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Clopidogrel Pharmacogenetics, resistance to antiplatelet therapy in ischemic stroke by Epigenome Wide Association Study (EWAS).

Cristina Gallego Fàbrega

Màster en Anàlisis de Dades Òmiques

Director/a: Dr. Israel, Fernandez Cadenas
Tutor/a: Dr. Jordi, Planas

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Introduction

Stroke or cerebrovascular disease (CVD) is the second leading cause of death worldwide, with 6.2 million deaths in 2008, and the leading cause of disability in developed countries. There are, in average, 200 new stroke cases per 100,000 people per year [1].

After a first stroke the cumulative risk of a new vascular event, know as recurrence, is 11.1% at first year, 26.4% over the next 5 years and 39.2% at 10 years[2]. As platelets play a major role in thrombus formation, to try to prevent new vascular episodes (secondary prevention), antithrombotic and antiplatelet drugs are used[3]. The most widely used antiplatelets are acetylsalicylic acid (ASA) and clopidogrel.

Despite the clear benefits demonstrated by antiplatelet therapy, platelet reactivity is variable and patients do not respond to this therapy evenly. Overall, patients mortality is reduced only by 18% and the number of patients with disability is reduced by 25% which are reductions rates far from satisfactory. In fact 10-20%[4] of patients treated with ASA or clopidogrel have new vascular events. This phenomenon may be due to drug resistance by the patient. Drug resistance is defined as a failure in platelet inhibition activity despite a correct antiplatelet therapy.

After a first stroke approximately 60% of patients receive antiplatelet therapy[5,6], for this reason, it's important to recognize the elements associated with drug resistance, in order to personalize the treatment to each patient. Therefore, in recent years, genetic studies have been performed to find genes associated with patient's resistance to antiplatelet treatment.

In the “omics” era Genome-wide association studies (GWAS) have been widely used to find polymorphisms associated with human diseases and pharmacogenetics. GWA studies have been more successful in finding new associations with complex diseases compared to classical candidate gene strategies. Pharmacogenetic studies in clopidogrel resistance, using candidate gene studies, have found polymorphisms in Cytochrome P450 genes mainly at 2C19 subtype (CYP2C19)[7] and some others. Later GWAS in Amish population[8] also found that CYP2C19*2 genotype was associated with diminished platelet response to clopidogrel treatment and poorer cardiovascular outcomes.

But phenotypes are not always defined only by the sequence of DNA sequence[9] and GWAS, despite being able to find Cytochrome P450 genes association, have not shown clear associations between polymorphisms and

vascular resistance. Epigenetic studies open a new field to identify new genes and biomarkers associated with antiplatelet resistance treatment.

Epigenetics refer to the stable, heritable and reversible modifications in DNA associated with transcriptional regulation without altering the nucleotide sequence[9]. Epigenetic processes such as DNA methylation (DNAm), histone acetylation/deacetylation, non coding mRNA expression and chromatin conformational changes[10], are essential for normal cellular development and differentiation. Also epigenetic changes can be modulated by environmental factors, and have been related to some monogenic and complex human diseases[11,12]. Most DNAm studies have mainly been performed in the context of Cancer research[12,13,14]. Other studies on epigenetics dial with are aging effects on methylation patterns and age related diseases[15,16], tobacco smoking impact on pregnancy and DNAm changes[17,18]. There are few studies in non-malignant common complex diseases there are few studies, obesity[19], diabetes mellitus[20], ulcerative colitis[21].

The major advances in epigenetics have been made in the field of DNA methylation. Epigenome-wide association studies (EWAS) use the same strategy of GWAS but for epigenetic modification. These studies use array-based genotyping technology, with a previous bisulfite conversion, to detect the methylation levels at CpG islands across genome. EWAS are hypothesis free approaches that work in a high throughput basis with methylation arrays which are currently able to analyze up to 450.000 CpG islands. EWA studies of human diseases are becoming increasingly common in the last few years.

The Illumina Infinium HumanMethylation450 BeadChip with over 450.000 methylation sites per sample at single-nucleotide resolution, more than 98% of reproducibility for technical replicates and a PCR-free protocol[22,23] is the most widely used technology for EWAS nowadays. The 450K BeadChip feature two different types of assays, Infinium I design employs two bead types per CpG site, one for the methylated and one for the unmethylated states, while Infinium II assay design uses just one bead type for both conditions. The methylation level is then estimated based on the measured intensities (Equation 1), ranging from 0 to 1 (β-value). Beta-values from 0.6 to 1 are considered as hypermethylated levels, and Beta-values from 0 to 0.4 are considered as hipomethylated levels[22,23,24].

$$\beta - value = \frac{\mathrm{Signal}_{\mathrm{methylated}}}{\mathrm{Signal}_{\mathrm{methylated}} + \mathrm{Signal}_{\mathrm{unmethylated}} + 100}$$

Equation 1: Methylation levels are measures by the ratio of the methylation probe intensity and the overall intensity

In the present study an epigenetic approach in pharmacogenetics, 42 patients with a previous stroke and clopidogrel administration, were assessed in order to find differential methylation levels between those who shoved resistance and those who did not.

