analysis workflow for one of them. The complete code can be found in QualityControl.R .

```
# Variable declaration to perform precision plot
medianSE <- numeric(length(files))
value_N <- numeric(length(files))
cohort_label <- character(length(files))

# Prepare output folder for results (create if not exists)
if(!dir.exists(file.path(getwd(), results_folder )))
   suppressWarnings(dir.create(file.path(getwd(), results_folder)))


# IMPORTANT FOR A REAL ANALYSIS :

# To show the execution flow we perform the analysis with only one data
# file. Normally, we have more than one data file to analyze, for that
# reason, we execute the code inside a loop and we follow the execution
# flow for each file defined in `files`
# So we need to uncomment the for instruction and remove i <- 1 assignment.

# for ( i in 1:length(files) )
# {

   # we force i <- 1 to execute the analysis only for the first variable
   # for real data we have to remove this line
   i <- 1
```

First, we need to read the content of a file with EWAS results,

```
# Read data.
cohort <- read.table(files[i], header = TRUE, as.is = TRUE)
print(paste0("Cohort file : ",files[i]," - readed OK", sep = " "))
## [1] "Cohort file : data/PROJ1_Cohort3_Model1_date_v2.txt - readed OK "
```

and store the content of the file in a `cohort` variable. After that, we perform a simple descriptive analysis, using the function `descriptives_CpGs`. This function needs the EWAS results to be analyzed (`cohort`), the fields for which we are interested to get descriptives, (BETA, SE and P_VAL (seq(2:4))), and a file name to write results. For the first file it would be: *QC_Results/PROJ1_Cohort3_A1_descriptives.txt*, at the end of each iteration we get the complete resume with before and after remove CpGs, the excluded CpGs, and the significative CpGs after p-value adjust by FDR and Bonferroni.

```
# Descriptives - Before CpGs deletion
descriptives_CpGs(cohort, seq(2,4), paste0(results_folder,'/',prefixes[i],
                                           '_descriptives_init.txt') )
```

Then, we test if there are any duplicate CpGs. If there are duplicated CpGs, these are removed using the function `remove_duplicate_CpGs`. In this function we must indicate what data have to be reviewed and the field that contains the CpG IDs. Optionally, we can write the duplicates and descriptives related to this duplicates in a file.

```
# Remove duplicates
cohort <- remove_duplicate_CpGs(cohort, "probeID",
                                paste0(results_folder,'/',prefixes[i],
                                       '_descriptives_duplic.txt'),
                                paste0(results_folder,'/',prefixes[i],
                                       '_duplicates.txt') )
```

To exclude CpGs that we are not interested in, we use the function `exclude_CpGs`. Here we use the parameters defined before in the `exclude` variable, which are the data, cohort, the CpG id field (can be the column number or the field name "probeId"), the filters to apply defined in `exclude` variable, and, optionally, a file name if we want to save excluded CpGs and the exclusion reason (in this case the file name will be *QC_Results/PROJ1_Cohort3_A1_excluded.txt*).

```
# Exclude CpGs not meet conditions
cohort <- exclude_CpGs(cohort, "probeID", exclude,
                       filename = paste0(results_folder,'/',prefixes[i],
                                         '_excluded.txt') )
```

After eliminating the inconsistent CpGs, we proceed to carry out another descriptive analysis,

```
# Descriptives - After CpGs deletion #
descriptives_CpGs(cohort, seq(2,4),
                  paste0(results_folder,'/',prefixes[i],
                         '_descriptives_last.txt') )
```

Now, we can get adjusted p-values by Bonferroni and False Discovery Rate (FDR). The function to get adjusted p-values is `adjust_data`, and we have to indicate in which column the p-value is and what adjustment we want. By default the function adjust data by Bonferroni (`bn`) and FDR (`fdr`). This function, returns the input data with two new columns corresponding to

these adjustments. As in other functions seen before, optionally, we can get a data summary with the number of significative values with bn, fdr, ... in a text file, (the generated file in the example is called *QC_Results/PROJ1_Cohort3_A1_ResumeSignificatives.txt*).

```
# data before adjustment
head(cohort)
##      probeID          BETA          SE     P_VAL CpG_chrm CpG_beg CpG_end
## 1 cg00002593 -0.0014173332 0.010439809 0.8920091     chr1 1333412 1333414
## 2 cg00009834 -0.0001004819 0.007697701 0.9895851     chr1 1412290 1412292
## 3 cg00014118 -0.0063691442 0.016149771 0.6933006     chr1 2004121 2004123
## 4 cg00040588  0.0010886197 0.013553046 0.9359805     chr1 1355331 1355333
## 5 cg00060374 -0.0178768165 0.030803617 0.5616800     chr1 1419854 1419856
## 6 cg00078456 -0.0104996986 0.008940391 0.2402302     chr1 1629041 1629043
##   MASK_snp5_EUR probeType Unrel_450_EPIC_blood MASK_mapping
## 1         FALSE        cg                FALSE        FALSE
## 2         FALSE        cg                FALSE        FALSE
## 3         FALSE        cg                FALSE        FALSE
## 4         FALSE        cg                FALSE        FALSE
## 5         FALSE        cg                FALSE        FALSE
## 6         FALSE        cg                FALSE        FALSE
##   MASK_typeINextBaseSwitch MASK_rmsk15 MASK_sub40_copy MASK_sub35_copy
## 1                    FALSE       FALSE           FALSE           FALSE
## 2                    FALSE        TRUE           FALSE           FALSE
## 3                    FALSE        TRUE           FALSE           FALSE
## 4                    FALSE       FALSE           FALSE           FALSE
## 5                    FALSE       FALSE           FALSE           FALSE
## 6                    FALSE       FALSE           FALSE           FALSE
##   MASK_sub30_copy MASK_sub25_copy MASK_snp5_common MASK_snp5_GMAF1p
## 1           FALSE           FALSE            FALSE            FALSE
## 2           FALSE           FALSE             TRUE            FALSE
## 3           FALSE           FALSE            FALSE            FALSE
## 4           FALSE           FALSE            FALSE            FALSE
## 5           FALSE           FALSE            FALSE            FALSE
## 6           FALSE           FALSE            FALSE            FALSE
##   MASK_extBase MASK_general Unrel_450_EPIC_pla_restrict Unrel_450_EPIC_pla
## 1        FALSE        FALSE                       FALSE              FALSE
## 2        FALSE        FALSE                       FALSE              FALSE
## 3        FALSE        FALSE                       FALSE              FALSE
## 4        FALSE        FALSE                       FALSE              FALSE
## 5        FALSE        FALSE                       FALSE              FALSE
## 6        FALSE        FALSE                       FALSE              FALSE

# Adjust data by Bonferroni and FDR
cohort <- adjust_data(cohort, "P_VAL", bn=TRUE, fdr=TRUE,
                      filename =  paste0(results_folder,'/',prefixes[i],
                                         '_ResumeSignificatives.txt')  )

# data after adjustment
head(cohort)
##         probeID        BETA          SE        P_VAL CpG_chrm CpG_beg CpG_end
## 609  cg10983617 -0.06967961 0.019136276 0.0002713369     chr1 1043283 1043285
```

```
## 409  cg07426077  0.01715768 0.004776728 0.0003282363     chr1 1553200 1553202
## 181  cg03538326 -0.02123421 0.006503119 0.0010937309     chr1 1440464 1440466
## 128  cg02630349 -0.05958242 0.018518028 0.0012929693     chr1 1043286 1043288
## 954  cg16679343 -0.02148449 0.006807270 0.0015988857     chr1 1117568 1117570
## 1018 cg17801765 -0.03068740 0.009899132 0.0019351477     chr1 1022893 1022895
##      MASK_snp5_EUR probeType Unrel_450_EPIC_blood MASK_mapping
## 609          FALSE        cg                FALSE        FALSE
## 409          FALSE        cg                FALSE        FALSE
## 181          FALSE        cg                FALSE        FALSE
## 128          FALSE        cg                FALSE        FALSE
## 954          FALSE        cg                FALSE        FALSE
## 1018         FALSE        cg                FALSE        FALSE
##      MASK_typeINextBaseSwitch MASK_rmsk15 MASK_sub40_copy MASK_sub35_copy
## 609                     FALSE       FALSE           FALSE           FALSE
## 409                     FALSE       FALSE           FALSE           FALSE
## 181                     FALSE       FALSE           FALSE           FALSE
## 128                     FALSE       FALSE           FALSE           FALSE
## 954                     FALSE       FALSE           FALSE           FALSE
## 1018                    FALSE       FALSE           FALSE           FALSE
##      MASK_sub30_copy MASK_sub25_copy MASK_snp5_common MASK_snp5_GMAF1p
## 609            FALSE           FALSE            FALSE            FALSE
## 409            FALSE            TRUE            FALSE            FALSE
## 181            FALSE           FALSE            FALSE            FALSE
## 128            FALSE           FALSE             TRUE            FALSE
## 954            FALSE            TRUE            FALSE            FALSE
## 1018           FALSE            TRUE             TRUE            FALSE
##      MASK_extBase MASK_general Unrel_450_EPIC_pla_restrict Unrel_450_EPIC_pla
## 609         FALSE        FALSE                       FALSE              FALSE
## 409         FALSE        FALSE                       FALSE              FALSE
## 181         FALSE        FALSE                       FALSE              FALSE
## 128         FALSE        FALSE                       FALSE              FALSE
## 954         FALSE        FALSE                       FALSE              FALSE
## 1018        FALSE        FALSE                       FALSE              FALSE
##      padj.bonf  padj.fdr
## 609         no 0.2420743
## 409         no 0.2420743
## 181         no 0.4716713
## 128         no 0.4716713
## 954         no 0.4716713
## 1018        no 0.4757238
```

Then EWAS results are annotated with the corresondign 450K or EPIC annotations and saved with the `write_QCData` function. The file generated by this function is the input for the meta-analysis with GWAMA. This data is stored with *_QC_Data.txt* sufix. In this function data is annotated before being written to the file,

```
# Write QC complete data to external file
write_QCData(cohort, paste0(results_folder,'/',prefixes[i]))
```

4.4 Quality Control - code for plots

To perform a graphical analysis we have different functions. We can easily generate a SE or p-value distribution plots with `plot_distribution` function

```
## Visualization - Plots

# Distribution plot
plot_distribution(cohort$SE,
                  main = paste('Standard Errors of', prefixes[i]),
                  xlab = 'SE')
```

Figure 2: SE distribution plot

```
## Visualization - Plots

plot_distribution(cohort$P_VAL,
                  main = paste('p-values of', prefixes[i]),
                  xlab = 'p-value')
```

```
# QQ plot.
qqman::qq(cohort$P_VAL,
          main = sprintf('QQ plot of %s (lambda = %f)', prefixes[i],
                         lambda = get_lambda(cohort,"P_VAL")))
```

```
# Volcano plot.
plot_volcano(cohort, "BETA", "P_VAL",
             main=paste('Volcano plot of', prefixes[i]) )
```

When we have the results for all models and cohorts, we can perform a Precision plot with `plot_precisionp` function,

```
plot_precisionp(precplot.data.n,
                paste(results_folder,  "precision_SE_N.png", sep='/'),
```

