TRAF3 Epigenetic Regulation Is Associated With Vascular Recurrence in Patients With Ischemic Stroke

Cristina Gallego-Fabrega, MSc; Caty Carrera, MD, MSc; Jean-Luc Reny, MD, PhD; Pierre Fontana, MD, PhD; Agnieszka Slowik, MD, PhD; Joanna Pera, MD, PhD; Alessandro Pezzini, MD; Gemma Serrano-Heras, PhD; Tomás Segura, MD, PhD; Joan Martí-Fàbregas, MD, PhD; Elena Muiño, MD; Natalia Cullell, MSc; Joan Montaner, MD, PhD; Jerzy Krupinski, MD, PhD; Israel Fernandez-Cadenas, PhD

Background and Purpose—Clopidogrel is one of the most used antiplatelet drugs in patients with cardiovascular disease. However, 16% to 50% of patients have a high on-clopidogrel platelet reactivity and an increased risk of ischemic events. The pathogenesis of high on-treatment platelet reactivity in patients with stroke is only partially explained by genetic variations. This study aims to find differentially methylated sites across the genome associated with vascular recurrence in ischemic stroke patients treated with clopidogrel.

Methods—From a cohort of 1900 patients with ischemic stroke, we selected 42 patients treated with clopidogrel, including 21 with a recurrent vascular event and 21 without vascular recurrence during the first year of follow-up. Over 480 000 DNA methylation sites were analyzed across the genome. Differentially methylated CpG sites were identified by nonparametric testing using R. Replication analysis was performed in a new cohort of 191 subjects and results were correlated with platelet reactivity in a subset of 90 subjects using light transmission aggregometry.

Results—A total of 73 differentially methylated CpG sites ($P<1\times10^{-05}$) were identified; 3 of them were selected for further replication: cg03548645 ($P=1.42\times10^{-05}$, *TRAF3*), cg09533145 ($P=7.81\times10^{-06}$, *ADAMTS2*), and cg15107336 ($P=1.89\times10^{-05}$, *XRCC1*). The cg03548645 CpG remained significant in the replication study ($P=0.034$), a deep analysis of this region revealed another methylation site associated with vascular recurrence, $P=0.037$. Lower cg03548645 (*TRAF3*) DNA methylation levels were correlated with an increased platelet aggregation ($\rho=-0.29$, $P=0.0075$).

Conclusions—This study suggests for the first time that epigenetics may significantly contribute to the variability of clopidogrel response and recurrence of ischemic events in patients with stroke. **(*Stroke*. 2016;47:1180-1186. DOI: 10.1161/STROKEAHA.115.012237.)**

Key Words: aspirin ■ clopidogrel ■ methylation ■ stroke ■ vascular resistance

Patients with Ischemic stroke are at high risk of having a new stroke or developing other vascular diseases such as acute myocardial infarction, or vascular death, known as vascular recurrence. A study in the South London Stroke Register described a cumulative risk of vascular recurrence after a first stroke of 8.0% at 1 year and 16.6% at 5 years.[1] To reduce vascular recurrence, the most prescribed treatment for secondary prevention of stroke is antiplatelet agents,[2] most widely used are acetylsalicylic acid, clopidogrel, or a combination of both. However 10% to 20% of patients treated with antiplatelet drugs have a new vascular event[3]; in addition, serious vascular events are reduced only by <25% compared with placebo.[4]

Pharmacogenetic studies have evaluated the relationship between genetic variants and high on-treatment platelet reactivity usually assessing platelet aggregation.[5] Mega et al[5] found an association between the CYP2C19 reduced-function allele and lower levels of the clopidogrel active metabolite, diminished platelet inhibition, and higher rates of major

Received November 25, 2015; final revision received February 29, 2016; accepted March 1, 2016.

From the Neuroscience Department, Stroke Pharmacogenomics and Genetics, Fundació Docència i Recerca Mutua Terrassa, Hospital Universitari Mútua de Terrassa, Terrassa (Barcelona), Spain (C.G.-F., J.M.-F., E.M., N.C., I.F.-C.); School of Medicine, University of Barcelona, Barcelona, Spain (C.G.-F.); Neurology Department, Neurovascular Research Laboratory, Vall d'Hebron Institute of Research (VHIR), Universitat Autònoma de Barcelona, Hospital Vall d'Hebron, Barcelona, Spain (C.C., J.M.); Division of Internal Medicine, and Rehabilitation, Trois-Chêne Hospital, University Hospitals of Geneva, Switzerland (J.-L.R.); Geneva Platelet Group, Faculty of Medicine, Geneva, Switzerland (J.-L.R., P.F.); Division of Angiology and Haemostasis, University Hospitals of Geneva, Geneva, Switzerland (P.F.); Department of Neurology, Jagiellonian University Medical College, Krakow, Poland (A.S., J.P.); Department of Clinical and Experimental Science, Neurological Clinic, Università degli Studi di Brescia, Brescia, Italy (A.P.); Neurology Department, Albacete Hospital, Albacete, Spain (G.S.-H., T.S.); Department of Neurology, Hospital de la Santa Creu i Sant Pau, IIB-Sant Pau, Barcelona, Spain (J.M.-F.); Neurology Service, Hospital Universitari Mútua Terrassa, Terrasa, (Barcelona), Spain (J.K.); and Neuroscience Department, School of Healthcare Science, Manchester Metropolitan University, Manchester, United Kingdom (J.K.).

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.115.012237/-/DC1.