Objectives

Main Objective:

Determine whether there is a different methylation pattern among ischemic stroke patients associated with resistance to clopidogrel treatment.

Secondary Objective:

1. Explore the altered metabolic pathways involved in clopidogrel resistance.
2. Standardize the epigenetic data analysis in our laboratory.
3. Selection of the genetic platform to perform the replication analysis.

Methods

Samples Selection.

In total, 42 individuals from the GRECAS study (Genotyping Risk and Efficacy of Clopidogrel or Aspirin following Stroke) were selected. The GRECAS individuals are patients with a previous ischemic stroke treated with clopidogrel or aspirin. The 21 cases and 21 controls were matched one-to-one by age, sex, TOAST classification, recruitment hospital and clopidogrel administration. All individuals were Caucasian, 35 of them were males and 7 were females (Table 1).

		Case	Controls
	N	21 (50%)	21 (50%)
	Age	70 ± 8	71 ± 9
Gender	Male	18 (42,9%)	17 (40,5%)
	Female	3 (7,1%)	4 (9,5%)
TOAST*	Aterothrombotic	11 (26,2%)	11 (262%)
	Undetermined	5 (12%)	5 (12%)
	Unknown	1 (2,4)	1 (2,4)
	Lacunar	3 (7,1%)	3 (7,1%)
	Hypertension	13 (31%)	16 (38,1%)
	Dyslipidemia	12 (28,6%)	8 (19%)
	Diabetes Mellitus	7 (16,7%)	8 (19%)

Table 1: Characteristics of the study population.
*TOAST classification of Ischemic stroke.

DNA purification.

Total genomic DNA was extracted from whole blood samples using the Gentra Puregene Blood Kid (Quiagen, Hilden, Germany) following the manufacturer's instructions. The samples were maintained at -20ºC until the EWAS analysis.

DNA concentration of each subject was determined individually, by measuring ultraviolet (UV) light absorption at 260nm, with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific , Redwood City, CA, USA). Finally 20µl at 50ng/µl of each samples were used to methylation analysis.

Methylation analysis.

Genome-wide DNA methylation was assessed using the Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego Ca). This array based study, quantitatively measures more than 450,000 CpG sites at single nucleotide resolution with a 99% coverage of RefSeq Gene.

First of all a Quality Control (QC) of all samples was performed to check the DNA integrity using Invitrogen E-Gel 1% Agarose Gels. No DNA samples showed fragmentation or poor quality.

Genomic DNA from 42 samples were bisulfite converted using Zymo EZ DNA Methylation^{TM} Kit (Zymo Research, Orange Ca) following the manufacturer's procedure with alternative incubations conditions suggested for the llumina Infinium Methylation Assay. All samples were processed in a single working batch using the Illumina Infinium MSA4 protocol that includes amplification, fragmentation, hybridization and BeadChip scan.

For QC of the process, fluorescence data generated for each CpG locus was analyzed with the Ilumina GenomeStudio software package. Methylation data (beta-values) is combined with gene expression (p-values) profiling experiments within the same GenomeStudio, considering values lower than 0.05 as of good quality.

Quality control and normalization of the EWAS results

All pre-processing, correction and normalization steps were implemented by R (versions 3.15.1 and 3.0.1) with Bioconductor packages. All plots were produced using R functions. The pipline is a sequence (Appendix A) of R scripts (Table 2) adapted from the methylumi (version 2.6.1), lumi (version 2.12.0), wateRmelon (version 1.0.3) and minfi (version 1.6.0).

R package	Instruction	Description
methylumi	*methylumiR*	Load Illumina methylation data into a MethyLumiSet object.
minfi	*densityPlot*	Density plots of methylation Beta values.
watRmelon	*pfilter*	Filter data sets based on bead count and detection p-values
minfi	*mdsPlot*	Multi-dimensional scaling (MDS) plots showing a 2-d projection of distances between samples.
watRmelon	*dasen*	Calculate normalized betas from Illumina 450K methylation arrays.
lumi	*estimateBetas*	Estimate methylation Beta-value matrix from eSet-class object (include methylated and unmethylated probe intensities)

Table 2: Specific instructions used from each R package .

CpG sites with detection p-value ≥ 0.05 in more than 1% of the samples and samples with detection p-value ≥ 0.05 in more than 1% of the CpG sites were excluded prior to data analysis. CpG sites with beadcount < 3 in 5% of samples were excluded too.

A cluster identification analysis was carried out to check if there was any subgroup on the sample or gender mismatch. 11.319 CpG islands being at X and Y chromosome and 65 SNP probes were excluded before quantile normalization of methylated and unmethylated results (β-values). After these quality checks, there were a total of 472.386 CpG islands and 41 samples.

Data analysis.

To find the differentially methylated CpG sites (DMCs), β-values among control and case samples were analyzed using the non parametric Mann-Whitney test for independent samples. We analyzed the 21 case samples (ischemic stroke subjects with resistance to clopidogrel) versus the 21 control samples (ischemic stroke subjects without resistance to clopidogrel).

Haploview software was used to create a Manhattan plot in other to find differentially methylated loci.