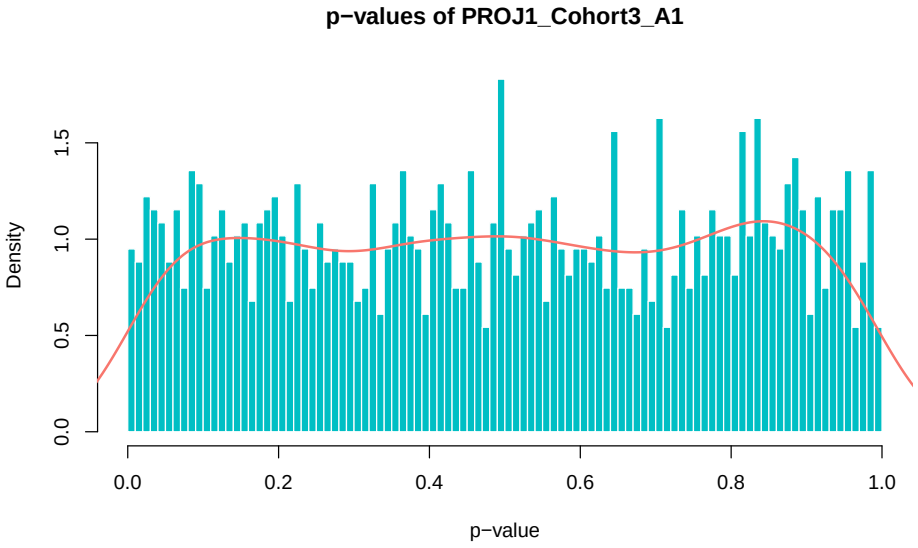


Figure 3: p-value distribution plot

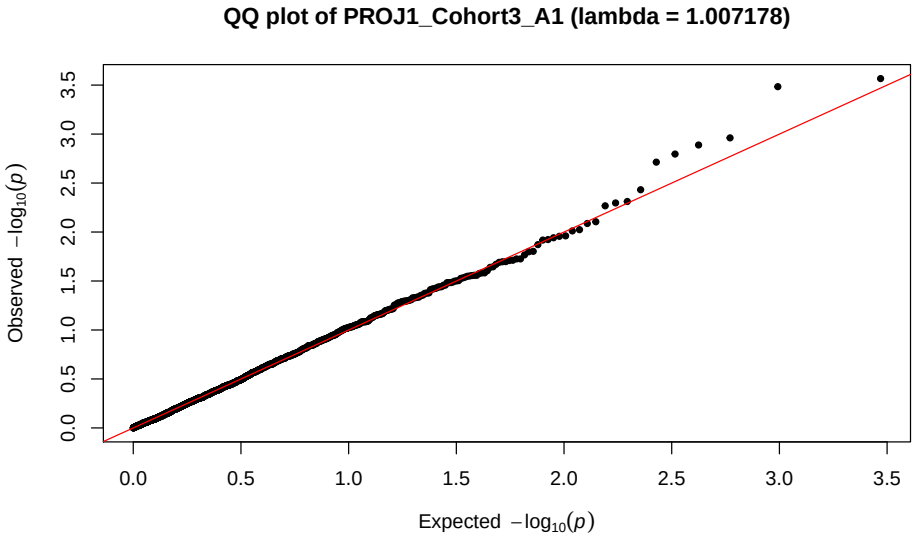


Figure 4: QQplot

```
                    main = "Subgroup Precision Plot -  1/median(SE) vs sqrt(n)")
```

this plot only makes sense if we have analyzed different models and cohorts. Here we show an plot example obtained with `EASIER` .

With all analysed data we can also plot the *betas boxplot* with `plot_betas_boxplot` function

```
plot_betas_boxplot(betas.data,
                   paste(results_folder, 'BETAS_BoxPlot.pdf', sep="/"))
```

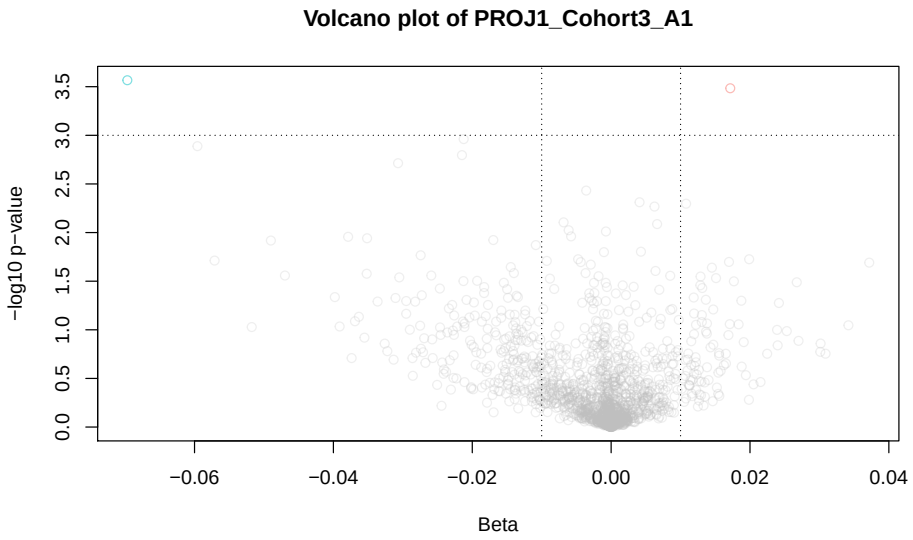


Figure 5: Volcano Plot

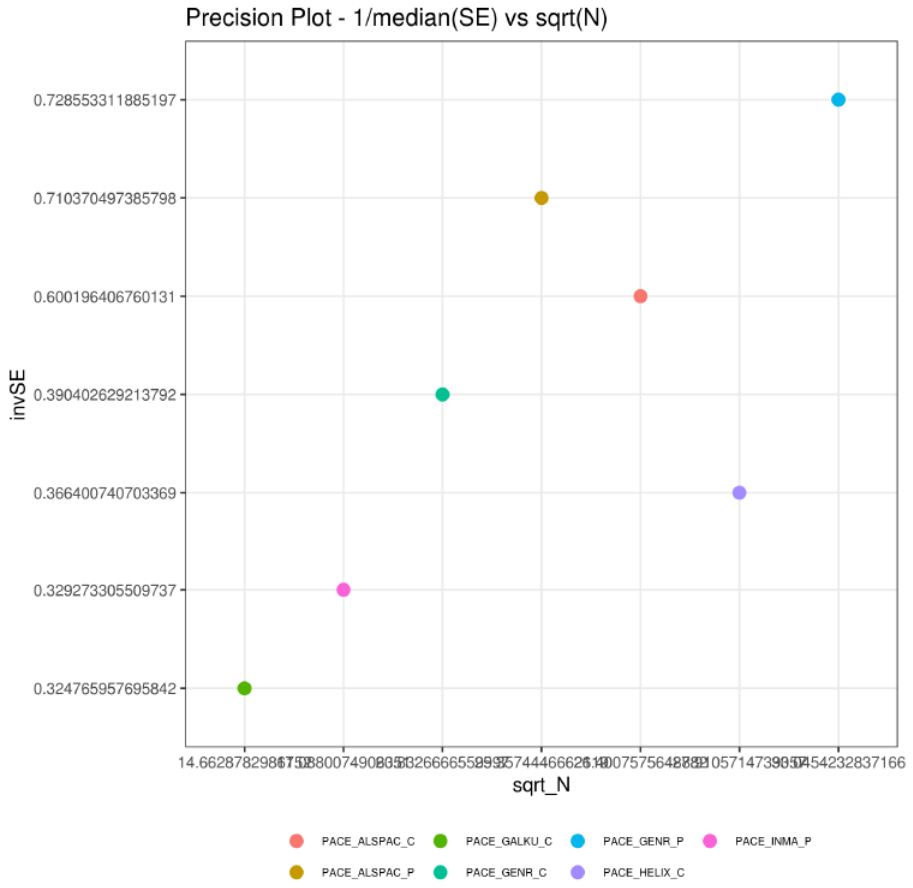


Figure 6: Precision plot for 7 different datasets

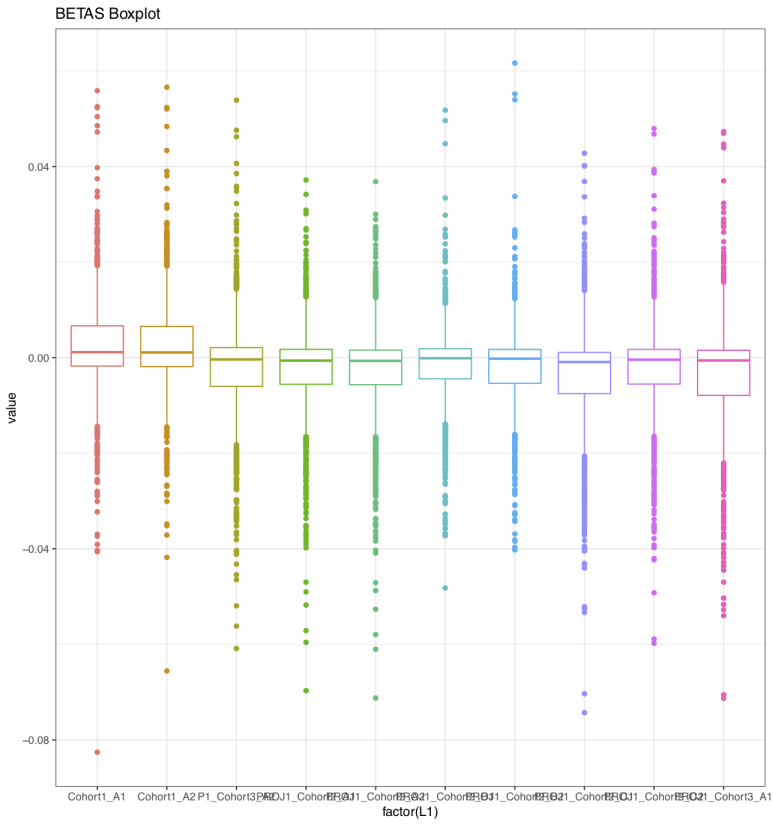


Figure 7: Betas Boxplot plot for 10 different datasets

5 Meta-Analysis with GWAMA

5.1 Meta-Analysis flowchart

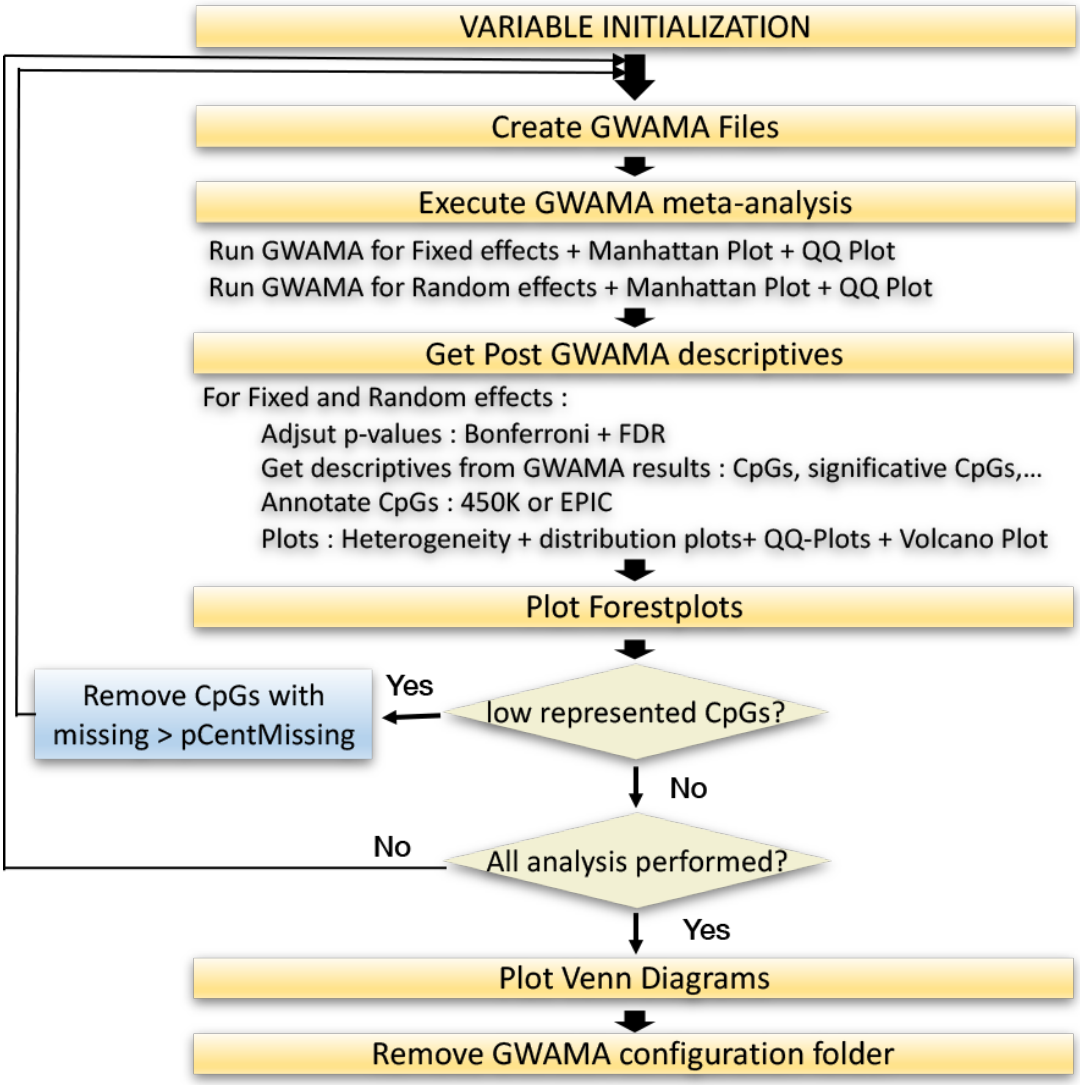


Figure 8: Meta-analysis flowchart
This flowchart is used in the script under test folder to perform the quality control (MetaAnalysis.R). The most important step in this workflow is the first step where we have to define the variables, if variables are well defined all the process is 'automatic'