Enrichment analysis

Finally, a basic biological function analysis was perform using NCBI data base, mainly PubMed (http://www.ncbi.nlm.nih.gov/pubmed), to find biological processes related to our findings in other studies.

Replication study.

A bibliographical research about sequencing technologies to methylation studies was performed using technological information from different providers and also, PubMed data base to obtain scientific results of the technologies.

Results

In total 485.577CpG sites were assessed by Illumnia BeadChip 450k across whole genome in each of the 42 individual samples. The methylation intensities showed bimodal distributions when displayed across all proves (Figure.1), none of the samples show a different behaviour.

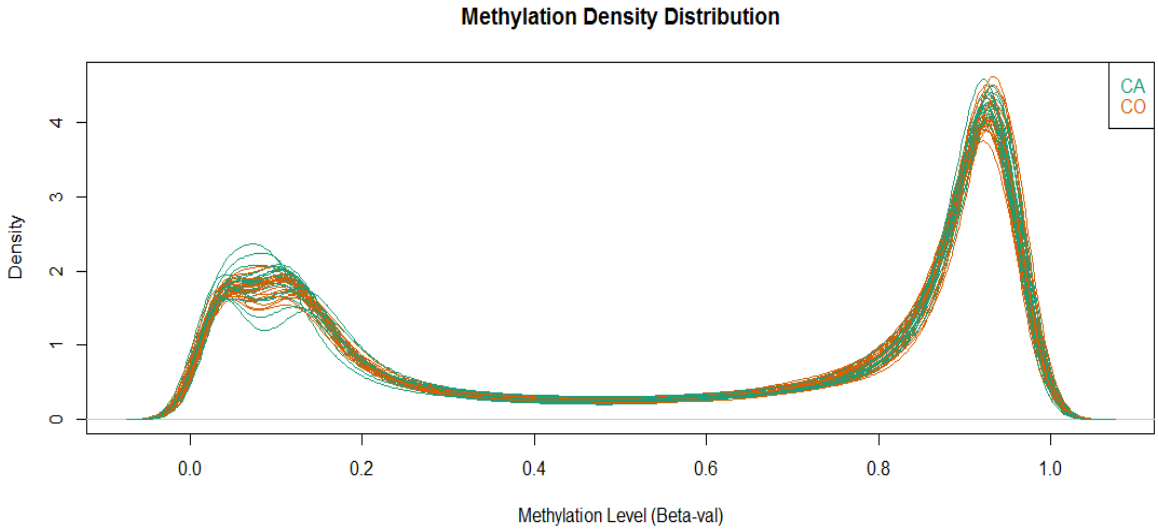

Figure 1: Density Plot of the Methylation Beta values for all samples colored by sample group. Cases: green lines and controls: orange lines. All samples follow the same distribution.

After filtering the data based on the bead count (< 3 in 5% of samples) and p-values (1% >0.05), 1.848 sites and zero samples were removed. Illumina 450k BeadChip have 65 SNP along the array, as quality controls, which were also removed. When performing a multidimensional scaling (MDS) no subgroups appeared in the sample (Figure.2), the graph show only two clusters representing males and females. One female sample that clustered into the males group, due to a genotyping error or a typing error, was removed from the study. Finally 11.278 CpG sites located at X and Y chromosomes were also removed before the quantile normalization step (Figure.3).

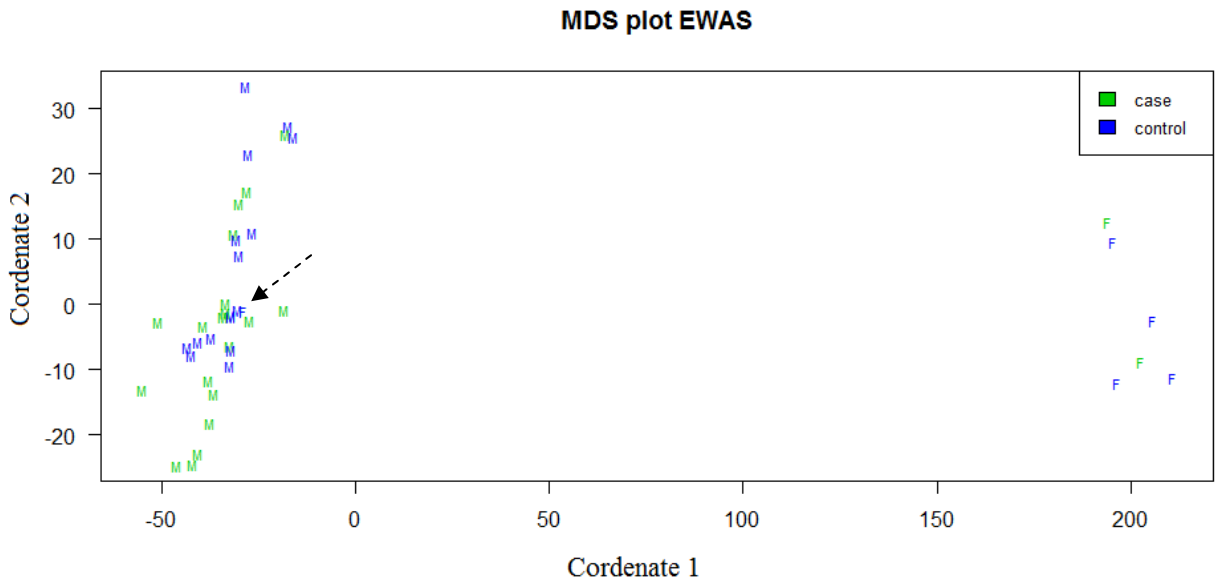

Figure 2: Multidimensional Scaling plot of samples. Cases are in green color and controls in blue. Letter 'M' indicates a male and letter 'F' indicates a female. Black arrow point a female sample clustered into male's group.

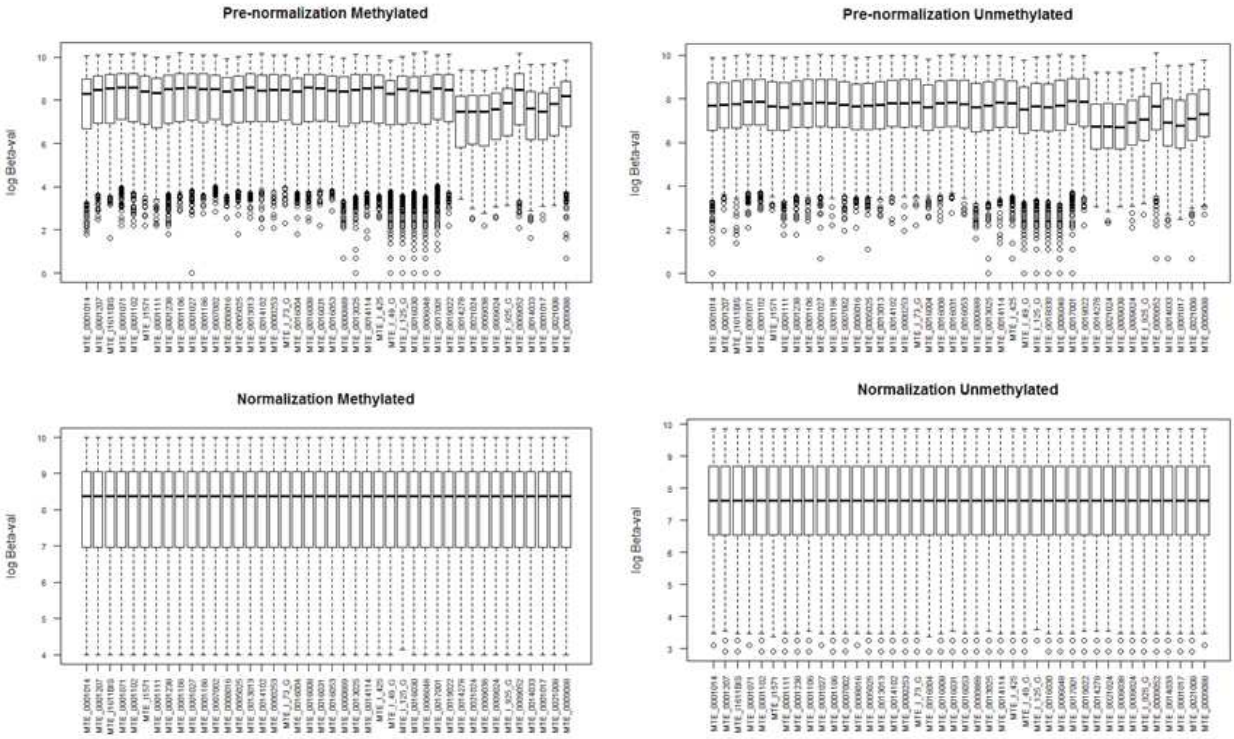

Figure 3: Box plot of methylated and unmethylated log beta-value, before and after quantile normalization step.

The analysis of differentially methylated CpG (DMCs) sites between cases and controls indicated a few number of CpG sites that were associated with new vascular events. In total 58 DMCs with p-val <0.05 were found (Table.3), none of them was significant after FDR correction. Distribution of recurrence related DMCs (Figure 4) showed different behaviors between cases and controls. Case samples had a slightly higher average of hiper-methylation comparing to the control samples, in the upper range area. P-values for each CpG site were plotted in a Manhattan Plot to show the most significant loci (Figure 5).

p-val	TargetID	Chr	UCSC Ref. Gene Name
8,54E-07	cg07925064	16	LOC100130015
3,03E-06	cg06726262	17	ZNF286A
6,97E-06	cg09332091	19	
6,97E-06	cg18002896	8	RHPN1
9,58E-06	cg03548645	14	TRAF3
1,12E-05	cg25236894	17	RPAIN
1,12E-05	ch,18,400468R	18	
1,31E-05	cg09533145	5	ADAMTS2
1,52E-05	cg18127204	17	MYOCD
1,77E-05	cg01348374	3	
2,05E-05	cg12181407	5	
2,05E-05	cg14630099	6	HLA-DOA
2,05E-05	cg18769818	11	ARAP1
2,38E-05	cg03654504	1	GRIK3
2,75E-05	cg10318528	4	SFRP2

Table 3: 15 most significant diferentially methylted CpG and related genes.