5.2 Initial Variables definition

Like in quality control analysis, in the meta-analysis we need to define some variables. One of this variables is the one that refers to the the QCed EWAS results that will be combine and anlayze in each meta-analysis. For example, in `metafiles` variable we have defined two different meta-analysis, MetaA1 and MetaA2 . In the first one, MetaA1, we have the datasets 'PROJ1_Cohort3_A1', 'PROJ1_Cohort2_A1' and 'Cohort1_A1', and we can use the simplified form to make all the study more understandable

We can also exclude those CpGs with low representation in meta-analysis, we can set the minimum percentage with `pcentMissing` variable. In this example, we take into account all CpGs present in at least 80% of the datasets of the meta-analysis. We execute the meta-analysis twice, one with all CpGs and other with only CpGs with presence higher than the indicated in `pcentMissing`.

```
## -- Variable definition for Meta-Analysis -- ##

# Array type, used : EPIC or 450K
artype <- '450K'

# Define data for each meta-analysis
metafiles <- list(
   'MetaA1' = c('Cohort1_A1','PROJ1_Cohort2_A1', 'PROJ1_Cohort3_A1' ),
   'MetaA2' = c('Cohort1_A2','PROJ1_Cohort2_A2', 'PROJ1_Cohort3_A2' ))

# Define maximum percent missing for each CpG
pcentMissing <- 0.8 # CpGs with precense lower than pcentMissing after EWAS
                    # meta-analysis will be deleted from the study.

# Paths with QCResults and path to store GWAMA results
results_folder <- 'QC_Results'
results_gwama <- '.'
```

Then, we define the GWAMA execution path for ISGlobal Servers ws05 and ws06.

```
## Create directory for GWAMA configuration files and GWAMA_Results
## inside the defined results_gwama variable defined before.
if(!dir.exists(file.path(getwd(), paste(results_gwama, "GWAMA", sep="/") )))
   suppressWarnings(dir.create(file.path(getwd(), paste(results_gwama, "GWAMA", sep="/"))))

## Create directory for GWAMA_Results
outputfolder <- paste0(results_gwama, "/GWAMA_Results")
if(!dir.exists(file.path(getwd(), outputfolder )))
   suppressWarnings(dir.create(file.path(getwd(), outputfolder)))

# We create a map file for GWAMA --> Used in Manhattan plots.
# We only need to indicate the array type
hapmapfile <- paste(results_gwama,"GWAMA", "hapmap.map" ,sep = "/")
generate_hapmap_file(artype, hapmapfile)
```

In this example we only run the first meta-analysis with all CpGs and with CpGs with missing data lower than `pcentMissing = 0.8` but in a complete script all meta-analyses are performed for both cases: **complete and lowCpGs**.

First, we must create the needed folders. In this example we create a `GWAMA` folder where we will put the input files for GWAMA, and `GWAMA_Results` folder where we will store all the results, when we finish the code execution the `GWAMA` folder with temporal configuration files is removed.

```
list.lowCpGs <- NULL

# Create folder for a meta-analysis in GWAMA folder, here we
# store the GWAMA input files for each meta-analysis,
# We create one for complete meta-analysis
if(!dir.exists(file.path(getwd(),
                         paste(results_gwama,"GWAMA", names(metafiles)[metf],
                               sep="/") )))
   suppressWarnings(dir.create(file.path(getwd(),
                                         paste(results_gwama,"GWAMA",
                                               names(metafiles)[metf],
                                               sep="/"))))
# We create another for meta-analysis without filtered CpGs with low
# percentage (sufix _Filtr)
if(!dir.exists(file.path(getwd(),
                         paste0(results_gwama,"/GWAMA/",
                                names(metafiles)[metf],
                                "_Filtr") )))
   suppressWarnings(dir.create(file.path(getwd(),
                                         paste0(results_gwama, "/GWAMA/",
                                                names(metafiles)[metf],
                                                "_Filtr"))))

# GWAMA File name base
inputfolder <- paste0(results_gwama,"/GWAMA/",  names(metafiles)[metf])

modelfiles <- unlist(metafiles[metf])

# Execution with all CpGs and without filtered CpGs
runs <- c('Normal', 'lowcpgs')
lowCpGs = FALSE;
outputfiles <- list()

outputgwama <- paste(outputfolder,names(metafiles)[metf],sep = '/')
```

To perform meta-analyses we use GWAMA, a Software tool for meta-analysis developed by Intitute of Genomics from University of Tartu. This software is available at https://genomics.ut.ee/en/tools/gwama-download. As mentioned before it must be installed on the computer where we are running analysis and the installation path must be defined in `gwama.dir` variable :

```
# GWAMA binary path  (GWAMA IsGlobal Server sw05 and sw06 installation)
gwama.dir <- paste0(Sys.getenv("HOME"),
                    "/data/EWAS_metaanalysis/1_QC_results_cohorts/GWAMA/")
```

5.2.1 Meta-Analysis - general code

Now we are ready to execute the meta-analysis. You can find the script to perform the full meta-analysis in MetaAnalysis.R file.

Like in Quality control , in GWAMA meta-analysis we created the script MetaAnalysis.R using the library functions to carry out the meta analysis process automatically only by defining the previous variables. The script follows the **Figure8** workflow. In this vignette we will explain how the script works to allow you to modify if necessary.

First of all, we need to generate files with predefined format by GWAMA. To do that, we use the function `create_GWAMA_files`. In this function we have to specify the gwama folder created before in `qcpath` parameter, a character vector with models present in meta-analysis (previously defined in metafiles variable), the folder with original data (these are the QC_Data output files from QC), the number of samples in the study, and we need to indicate if this is the execution with all CpGs or not (if not, we indicate the list with excluded CpGs, which can be obtained with `get_low_presence_CpGs` function.

`create_GWAMA_files` function takes the original files and converts them to the GWAMA format, it also creates the .ini file necessary to run GWAMA

When we have all the files ready to execute GWAMA, we proceed to its execution with `run_GWAMA_MetaAnalysis` function. This function needs to know:

- the folder with data to be analysed, (this is the GWAMA folder),
- where to store the results (by default this function creates a subfolder with meta-analysis name and stores all the results together),
- the meta-analysiss name,
- where is the GWAMA binary installed,

GWANA is executed by fixed and random effects. The function `run_GWAMA_MetaAnalysis` function generates one .out file with meta-analysis results, and the associated Manhattan plots and QQ plots, one for fixed effects and another for random effects.

```
for(j in 1:length(runs))
{
   if(runs[j]=='lowcpgs') {
      lowCpGs = TRUE
      # Get low presence CpGs in order to exclude this from the new meta-analysis
      list.lowCpGs <- get_low_presence_CpGs(outputfiles[[j-1]], pcentMissing)
      inputfolder <- paste0(results_gwama,"/GWAMA/",  names(metafiles)[metf], "_Filtr")
      outputgwama <- paste0(outputgwama,"_Filtr")
   }

   # Create a GWAMA files for each file in meta-analysis and one file with
```

```
# gwama meta-analysis configuration
for ( i in 1:length(modelfiles) )
   create_GWAMA_files(results_folder,  modelfiles[i],
                      inputfolder, N[i], list.lowCpGs )

# Execute GWAMA meta-analysis and manhattan-plot, QQ-plot and a file
# with gwama results.
outputfiles[[runs[j]]] <- run_GWAMA_MetaAnalysis(inputfolder,
                                                 outputgwama,
                                                 names(metafiles)[metf],
                                                 gwama.dir)
```

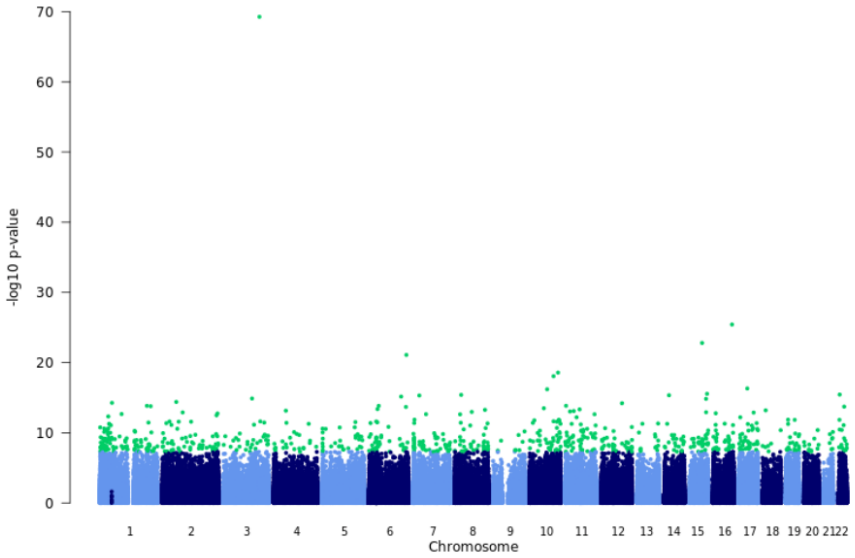


Figure 9: Manhattan plot obtained with GWAMA

After getting the GWAMA results, we perform an analysis with `get_descriptives_postGWAMA` function (similar to what was done in the quality control procedure but with meta-analysis results). This function adjusts p-values, annotates CpGs, and generates a file with descriptive results and plot heterogeneity distribution, SE distribution, p-values distribution, QQ-plot with lambda, and the volcano plot.