.

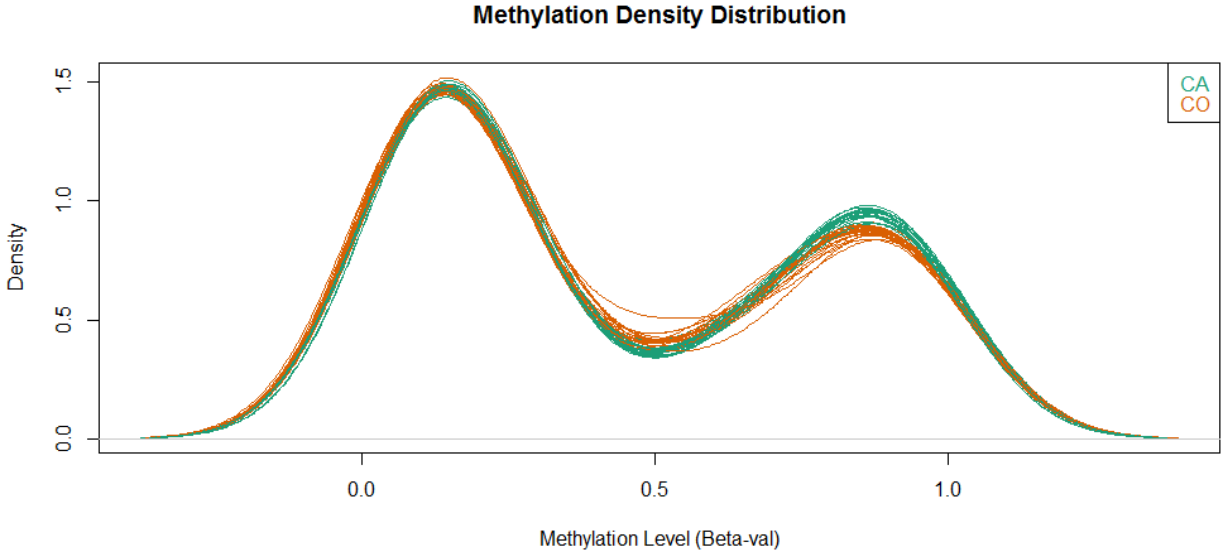

Figure 4: Density Plot of the Methylation Beta values for 58 most significant differentially methylated CpGs. . The arrow indicates the hipermethylation of CpG sites of cases compared to controls.

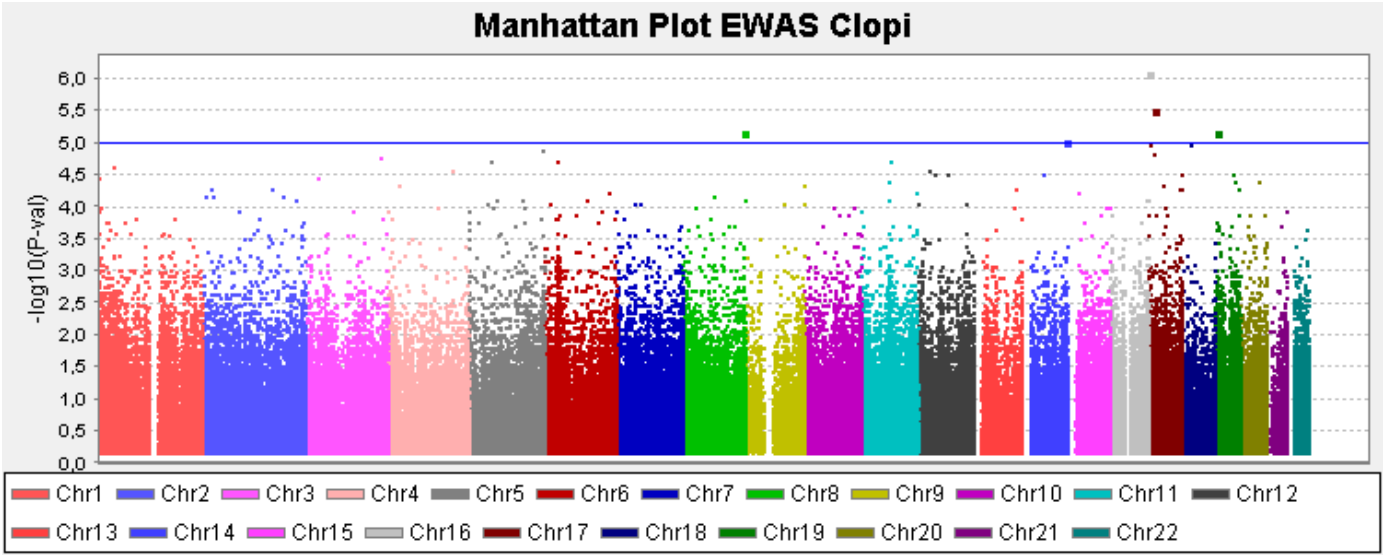

Figure 4: Manhattan Plot of DMCs P-values across genome. Each spot represent a p-val and each color represents one chromosome.

The two most remarkable genes founds where, TRAF3 gene (cg03548645, p-val= $9{,}58e^{-06}$), and ADAMTS2 (cg09533145, p-val=$1{,}31e^{-05}$).

Replication.

To validate the 450k BeadChip data results, some sequencing technologies were assessed. The bibliographic research about, Sequenom - EpiTyper DNA methylation, Pyrosequencing for bisulfite converted samples and NGS for methylation studies, revealed no clear benefits for any of these technologies over the rest, and really all require high budgets. So the Sequenom - Epityper DNA methylation was chosen by economical conveniences.

Discursion

Clopidogrel, was ranked seventh among the most prescribed medications in United States in 2011, with more than 28 million prescriptions[9,25]. This drug is commonly prescribed in secondary prevention after stroke, however 21% of patients suffer experience recurrent ischemic events despite a good medication acherence[26]. Clopidogrel metabolism is highly variable and it has been related with patient genetic background[27]. Increase our knowledge about Clopidogrel function and prevention of new vascular events promise to have a global impact on health promotion.

Pharmacogenetics offers the hope of greater personalized medicine, prescribing medications to those most likely to be benefit and avoid the use of certain medications in those most likely to be harmed by them. In addition, when several medication options are available, pharmacogenetics could help us to choose the one most appropriate for a particular individual. Good evidence of clinical usefulness of pharmacogenetics has been described for several treatments and diseases. Recently the Clinical Pharmacogenetics Implementation Consortium (CPIC)[28], as the Food and Drugs Administration (FDA)[29,30], proposed to genotype CYP2C19 before clopidogrel prescription. In addition, other Cytochrome P450 polymosrphidms have been recommended to be genotyped before drug administration[31].

In the present study, we have performed an epigenetic study to determine altered methylation CpG sites associated with clopidogrel resistant. We analyzed ischemic stroke patients treated with clopidogrel who presented new vascular events (cases) and those who do not had new vascular events (controls).

A group of 58 CpG sites were found significantly differentially methylated between cases and controls (Figure 4), although did not appear a clear locus that could indicate an affected gene (Figuere 5). However two of the most significant DMCs sites were next to *TRAF3* and *ADAMTS2* genes.

TRAF3 gene, TNF receptor-associated factor 3, encodes a protein member of the TNF receptor associated factor (TRAF) family. These proteins participates in the signal transduction of CD40 and TNFR important to de activation of immune response. It has been shown that patients suffering from cardiovascular disease exhibit increased levels of circulating and soluble CD40 ligand[32,33]. Zifang Song,et all described association between TRAF3 gene expression and CD40 levels in arterial injury[34]. Del Río-Espínola A. et all, also found an association between CD40 and the reclusion risk after fibrinolytic therapy during the acute phase of ischemic stroke[35]. Our results indicate that an increased expression of this gene

could be associated with a higher risk of new vascular events in ischemic stroke patients treated with clopidogrel.

ADAMTS2 gene, ADAM metallopeptidase with thrombospondin type 1 motif 2, encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family. ADAMTS designates a family of 19 secreted enzymes, that have a role in extracellular matrix degradation has previously been involved in various human biological processes, including connective tissue structure, cancer, coagulation, arthritis, angiogenesis and cell migration[36]. Mutations in ADAMTS13 gene lead to endothelial von Willebrand factor (VWF) platelet aggregation and then abnormal coagulation in targeted organs[37]. In relation to stroke a study of Arning and collegues observed a clustering of association signals in 4 genes belonging to ADAMTS family of genes, including ADAMTS2, with pediatric stroke[38].

The 'omics' studies require replication analysis to validate the results obtained in the discovery stage.. Therefore a replication study will be performed, using a new cohort of samples (n=300) in order to validate the results of the EWAS. The replication study will be performed using a different technology. We chose EpiTyper DNA methylation (Sequenom) due to it was a consistently technology with a low cost.. We will analyze 3-5 CpG sites in the new cohort of 300 patients.

As a summary we found several interested genes that could be associated with clopidogrel resistance in ischemic stroke patients. To validate these results new studies will be performed in a new cohort of patients.

Conclusions

The present study has reported a group of CpG sites in clopidogrel resistance patients with higher percentage of methylation compared to non resistant patients. Two of the differentially methylated CpG sites found, are next to two genes associated with vascular diseases and inflammation (*TRAF3* and *ADAMTS2*). To confirm these results, a new study in a different cohort is needed.

The customized R pipeline used for this study had showed good results compared to use one unique R package. Further EWAS in our lab will use this script as a standard.