To finish we generate the ForestPlot associated to the most significative CpGs, by default we get the 30 top significative CpGs.

```
# Post-metha-analysis QC --- >>> adds BN and FDR adjustment
dataPost <- get_descriptives_postGWAMA(outputgwama,
                                       outputfiles[[runs[j]]],
                                       modelfiles,
                                       names(metafiles)[metf],
                                       artype,
```

```
                                        N[which(prefixes %in% modelfiles)] )

    # Forest-Plot
    plot_ForestPlot( dataPost, metafiles[[metf]], runs[j],
                     inputfolder, names(metafiles)[metf], files, outputgwama  )

}
```

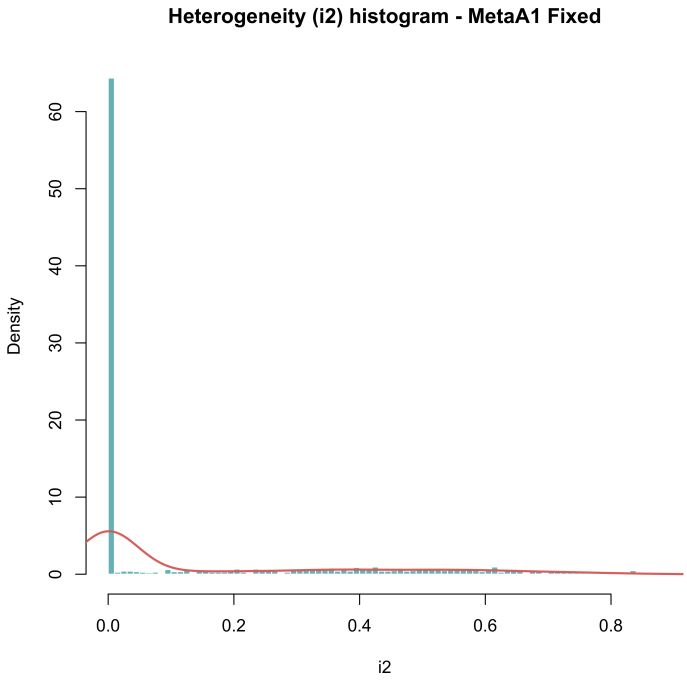


Figure 10: Heterogeneity distribution plot (i2)

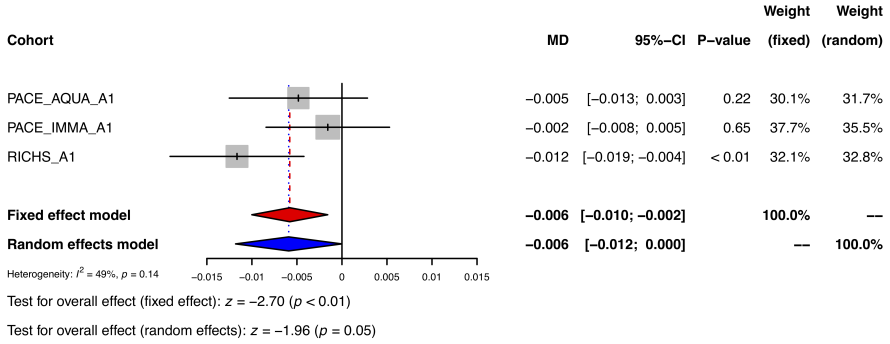


Figure 11: Forest plot for cpg22718050

Wen we have all the meta-analysis we create the venn diagrams defined in `venn_diagrams` variable with `plot_venndiagram` function for FDR and Bonferroni significative CpGs.

```
for (i in 1:length(venn_diagrams))
   plot_venndiagram(venn_diagrams[[i]], qcpath = outputfolder, plotpath =  paste0(results_gwama, "/GWAMA_Resu
                    pattern = '_Fixed_Modif.out',bn='Bonferroni', fdr='FDR')
```

this is venn diagram output example

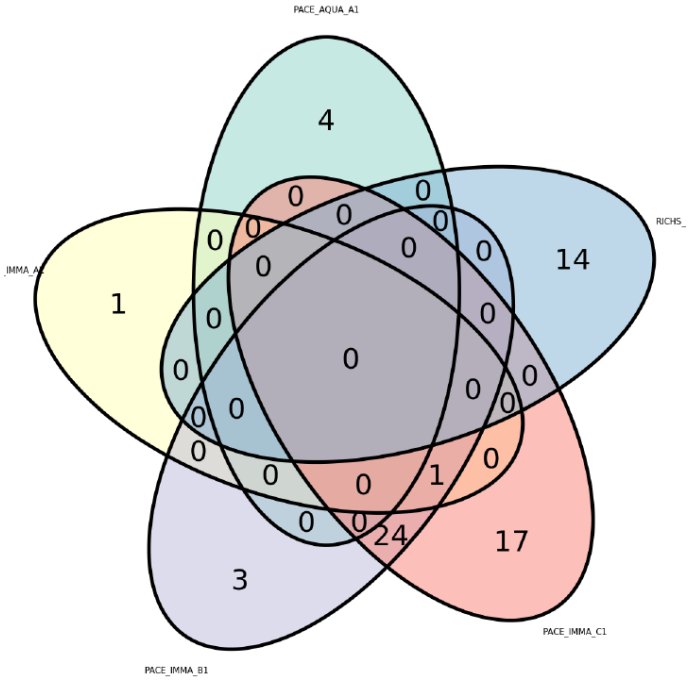


Figure 12: Venn diagram example

6 Enrichment

6.1 Enrichment Flowchart

Like Quality Control and Meta-analysis, we have created a script Enrichment.R using the library functions to carry out the *enrichment process* automatically only by defining some variables. The script follows the **Figure13** workflow.

The first step is define variables, after variable declaration, we read the files with CpGs, the file can contain only a list of CpGs, wit no mre data than the CpG name, can contain the results from GWAMA, depending if we have only a CpG list or the results from GWAMA, the enrichment is different, the differences are specified in next slides.

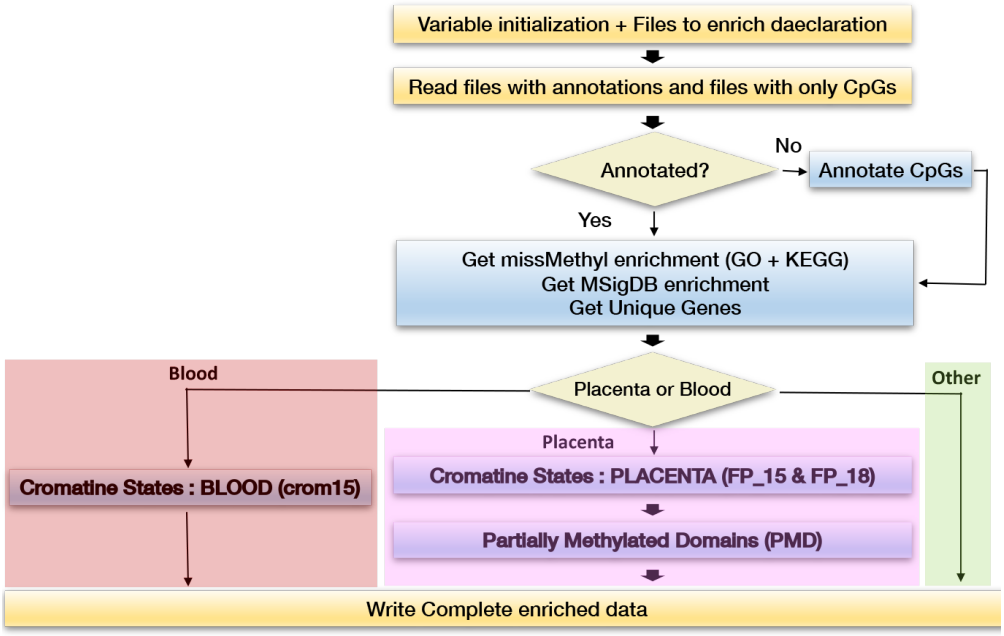


Figure 13: Enrichment flowchart
This flowchart is used in the script under test folder to perform the enrichment (Enrichment.R). The most important step in this workflow is the first step where we have to define the variables, if variables are well defined all the process is 'automatic'

6.2 Variables definition

First, we need to set up the working directory, in our case, we set the working directory to the metaanallysis folder, after that, we need to define the route to the files with the data to enrich in `FilesToEnrich` variable, this files could be files with only CpGs (a nude CpG list) or the results from GWAMA meta-analysis with p-values and other related data to this CpGs.

```
# Set working directory to metaanalysis folder
setwd("<path to metaanalysis folder>/metaanalysis")

# Files with CpG data to enrich may be a CpGs list or annotated GWAMA output
FilesToEnrich <- c('toenrich/CpGstoEnrich.txt',
                   'GWAMA_Results/MetaA1/MetaA1_Fixed_Modif.out'
                    )
```

After that, with those files with p-value information, we need to define which CpGs should be used for enrichment, * `BN` : CpGs that accomplish with Bonferroni criteria, possible values : TRUE or FALSE * `FDR` : at what significance level based on FDR we want to take in to account CpGs, this value should be smaller or equal to 0.05, if FDR = NA, FDR is not taken in to a ccount * `pvalue` : at what significance level based we want to take in to account CpGs, this value should be smaller or equal to 0.05, if pvalue = NA, FDR is not taken in to a

```
# Values for adjustment
BN <-  TRUE       # Use Bonferroni ?
FDR <- 0.7        # significance level for adjustment, if NA FDR is not used
pvalue <- 0.05    # significance level for p-value, if NA p-value is not used
```

Like in previous steps, we define the artype and the folders used to store results in quality control and meta-analysis (needed in some enrichment steps if we are enriching GWAMA results) and the folder to store enrichment results.

```
# Array type, used : EPIC or 450K
artype <- '450K'
# Result paths definition for QC, Meta-Analysis and Enrichment
results_folder <- 'QC_Results'
results_gwama <- '.'
results_enrich <- 'Enrichment'
```

Next variable `enrichtype` is related to enrichment type, enrichment for blood, placenta or a general enrichment, we can observe de differences between them in **Figure**13 if we are performing a placenta enrichment we must also define `enrichFP18 = TRUE` if we wan to use Fetal placenta States 18. For all branches, if we have the p-values we can indicate what type of statistical test we want to use, hypergeometric or Fisher if no test is defined, by default Fisher test is used.

```
# Enrichment type :  'BLOOD' or 'PLACENTA'
#     if enrichtype <- 'BLOOD' => enrichment with : Cromatine States : BLOOD (crom15)
#                                                   (To be implemented in future) Partially Methylated Domai
#     if enrichtype <- 'PLACENTA' => enrichment with: Cromatine States : PLACENTA (FP_15) optionally (FP_18)
#                                                     Partially Methylated Domains (PMD) for Placenta
#     if enrichtype is different from 'BLOOD' and 'PLACENTA' we only get the missMethyl and MSigDB enrichment
enrichtype <- 'PLACENTA'

# Cromatine States Placenta Enrichment FP_18
# if enrichFP18 = TRUE the enrichment is performed wit FP_15 and FP_18
enrichFP18 <- FALSE

# Test to be used : 'Fisher' or 'Hypergeometric' if testdata is different no test will be performed
testdata <- 'Fisher'
```

Prepare output folcers to sotre data

```
## Check if we have any files to enrich and if these files exists
if (length(FilesToEnrich)>=1 & FilesToEnrich[1]!='') {
   for ( i in 1:length(FilesToEnrich))
      if (!file.exists(FilesToEnrich[i])) stop(paste0('File ',FilesToEnrich[i],' does not exsits, please chec
}

## Check variables

if( ! toupper(enrichtype) %in% c('PLACENTA','BLOOD') )
   warning('Only enrichment with MyssMethyl and MSigDB will be done')

if( ! tolower(testdata) %in% c('fisher','hypergeometric') )
   warning('Wrong value for testdata variable, values must be "Fisher" or "Hypergeometric". No test will be p
```

```
# Convert relative paths to absolute paths for FilesToEnrich
FilesToEnrich <- unlist(sapply(FilesToEnrich, function(file) { if(substr(file,1,1)!='.' & substr(file,1,1)!=
FilesToEnrich <- sapply(FilesToEnrich, file_path_as_absolute)

if(results_enrich!='.'){
   outputfolder <- file.path(getwd(), results_enrich )
}else{
   outputfolder <- file.path(getwd() )}


# Create dir to put results from enrichment
if(!dir.exists(outputfolder))
   suppressWarnings(dir.create(outputfolder))

setwd( outputfolder)
```

6.3 Common enrichment for Blood and Placenta

The procedure that we will detail is executed for each one of the files entered in the variable `FilesToEnrich`, we show how it works with CpG nude list (first file in `FilesToEnrich` variable) and wiht GWAMA output results (second file in `FilesToEnrich` variable).