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Appendix

Appendix A:

```
# Paquetes #
library("wateRmelon", lib.loc="/usr/local/lib/R/site-library")
library("methylumi", lib.loc="/usr/local/lib/R/site-library")
library("minfi", lib.loc="/usr/local/lib/R/site-library")
library("lumi", lib.loc="/usr/local/lib/R/site-library")

#Crear Objeto#
EWAS <- methylumiR('FinalReport', sampleDescriptions=SamplesTable)

EWAS # 485577 x 48

## Eliminando Controles Sanos y Pools ##
Eliminar<- c("MTE_CONIC97","MTE_CONIC112", "MTE_PoolA",
"MTE_PoolB","MTE_PoolC","MTE_PoolD")
EWAS <- EWAS[,!sampleNames(EWAS) %in% Eliminar]
ewas <- as(EWAS, "MethylSet")
data <- pData(EWAS)

## Distribució de les Betas ##
densityPlot(ewas, sampGroups = data$Group, main = "Methylation Density Distribution",
xlab = "Methylation Level (Beta-val)")
#qcReport(EWAS, sampNames=data$sampleID, sampGroups=data$Group, pdf="qcReport
EWAS.pdf")

# QC 1 #
EWAS <- pfilter(EWAS)

# Eliminar els 'proves' que son SNPs #
rs <- c(grep("rs", featureNames(EWAS)))
rs <- fData(EWAS)$TargetID[rs]
EWAS <- EWAS[!fData(EWAS)$TargetID %in% rs,]
ewas <- as(EWAS, "MethylSet")

# QC 2#
MDS <- cmdscale(dist(t(exprs(EWAS)[fData(EWAS)$CHR=='X',])),2)
plot(MDS,main="MDS plot EWAS", pch=as.character(pData(EWAS)$Gender), las=1, cex=0.7,
col=as.numeric(pData(EWAS)$Group)+2, xlab="V1",ylab="V2")
legend("topright",c("case","control"),fill=c(3,4),cex=0.8)
#####################################################
#ewas <- as(EWAS, "MethylSet")
#data <- pData(EWAS)
#mdsPlot(ewas, numPositions = 1000, sampGroups = data$Group,sampNames = data$Index)
#mdsPlot(ewas, numPositions = 1000, sampGroups = data$Gender,sampNames = data$Index)
#avgPval <- colMeans(pvals(EWAS))
#barplot(avgPval, ylab="P-Value",las=2, cex.axis=0.6, main="Average p-val EWAS")
#####################################################
```

```
rm_mostres<- c("MTE_0001207")
EWAS<- EWAS[,!sampleNames(EWAS) %in% rm_mostres]
data <- pData(EWAS)

# Eliminar CRH X e Y #
XY_cpgs<-fData(EWAS)[fData(EWAS)$CHR=="X" | fData(EWAS)$CHR=="Y",]
XY_cpgs<-XY_cpgs$TargetID
EWAS <- EWAS[!fData(EWAS)$TargetID %in% XY_cpgs,]

# Normalizacion
boxplot(log(methylated(EWAS)), las=2, cex.axis=0.7, ylab="log Beta-val", main="Pre-
normalization Methylated" )
boxplot(log(unmethylated(EWAS)), las=2, cex.axis=0.7, ylab="log Beta-val", main="Pre-
normalization Unmethylated")
EWAS_D <-  dasen(EWAS)
boxplot(log(methylated(EWAS_D)), las=2, cex.axis=0.7,ylab="log Beta-val",
main="Normalization Methylated"  )
boxplot(log(unmethylated(EWAS_D)), las=2, cex.axis=0.7,ylab="log Beta-val",
main="Normalization Unmethylated"  )

# Estimar Betas #
manifest<- fData(EWAS_D)

betas <- estimateBeta(EWAS_D,returnType="matrix")
betas<-as.data.frame(betas)

dat<- pData(EWAS_D)

# Establecer Casos y controles #
case<-colnames(betas)[grep("CA",dat$Group)]
cases<-which(colnames(betas) %in% case)
controls<-which(!colnames(betas) %in% case)

# Diferencias entre dos grupos con variable Continua # Man-withney
betas<- data.frame(betas,wilcox.npaired=apply(betas,1,function(y) {
  y <-
try(wilcox.test(as.numeric(y[controls]),as.numeric(y[cases]),paired=F)$p.value)
  return(ifelse(class(y)=="numeric",y,1))
}))

# Corrección estadística de la significacncia de las diferencias encontradas#
betas<- data.frame(betas,bonfe=p.adjust(betas$wilcox.npaired,"bonferroni"))
selected_cpgs_bonfe<-subset(betas, betas$bonfe<0.05)
betas<- data.frame(betas,FDR=p.adjust(betas$wilcox.npaired,"fdr"))
selected_cpgs_FDR<-subset(betas, betas$FDR<0.05)

#############################################################################
ewas_D <- as(EWAS_D, "MethylSet")
cpgs <- rownames(gene_ord30)[1:6]
par(mfrow=c(2,3))
plotCpg(ewas_D, cpg=cpgs, pheno=dat$Group)
#############################################################################
```

```
# Selección de los mejores p-val #
pval<-data.frame(betas$wilcox.npaired)
genes<-data.frame(pval$betas.wilcox.npaired,
fData(EWAS_D)$TargetID,fData(EWAS_D)$CHR,
                  fData(EWAS_D)$MAPINFO, fData(EWAS_D)$UCSC_REFGENE_NAME,
                  fData(EWAS_D)$REGULATORY_FEATURE_GROUP)

pval_ord<-order(genes$pval.betas.wilcox.npaired)
gene_ord<-genes[pval_ord,]
gene_ord30 <- gene_ord [1:30,]
gene_ord_sig <- gene_ord [1:58,]

manhatan<-
data.frame(genes$fData.EWAS_D..TargetID,genes$fData.EWAS_D..CHR,genes$fData.EWAS_D..M
APINFO, genes$pval.betas.wilcox.npaired)
map<-data.frame(genes$fData.EWAS_D..CHR,genes$fData.EWAS_D..TargetID,
"0",genes$fData.EWAS_D..MAPINFO)

write.table(manhatan, file="manhattan.txt", row.names = FALSE,
            col.names = FALSE, quote=FALSE, sep="\t")
write.table(map, file="map.map", row.names = FALSE,
            col.names = FALSE, quote=FALSE, sep="\t")
write.table(gene_ord30, file="genes_ord30.txt",quote=FALSE, sep="\t")
write.table(gene_ord_sig, file="genes_ord_sig.txt",quote=FALSE, sep="\t")

#########################################################################

Manterner<-
c("cg07925064","cg06726262","cg09332091","cg18002896","cg03548645","cg25236894","ch.1
8.400468R","cg09533145","cg18127204","cg01348374","cg12181407","cg14630099","cg187698
18","cg03654504","cg10318528","cg14258853","cg15107336","cg17737641","cg20908919","cg
25146814","cg26322913","cg21285198","cg27093242","cg16126516","cg16338278","cg2284954
3","cg05036153","cg06419964","cg18997433","cg00660096","cg03523799","cg09131511","cg1
1539992","cg19113641","cg19861460","cg12096506","cg27073311","cg27553667","cg00571292
","cg16149238","cg16575530","cg24997845","cg03331123","cg07480754","cg11841562","cg17
761538","cg24083496","cg24532669","cg26243551","cg26529771","cg00048178","cg05141341"
,"cg09156140","cg12624825","cg14235701","cg14793406","cg22296322","cg25629418")
EWAS_D_mantener <- EWAS_D[featureNames(EWAS_D) %in% Manterner,]
ewas <- as(EWAS_D_mantener, "MethylSet")
data <- pData(EWAS_D_mantener)
densityPlot(ewas, sampGroups = data$Group, main = "Methylation Density Distribution",
xlab = "Methylation Level (Beta-val)")
```

Appendix B:

pval	TargetID	CHR	UCSC Ref. Gene Name
8,54E-07	cg07925064	16	LOC100130015
3,03E-06	cg06726262	17	ZNF286A
6,97E-06	cg09332091	19	
6,97E-06	cg18002896	8	RHPN1
9,58E-06	cg03548645	14	TRAF3
1,12E-05	cg25236894	17	RPAIN
1,12E-05	ch.18.400468R	18	
1,31E-05	cg09533145	5	ADAMTS2
1,52E-05	cg18127204	17	MYOCD
1,77E-05	cg01348374	3	
2,05E-05	cg12181407	5	
2,05E-05	cg14630099	6	HLA-DOA
2,05E-05	cg18769818	11	ARAP1
2,38E-05	cg03654504	1	GRIK3
2,75E-05	cg10318528	4	SFRP2
2,75E-05	cg14258853	12	TMTC1
3,18E-05	cg15107336	19	XRCC1
3,18E-05	cg17737641	12	LGR5
3,18E-05	cg20908919	12	PRICKLE1
3,18E-05	cg25146814	17	
3,18E-05	cg26322913	14	MAP4K5; ATL1
3,66E-05	cg21285198	1	TMEM88B
3,66E-05	cg27093242	3	TRIM71
4,21E-05	cg16126516	19	PPP5C
4,21E-05	cg16338278	11	ALDH3B2
4,21E-05	cg22849543	20	LOC284749
4,84E-05	cg05036153	9	SLC34A3
4,84E-05	cg06419964	17	MRM1
4,84E-05	cg18997433	4	DHX15
5,55E-05	cg00660096	17	
5,55E-05	cg03523799	13	FARP1
5,55E-05	cg09131511	2	FAP
5,55E-05	cg11539992	17	RHBDF2
5,55E-05	cg19113641	2	GDF7
5,55E-05	cg19861460	19	ZNF581; ZNF580
6,36E-05	cg12096506	11	
6,36E-05	cg27073311	6	RBM16
6,36E-05	cg27553667	15	GABRG3
7,27E-05	cg00571292	8	UBE2W
7,27E-05	cg16149238	2	KLHL29
7,27E-05	cg16575530	2	LOC339788

7,27E-05	cg24997845	2	STAT1
8,30E-05	cg03331123	5	
8,30E-05	cg07480754	16	
8,30E-05	cg11841562	5	SLC27A6
8,30E-05	cg17761538	8	MGC70857; LRRC24
8,30E-05	cg24083496	2	
8,30E-05	cg24532669	11	BRMS1
8,30E-05	cg26243551	6	SFRS18
8,30E-05	cg26529771	16	CDH15
9,46E-05	cg00048178	6	GFOD1
9,46E-05	cg05141341	7	ZMIZ2
9,46E-05	cg09156140	5	C5orf34
9,46E-05	cg12624825	9	LOC100128076
9,46E-05	cg14235701	9	FAM69B
9,46E-05	cg14793406	12	
9,46E-05	cg22296322	7	
9,46E-05	cg25629418	12	LRTM2