CpG Nude List without p-values and annotattion

After define the variables we read the file content and test if data is a nude CpG list or a resulting file from GWAMA. If the input file is a nude CpG list we first of all enrich this data with illumina annotation for EPIC or 450K with `get_annotattions` function (GWANA files are previously annotated in meta-analysis), in `get_annotattions` we have as a parameter the array type, and the data to enrich.

```
i <- 1 # We get first file in FilesToEnrich

# Enrich all CpGs
allCpGs <- FALSE

# Get data
data <- NULL
data <- read.table(FilesToEnrich[i], header = TRUE, sep = "", dec = ".", stringsAsFactors = FALSE)

# Is a CpG list only ? then read without headers and annotate data
if(dim(data)[1] <= 1 | dim(data)[2] <= 1) {
   data <- read.table(FilesToEnrich[i], dec = ".") # Avoid header
   data <- as.vector(t(data))
   head(data)
   data <- get_annotattions(data, artype, FilesToEnrich[i], outputfolder )

   head(data)

   allCpGs <- TRUE
   data$chromosome <- substr(data$chr,4,length(data$chr))
```

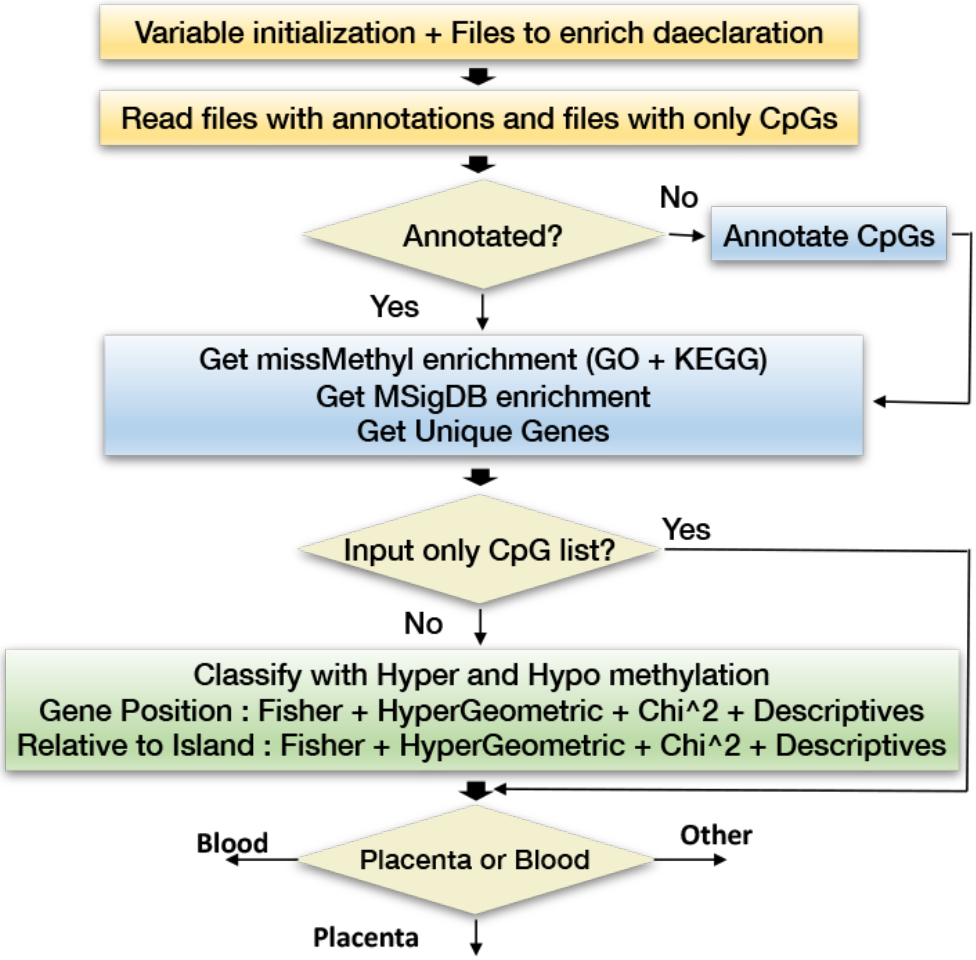


Figure 14: Enrichment flowchart
Detailed common enrichment for blood, placenta and other

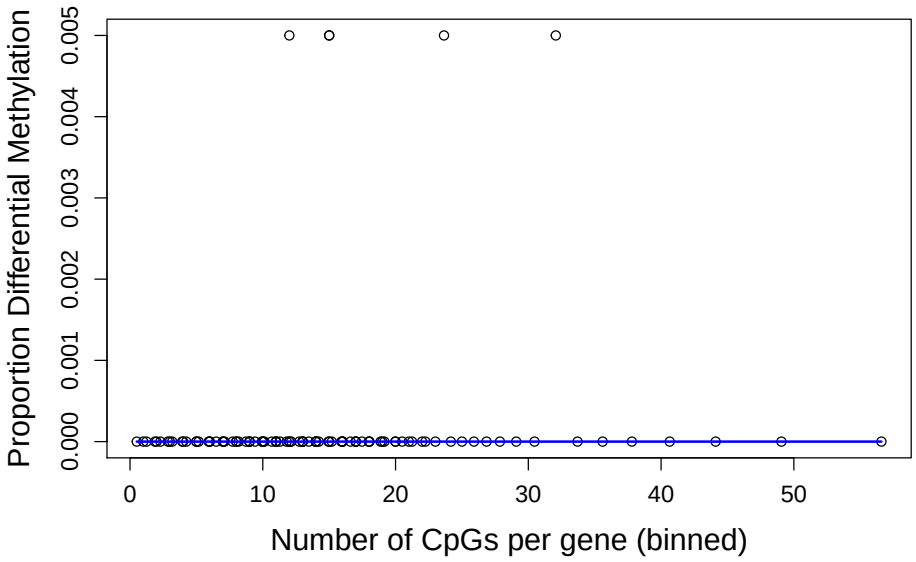
```
    data$rs_number <- data$CpGs
}
```

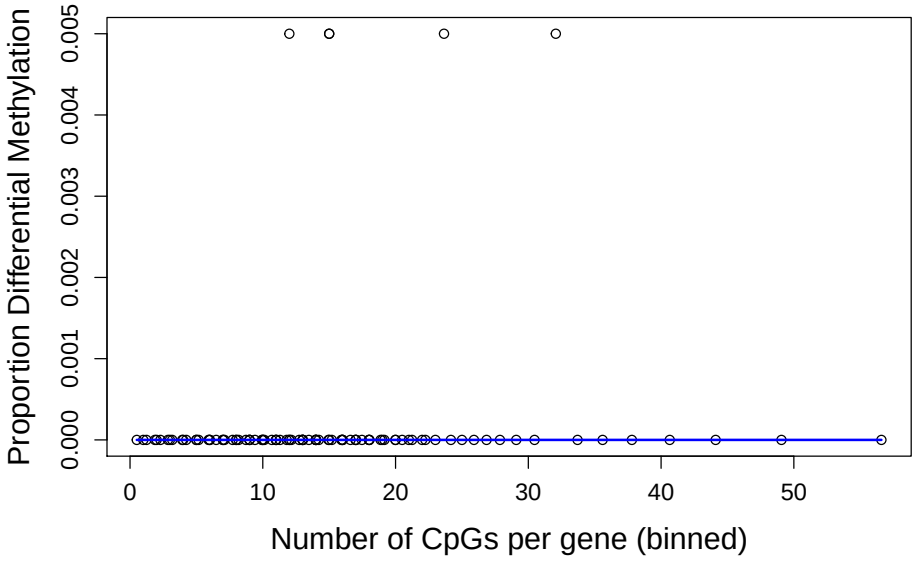
6.3.1 GO and KEGG and MolSig

When all data is annotated we enrich data with `missMethyl_enrichment` function, to this function we have to inform the paramteres to decide if the CpG is significative or not, we do that with previously defined variables FDR, Bonferroni and pval, this only work with GWAMA results or with files that contains this information, if we are working with only CpG list, all the CpGs are enriched. missMethyl_enrichment function performs the enrichment with GO and KEGG ,

```
## -- Functional Enrichmnet
## ------------------------
```

```
# Enrichment with missMethyl - GO and KEGG --> Writes results to outputfolder
miss_enrich <- missMethyl_enrichment(data, outputfolder, FilesToEnrich[i], artype, BN, FDR, pvalue, allCpGs,
## [1] "/Users/mailos/Library/Mobile Documents/com~apple~CloudDocs/PROJECTES/Treballant/EASIER/vignettes/Enri
```






```
head(miss_enrich$GO)
##            ONTOLOGY                                                TERM    N DE
## GO:0000002       BP                  mitochondrial genome maintenance   30  0
## GO:0000003       BP                                        reproduction 1402  0
## GO:0000009       MF            alpha-1,6-mannosyltransferase activity    1  0
## GO:0000010       MF         trans-hexaprenyltranstransferase activity    2  0
## GO:0000012       BP                           single strand break repair   10  0
## GO:0000014       MF single-stranded DNA endodeoxyribonuclease activity   10  0
##            P.DE FDR
## GO:0000002    1   1
```

```
## GO:0000003    1   1
## GO:0000009    1   1
## GO:0000010    1   1
## GO:0000012    1   1
## GO:0000014    1   1
head(miss_enrich$KEGG)
##                                          Description  N DE P.DE FDR
## path:hsa00010            Glycolysis / Gluconeogenesis 66  0    1   1
## path:hsa00020               Citrate cycle (TCA cycle) 30  0    1   1
## path:hsa00030               Pentose phosphate pathway 29  0    1   1
## path:hsa00040 Pentose and glucuronate interconversions 33  0    1   1
## path:hsa00051          Fructose and mannose metabolism 33  0    1   1
## path:hsa00052                     Galactose metabolism 29  0    1   1
```

6.3.2 Pathways with Molecular Signatures Database (MSigDB)

We can get the Molecular Signatures Database (MSigDB) enrichment with `MSigDB_enrichment` function, this functions needs the list of CpGs or the output data from GWAMA, in that case also needs the parameters to take in to account to decide whether CpG is or not significative.

```
## -- Molecular Enrichmnet
## ----------------------

# Molecular Signatures Database enrichment
msd_enrich <- MSigDB_enrichment(data, outputfolder, FilesToEnrich[i], artype, BN, FDR, pvalue, allCpGs)
## [1] "/Users/mailos/Library/Mobile Documents/com~apple~CloudDocs/PROJECTES/Treballant/EASIER/vignettes/Enri

head(msd_enrich$MSigDB)
##                                              N DE P.DE FDR
## KEGG_GLYCOLYSIS_GLUCONEOGENESIS             62  0    1   1
## KEGG_CITRATE_CYCLE_TCA_CYCLE                30  0    1   1
## KEGG_PENTOSE_PHOSPHATE_PATHWAY              26  0    1   1
## KEGG_PENTOSE_AND_GLUCURONATE_INTERCONVERSIONS 27  0    1   1
## KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM        34  0    1   1
## KEGG_GALACTOSE_METABOLISM                   26  0    1   1
```

6.3.3 Get unique genes

To get the list with unique genes related to significative CpGs

```
      # get unique genes from data
geneUniv <- lapply( lapply(miss_enrich[grepl("signif", names(miss_enrich))],
                           function(cpgs) { data[which(as.character(data$CpGs) %in% cpgs),]$UCSC_RefGene_Name
                    getUniqueGenes)

geneUniv
## $signif
##   ZNF843    EXPH5   CREBZF   ENTPD4     LAG3
## "283933"  "23086"  "58487"   "9583"   "3902"
```

6.3.4 Gene relative position

When we have p-values we can apply statistical tests 'Fisher' or 'Hypergeometric', depends on variable `testdata` defined previously. We apply the test for FDR and Bonferroni significative CpGs (taking in to account the initial definition for FDR and Bonferroni) and for Hyper and Hypo methylated.

First of all, we have to classify CpGs in Hyper and Hypo methylated, to do that, we apply the function `getHyperHypo` to beta values, we also get a binary classification ('yes', 'no') for FDR taking in to account the significance level declared before.

```
if("FDR" %in% colnames(data) & "Bonferroni" %in% colnames(data))
{

   ## -- Prepare data
   ## ---------------

   # Add column bFDR to data for that CpGs that accomplish with FDR
    # Classify fdr into "yes" and no taking into account FDR significance level
   data$bFDR <- getBinaryClassificationYesNo(data$FDR, "<", FDR)

   # Classify by Hyper and Hypo methylated
   data$meth_state <- getHyperHypo(data$beta) # Classify methylation into Hyper and Hypo

   # CpGs FDR and Hyper and Hypo respectively
   FDR_Hyper <- ifelse(data$bFDR == 'yes' &
                         data$meth_state=='Hyper', "yes", "no")
   FDR_Hypo <- ifelse(data$bFDR == 'yes' &
                        data$meth_state=='Hypo', "yes", "no")

   # CpGs Bonferroni and Hyper and Hypo respectively
   BN_Hyper <- ifelse(data$Bonferroni == 'yes' &
                        data$meth_state=='Hyper', "yes", "no")
   BN_Hypo <- ifelse(data$Bonferroni == 'yes' &
                       data$meth_state=='Hypo', "yes", "no")
```

Now, we get all descriptive data related to Gene positions for significative CpGs an all fisher test with `getAllFisherTest` or all Hypergeometric test with `getAllHypergeometricTest` for all Gene positions, this functions write all results in `outputfile` inside `outputdir` parameters

```
## --  CpG Gene position
## --------------------

# Get descriptives
get_descriptives_GenePosition(data$UCSC_RefGene_Group,
                              data$Bonferroni,
                              "Bonferroni",
                              outputdir = "GenePosition/Fisher_BN_Desc",
                              outputfile = FilesToEnrich[i])
get_descriptives_GenePosition(data$UCSC_RefGene_Group, d
                              ata$bFDR , "FDR",
                              outputdir = "GenePosition/Fisher_FDR_Desc",
                              outputfile = FilesToEnrich[i])
```

```
if( tolower(testdata) =='fisher') {
   ## --  Fisher Test - Gene position - FDR, FDR_hyper and FDR_hypo
   GenePosition_fdr <- getAllFisherTest(data$bFDR,
                                  data$UCSC_RefGene_Group,
                                  outputdir = "GenePosition/Fisher_FDR",
                                  outputfile = FilesToEnrich[i],
                                  plots = TRUE )
   GenePosition_fdr_hyper <- getAllFisherTest(FDR_Hyper,
                                  data$UCSC_RefGene_Group,
                                  outputdir = "GenePosition/Fisher_FDRHyper",
                                  outputfile = FilesToEnrich[i],
                                  plots = TRUE )
   GenePosition_fdr_hypo <- getAllFisherTest(FDR_Hypo,
                                    data$UCSC_RefGene_Group,
                                    outputdir = "GenePosition/Fisher_FDRHypo",
                                    outputfile = FilesToEnrich[i], plots = TRUE )
}
else if ( tolower(testdata) =='hypergeometric') {
   ## --  HyperGeometric Test - Island relative position -
   ## FDR, FDR_hyper and FDR_hypo (for Depletion and Enrichment)
   GenePosition_fdr <- getAllHypergeometricTest(data$bFDR,
                                    data$UCSC_RefGene_Group,
                                    outputdir = "GenePosition/HyperG_FDR",
                                    outputfile = FilesToEnrich[i])
   GenePosition_fdr_hyper <- getAllHypergeometricTest(FDR_Hyper,
                                    data$UCSC_RefGene_Group,
                                    outputdir = "GenePosition/HyperG_FDRHyper",
                                    outputfile = FilesToEnrich[i])
   GenePosition_fdr_hypo <- getAllHypergeometricTest(FDR_Hypo,
                                  data$UCSC_RefGene_Group,
                                  outputdir = "GenePosition/HyperG_FDRHypo",
                                  outputfile = FilesToEnrich[i])
}
```

we can also get a collapsed plot with all results regardless if the applied test has been fisher or hypergeometric with `plot_TestResults_Collapsed` function.

```
        plot_TestResults_Collapsed(list(fdr = GenePosition_fdr,
                                        fdr_hypo = GenePosition_fdr_hypo,
                                        fdr_hyper = GenePosition_fdr_hyper),
                                    outputdir = "GenePosition",
                                    outputfile = FilesToEnrich[i], main = )
```

6.3.5 CpG island relative position

We can also proceed with the same steps with CpGs Island relative position, in that case, we get the descriptives to Relative to Island position with `get_descriptives_RelativetoIsland` function, the procedure to get we also get a plot with statistical results.

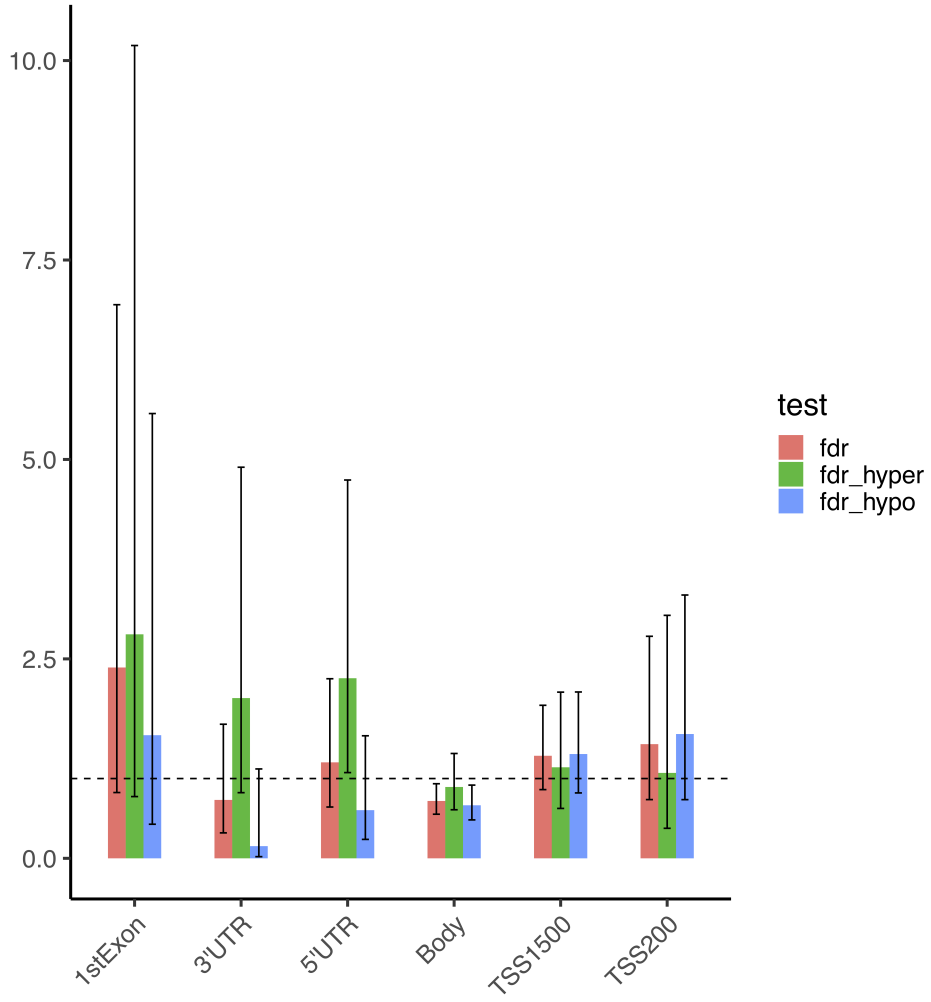


Figure 15: Gene position with Fisher test for Hyper and Hypo methylated CpGs

```
## --  CpG Island relative position
## --------------------------------

# Get descriptives
get_descriptives_RelativetoIsland(data$Relation_to_Island,
                            data$Bonferroni,
                            "Bonferroni",
                            outputdir = "RelativeToIsland/Fisher_BN_RelativeToIsland",
                            outputfile = FilesToEnrich[i])
get_descriptives_RelativetoIsland(data$Relation_to_Island,
                            data$bFDR ,
                            "FDR",
                            outputdir = "RelativeToIsland/Fisher_FDR_RelativeToIsland",
                            outputfile = FilesToEnrich[i])
```

```
if( tolower(testdata) =='fisher') {
   ## --  Fisher Test - Position Relative to Island - FDR, FDR_hyper and FDR_hypo
   relative_island_fdr <- getAllFisherTest(data$bFDR,
                                  data$Relation_to_Island,
                                  outputdir = "RelativeToIsland/Fisher_FDR",
                                  outputfile = FilesToEnrich[i], plots = TRUE )
   relative_island_fdr_hyper <- getAllFisherTest(FDR_Hyper,
                                  data$Relation_to_Island,
                                  outputdir = "RelativeToIsland/Fisher_FDRHyper",
                                  outputfile = FilesToEnrich[i], plots = TRUE )
   relative_island_fdr_hypo <- getAllFisherTest(FDR_Hypo,
                                       data$Relation_to_Island,
                                       outputdir = "RelativeToIsland/Fisher_FDRHypo",
                                       outputfile = FilesToEnrich[i], plots = TRUE )
}
```

6.4 Specific Blood

As we show in **Figure17** blood enrichment is done with the chromatine states 15, in that case we also perform an statistical analysis applying Fisher or Hypergeometric and Hyper and Hypo methylated CpGs.

6.4.1 15 ROADMAP chromatine states

```
## --  ROADMAP  -  Metilation in Cromatine States - BLOOD
## ------------------------------------------------------
##  Analysis of methylation changes in the different chromatin
##  states (CpGs are diff meth in some states and others don't)

# Prepare data
# Adds chromatine state columns
crom_data <- addCrom15Columns(data, "CpGId")

if("FDR" %in% colnames(data) & "Bonferroni" %in% colnames(data))
{

   # Columns with chromatin status information :
   ChrStatCols <- c("TssA","TssAFlnk","TxFlnk","TxWk","Tx","EnhG",
                    "Enh","ZNF.Rpts","Het","TssBiv","BivFlnk",
                    "EnhBiv","ReprPC","ReprPCWk","Quies")

   if( !is.na(FDR) ) {
      chrom_states_fdr <- getAllChromStateOR( crom_data$bFDR,
                                  crom_data[,ChrStatCols],
```

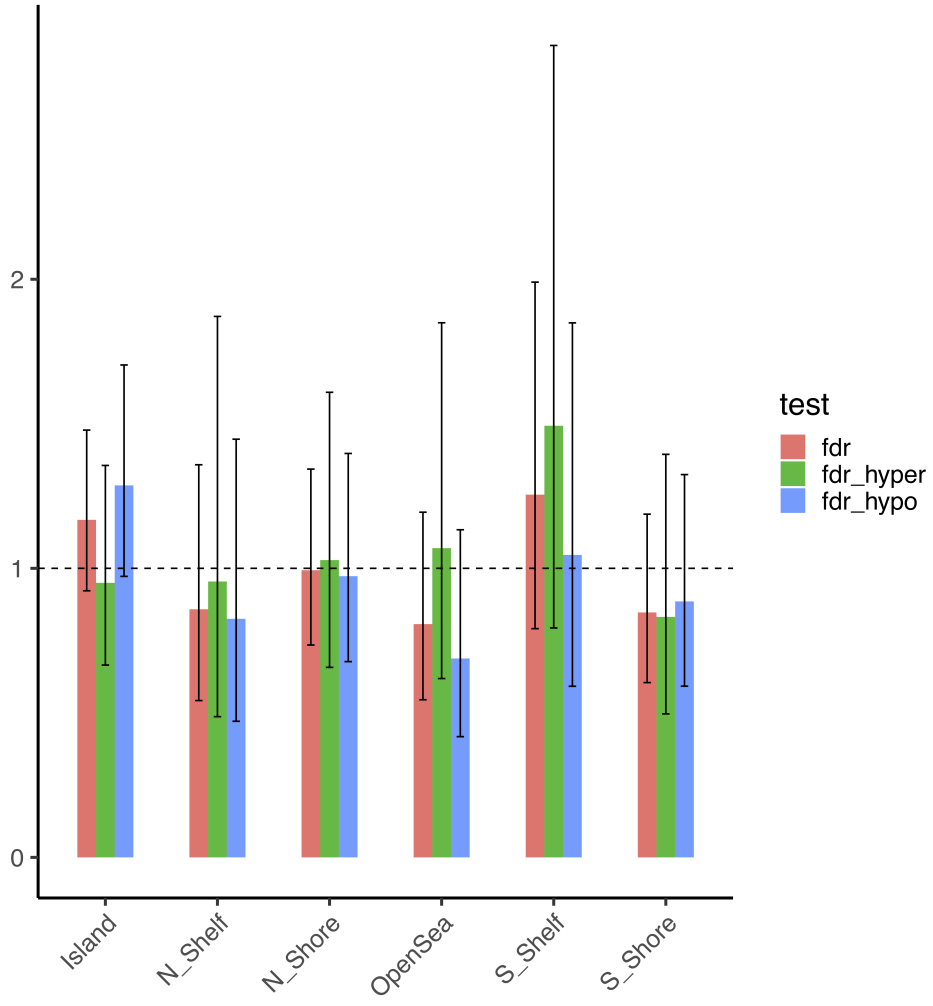


Figure 16: Gene position with Fisher test for Hyper and Hypo methylated CpGs

```
                                outputdir = "CromStates/OR_FDR",
                                outputfile = FilesToEnrich[i],
                                plots = TRUE )
    chrom_states_fdr_hyper <- getAllChromStateOR( FDR_Hyper,
                                crom_data[,ChrStatCols],
                                outputdir = "CromStates/OR_FDRHyper",
                                outputfile = FilesToEnrich[i],
                                plots = TRUE )
    chrom_states_fdr_hypo <- getAllChromStateOR( FDR_Hypo,
                               crom_data[,ChrStatCols],
                               outputdir = "CromStates/OR_FDRHypo",
                               outputfile = FilesToEnrich[i],
                               plots = TRUE )
}
```

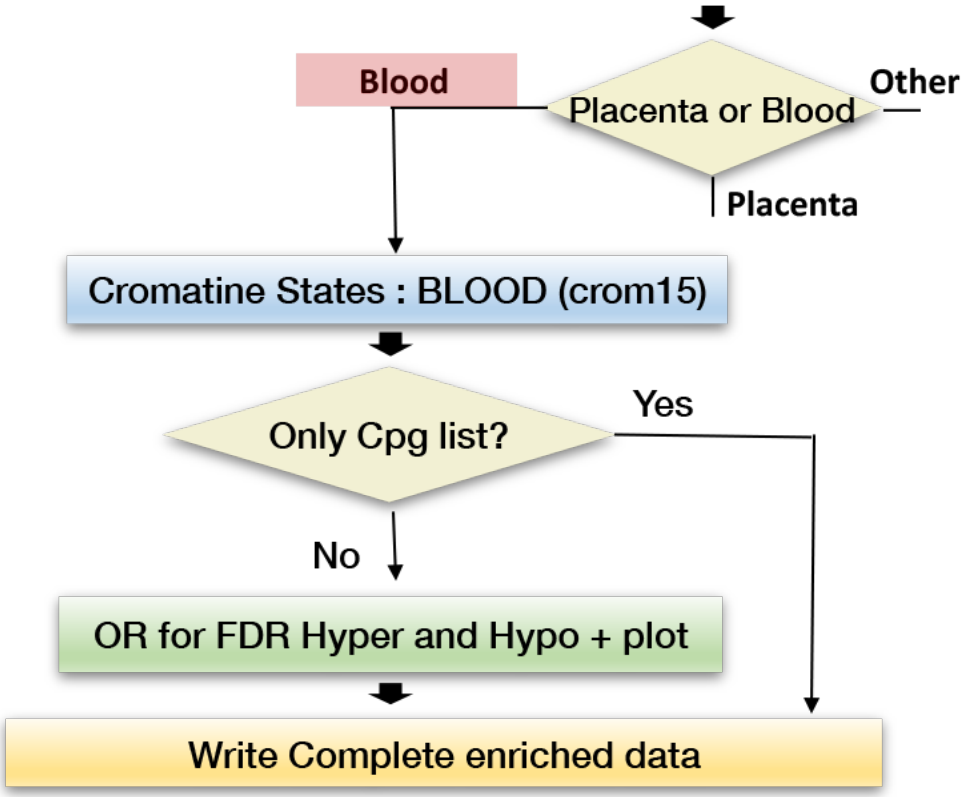


Figure 17: Enrichment flowchart
Detailed Blood enrichment

```
        if ( BN == TRUE) {
           chrom_states_bn <- getAllChromStateOR( crom_data$Bonferroni,
                                    crom_data[,ChrStatCols],
                                    outputdir = "CromStates/OR_BN",
                                    outputfile = FilesToEnrich[i],
                                    plots = TRUE )
           chrom_states_bn_hyper <- getAllChromStateOR( BN_Hyper,
                                    crom_data[,ChrStatCols],
                                    outputdir = "CromStates/OR_BNHyper",
                                    outputfile = FilesToEnrich[i],
                                    plots = TRUE )
           chrom_states_bn_hypo <- getAllChromStateOR( BN_Hypo,
                                   crom_data[,ChrStatCols],
                                   outputdir = "CromStates/OR_BNHypo",
                                   outputfile = FilesToEnrich[i],
                                         plots = TRUE )
        }
      }
```

6.5 Specific Placenta

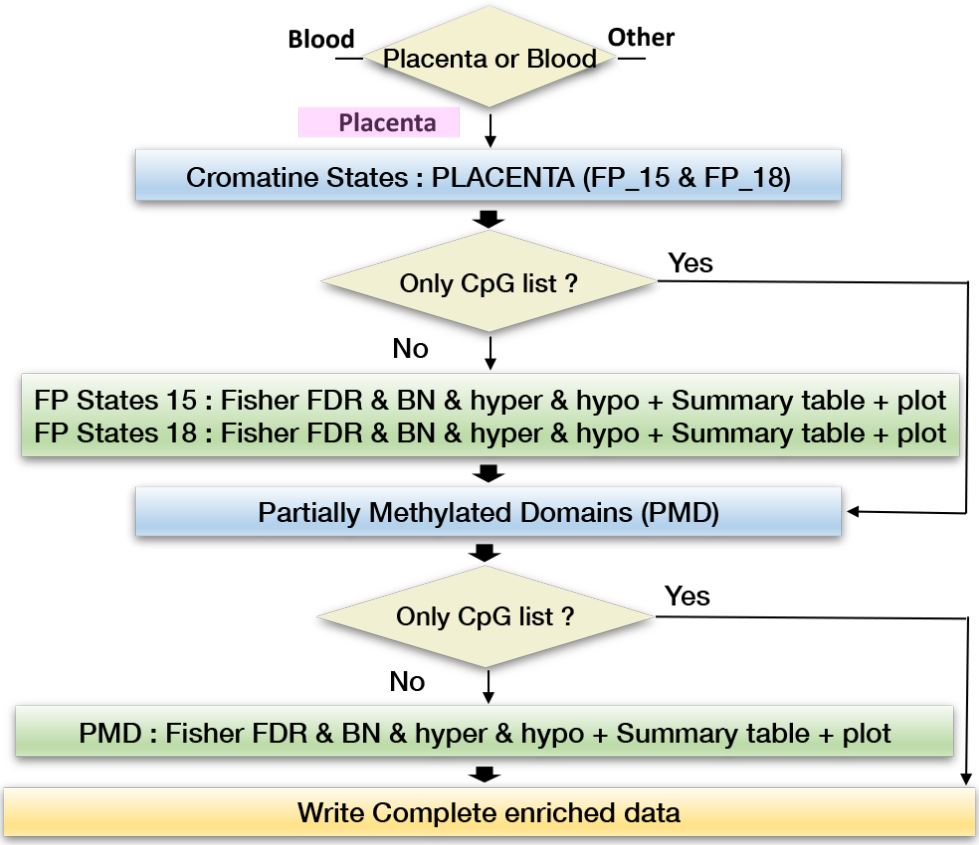


Figure 18: Enrichment flowchart
Detailed Placenta enrichment

6.5.1 ROADMAP chromatine states Fetal Placenta 15 and 18

```
## -- ROADMAP  -  Regulatory feature enrichment analysis - PLACENTA
## ------------------------------------------------------------------

# Convert to Genomic Ranges
data.GRange <- GRanges(
   seqnames = Rle(data$chr),
   ranges=IRanges(data$pos, end=data$pos),
   name=data$CpGs,
   chr=data$chromosome,
   pos=data$pos
)
names(data.GRange) <- data.GRange$name
```

```
# Find overlaps between CpGs and Fetal Placenta (States 15 and 18)
over15 <- findOverlapValues(data.GRange, FP_15_E091 )

if (enrichFP18 == TRUE){
   over18 <- findOverlapValues(data.GRange, FP_18_E091 )
   # Add states 15 and 18 to data.GRange file
   # and write to a file : CpGs, state15 and state18
   data.chrstates <- c(mcols(over15$ranges), over15$values, over18$values)
   colnames(data.chrstates)[grep("States",colnames(data.chrstates))] <-
      c("States15_FP", "States18_FP")
} else {
   # Add states 15 to data.GRange file and write to a file : CpGs, state15
   data.chrstates <- c(mcols(over15$ranges), over15$values)
   colnames(data.chrstates)[grep("States",colnames(data.chrstates))] <-
      c("States15_FP")
}

# Merge annotated data with chromatine states with states with data
crom_data <- merge(data, data.chrstates, by.x = "CpGs", by.y = "name" )

fname <- paste0("ChrSates_Pla_data/List_CpGs_",
                tools::file_path_sans_ext(basename(FilesToEnrich[i])),
                "_annot_plac_chr_states.txt")
dir.create("ChrSates_Pla_data", showWarnings = FALSE)
write.table( crom_data, fname, quote=F, row.names=F, sep="\t")

## --  Fisher Test - States15_FP - BN,  BN_hyper and BN_hypo
## (Depletion and Enrichment)
States15FP_bn <- getAllFisherTest(crom_data$Bonferroni,
                                  crom_data$States15_FP,
                                  outputdir = "ChrSates_15_Pla/Fisher_BN",
                                  outputfile = FilesToEnrich[i])
States15FP_bnhyper <- getAllFisherTest(BN_Hyper,
                                    crom_data$States15_FP,
                                    outputdir = "ChrSates_15_Pla/Fisher_BNHyper",
                                    outputfile = FilesToEnrich[i])
States15FP_bnhypo <- getAllFisherTest(BN_Hypo,
                                  crom_data$States15_FP,
                                  outputdir = "ChrSates_15_Pla/Fisher_BNHypo",
                                  outputfile = FilesToEnrich[i])


## --  Plot collapsed data HyperGeometric Test - States15_FP - BN
plot_TestResults_Collapsed(list(bn = States15FP_bn,
                                  bn_hypo = States15FP_bnhypo,
                                  bn_hyper = States15FP_bnhyper),
                           outputdir = "ChrSates_15_Pla",
                           outputfile = FilesToEnrich[i])
```

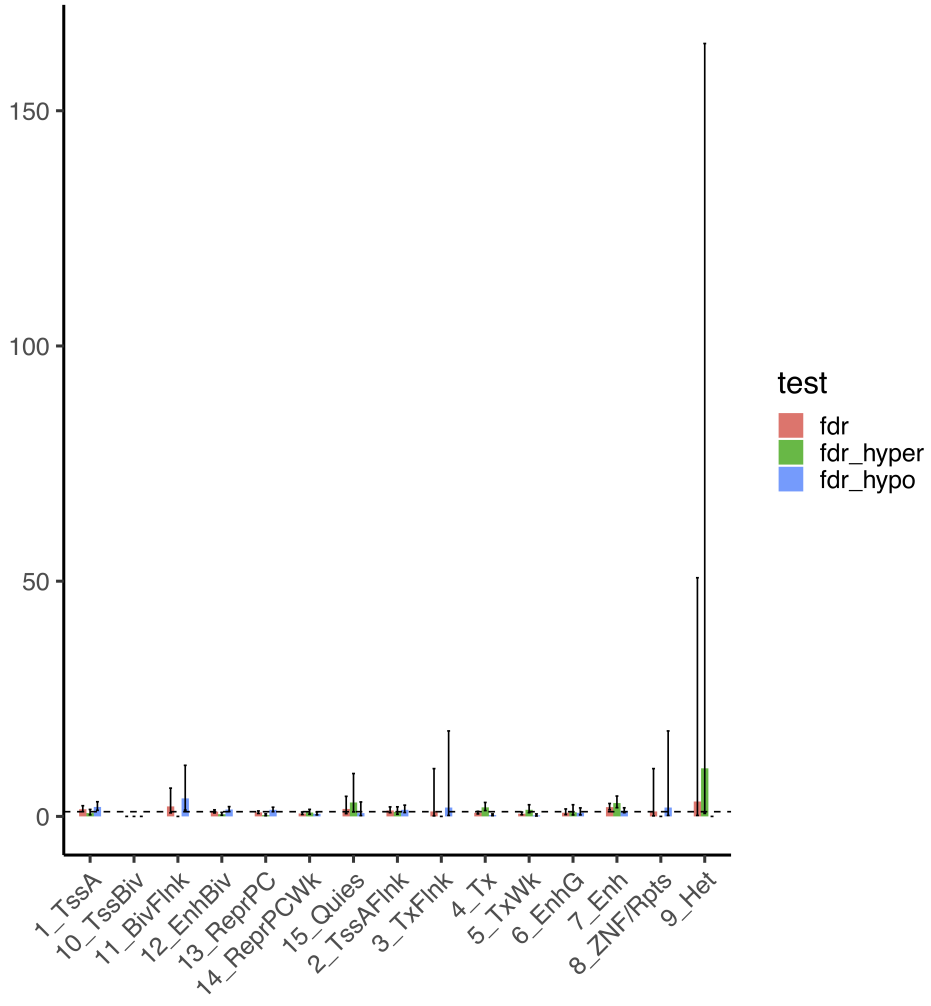


Figure 19: Chromatine states 15 for placenta - Fisher test for Hyper and Hypo methylated CpGs

6.5.2 Partially methylated domains (PMDs) – Paper

```
 ## -- Partially Methylated Domains (PMDs) PLACENTA
## ------------------------------------------------

# Create genomic ranges from PMD data
PMD.GRange <- getEnrichGenomicRanges(PMD_placenta$Chr_PMD,
                                     PMD_placenta$Start_PMD,
                                     PMD_placenta$End_PMD)

# Find overlaps between CpGs and PMD (find subject hits, query hits )
overPMD <- findOverlapValues(data.GRange, PMD.GRange )

#Create a data.frame with CpGs and PMDs information
```

```
mdata <- as.data.frame(cbind(DataFrame(CpG = data.GRange$name[overPMD$qhits]),
                             DataFrame(PMD = PMD.GRange$name[overPMD$shits])))

# Merge with results from meta-analysis (A2)
crom_data <- merge(crom_data, mdata, by.x="CpGs", by.y="CpG",all=T)
# crom_data <- crom_data[order(crom_data$p.value),]

# CpGs with PMD as NA
PMD_NaN <- ifelse(is.na(crom_data$PMD),'IsNA','NotNA' )


## --  Fisher Test - PMD - BN,  BN_hyper and BN_hypo
## (Full data ) (Depletion and Enrichment)
PMD_bn <- getAllFisherTest(crom_data$Bonferroni,
                           PMD_NaN,
                           outputdir = "PMD_Pla/Fisher_BN",
                           outputfile = FilesToEnrich[i])
PMD_bnhyper <- getAllFisherTest(BN_Hyper,
                                PMD_NaN,
                                outputdir = "PMD_Pla/Fisher_BNHyper",
                                outputfile = FilesToEnrich[i])
PMD_bnhypo <- getAllFisherTest(BN_Hypo,
                               PMD_NaN,
                               outputdir = "PMD_Pla/Fisher_BNHypo",
                               outputfile = FilesToEnrich[i])

 ## --  Plot collapsed data HyperGeometric Test - States15_FP - BN
plot_TestResults_Collapsed(list(bn = PMD_bn,
                                bn_hypo = PMD_bnhypo,
                                bn_hyper = PMD_bnhyper),
                           outputdir = "PMD_Pla",
                           outputfile = FilesToEnrich[i])
```

6.5.3 Impreinted regions - Paper

```
## -- Imprinting Regions PLACENTA
## ------------------------------------------------

# Create genomic ranges from DMR data
DMR.GRange <- getEnrichGenomicRanges(IR_Placenta$Chr_DMR,
                                     IR_Placenta$Start_DMR,
                                     IR_Placenta$End_DMR)

# Find overlaps between CpGs and DMR (find subject hits, query hits )
overDMR <- findOverlapValues(data.GRange, DMR.GRange )

#Create a data.frame with CpGs and DMRs information
mdata <- as.data.frame(cbind(DataFrame(CpG = data.GRange$name[overDMR$qhits]),
                             DataFrame(DMR = DMR.GRange$name[overDMR$shits])))
```

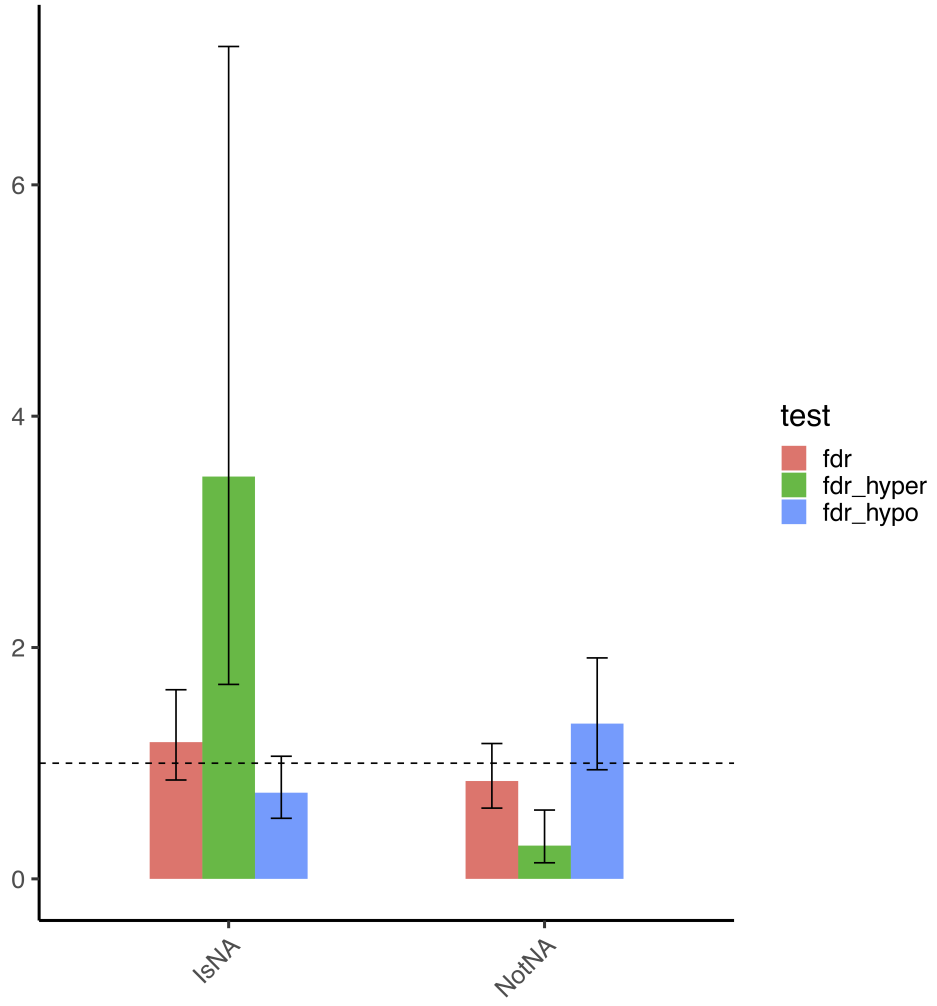


Figure 20: Partial Metilated Domains for placenta - Fisher test for Hyper and Hypo methylated CpGs

```
# Merge with results from meta-analysis (A2)
crom_data <- merge(crom_data, mdata, by.x="CpGs", by.y="CpG",all=T)

# CpGs with DMR as NA
DMR_NaN <- ifelse(is.na(crom_data$DMR.y),'IsNA','NotNA' )

## --  Fisher Test - DMR - BN,  BN_hyper and BN_hypo
## (Full data ) (Depletion and Enrichment)
DMR_bn <- getAllFisherTest(crom_data$Bonferroni,
                           DMR_NaN,
                           outputdir = "DMR_Pla/Fisher_BN",
                           outputfile = FilesToEnrich[i])
DMR_bnhyper <- getAllFisherTest(BN_Hyper,
                                DMR_NaN,
```

```
                                    outputdir = "DMR_Pla/Fisher_BNHyper",
                                    outputfile = FilesToEnrich[i])
        DMR_bnhypo <- getAllFisherTest(BN_Hypo,
                                    DMR_NaN,
                                    outputdir = "DMR_Pla/Fisher_BNHypo",
                                    outputfile = FilesToEnrich[i])

         ## --  Plot collapsed data HyperGeometric Test - States15_FP - BN
        plot_TestResults_Collapsed(list(bn = DMR_bn,
                                    bn_hypo = DMR_bnhypo,
                                    bn_hyper = DMR_bnhyper),
                                outputdir = "DMR_Pla", outputfile = FilesToEnrich[i])
```

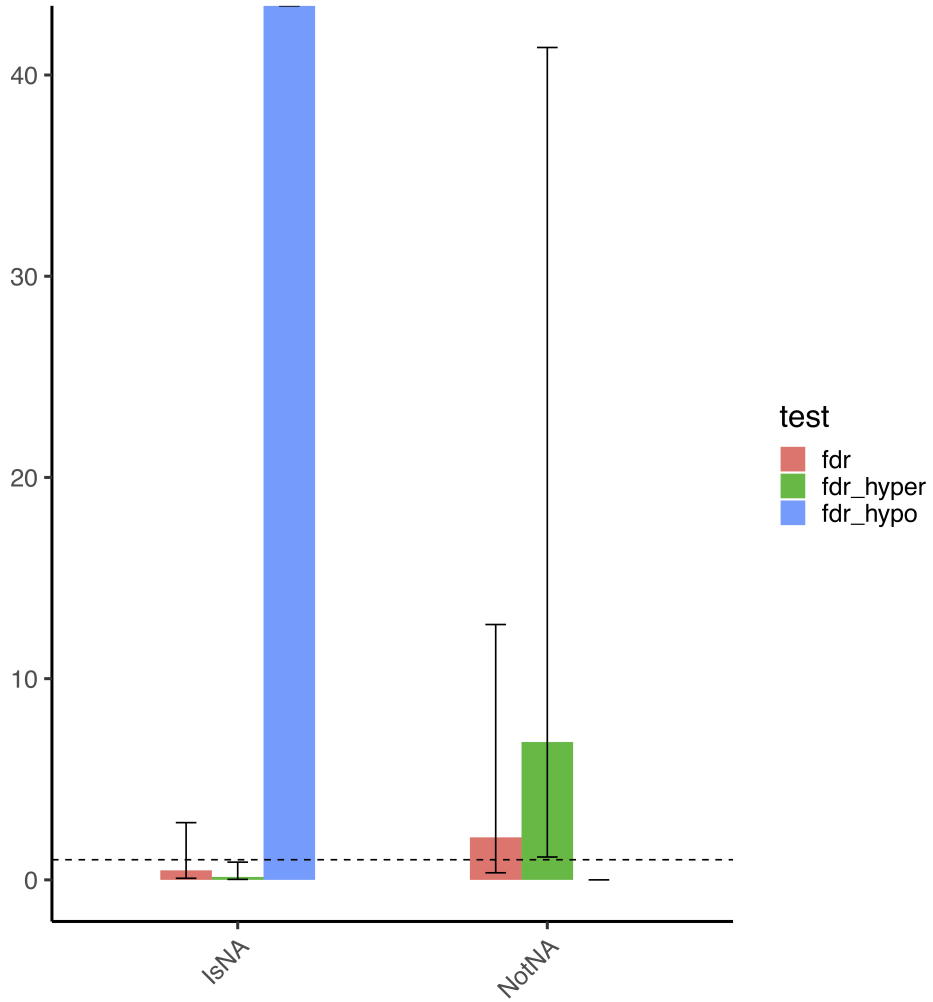


Figure 21: Imprinted Regions for placenta - Fisher test for Hyper and Hypo methylated CpGs

Session info