Correspondence to Israel Fernandez-Cadenas, PhD, Stroke Pharmacogenomics and Genetics, Fundació Docència i Recerca Mutua Terrassa, C/ Sant Antoni 19, 08221 Terrassa, Spain. E-mail israelcadenas@yahoo.es

adverse cardiovascular events. Shuldiner et al[6] also found the CYP2C19*2 allele to be associated with diminished platelet response to clopidogrel treatment and poorer cardiovascular outcomes. Furthermore, Holmes et al[7] found an association between the CYP2C19 genotype and clopidogrel responsiveness, although there was no significant association of genotype with cardiovascular events.

Pharmacogenetic studies have found single-nucleotide polymorphisms associated with platelet aggregation in clopidogrel-treated patients; however, they have failed to find a genetic predisposition associated with new vascular events. Therefore new approaches, such as epigenetic studies, may contribute to finding the underlying cause of the occurrence of new vascular events after clopidogrel treatment. Epigenetics refers to DNA modifications affecting gene expression and chromatin structure without altering the nucleotide sequence. Epigenetic modifications are stable, reversible, and heritable and can be modulated by many factors including physiological and pathological conditions and by the environment. Epigenetic processes are involved in numerous cellular processes, and also related to some monogenic and complex human diseases.[8]

Recent studies have observed epigenetic mechanisms involved in the pathogenesis of atherosclerosis.[9,10] Su et al[11] observed that lower *P2Y12* gene promoter DNA methylation (DNAm) was associated with an increased risk of clopidogrel high on-treatment platelet reactivity in patients with albumin ≤35 g/L, currently smoking, or abusing alcohol, suggesting a potential role for epigenetics in clopidogrel high on-treatment platelet reactivity and vascular events after clopidogrel treatment.

The aim of this study is to analyze the whole epigenome of ischemic stroke patients treated with clopidogrel with an Epigenome-Wide Association Study (EWAS) to find altered methylation sites associated with new ischemic events (vascular recurrence) after first ischemic stroke.

Materials and Methods

Clinical Protocol

From a cohort of 1900 patients with stroke from Vall d'Hebron University Hospital (Barcelona, Spain), 42 subjects were selected. First, 21 subjects with ischemic stroke, who were treated with clopidogrel after the first stroke and had a new vascular event (defined as new ischemic stroke, myocardial infarction, peripheral vascular disease, or cardiovascular death), were selected. The remaining 21 subjects were selectively matched one-by-one with the first 21 subjects (ischemic stroke patients treated with clopidogrel after the ischemic stroke event and without a new vascular event). Matching variables were age (±7 years), sex, Trial of Org 10172 in Acute Stroke Treatment (TOAST)[12] classification, and clopidogrel administration (Table 1). Ethical committee approved the study (PR(AG) 03/2007). All patients were provided with oral and written information about the project, and signed the informed consent.

The study cases were defined as being patients with ischemic stroke who started clopidogrel treatment after stroke and who had a vascular recurrence in the first year of follow-up with a good adherence to the treatment as measured by the Morisky–Green test.[13] Controls were defined as patients with ischemic stroke who started clopidogrel treatment after stroke with a good adherence to the treatment but without vascular recurrence during the first year of follow-up. Vascular recurrence was described as new ischemic stroke, myocardial infarction, peripheral vascular disease, or cardiovascular death and was detected through telephone calls every 3 months or direct clinical visit.

Replication analysis was performed on 191 new samples from 3 cohorts, 2 ischemic stroke patients' cohorts and 1 cardiovascular disease patients' cohort, for further information about sample selection and cohorts see Table I in the online-only Data Supplement. The replication cohort included 29 patients with cardiovascular disease (ischemic stroke and myocardial infarction) treated with clopidogrel with a new vascular event and 162 patients with cardiovascular disease treated with clopidogrel without a new vascular event during the first year of follow-up (Table 1).

DNA Preparation and Bisulfite Conversion

Total genomic DNA was extracted from whole blood samples obtained during the first 24 hours after stroke onset before clopidogrel initial administration using the Gentra Puregene Blood Kid (Quiagen, Hilden, Germany) following the manufacturer's instructions.

Infinium HumanMethylation450 BeadChip Discovery Assay

Genome-wide DNAm was assessed using the Infinium HumanMethylation450 BeadChip (Illumina Inc, San Diego, CA). All samples were processed in a single working batch.

All preprocessing, correction, normalization steps, and plots were implemented using the R statistical computing environment (3.1.3 version) with Bioconductor packages (Table II in the online-only Data Supplement). Quality control metrics were examined to determine the success of the bisulfite conversion and subsequent array hybridization. Fluorescence intensities were imported from GenomeStudio, then probe filtering was performed to remove probes that have failed to hybridize (detection $P>0.05$) and that are not represented by a minimum of 3 beads on the array, as described elsewere.[14,15] CpG sites containing documented single-nucleotide polymorphisms were also excluded.[16] Multidimensional scaling plots were used to evaluate sex outliers based on chromosome X data. Multidimensional scaling and principal components were also used to check unknown population structures. Probes mapping to sex chromosomes were removed. We also checked the white cell count (neutrophils, lymphocytes, and monocytes) as a possible confounding factor. Finally, a subset quantile normalization was performed using a background adjustment between-array normalization and a dye bias correction, following previous recommendations.[15]

The methylation level of each cytosine was expressed as a β-value, which ranged between 0 and 1, unmethylated to completely methylated, respectively. Differentially methylated CpG (DMCs) sites were analyzed using the nonparametric Mann–Whitney *U* test for independent samples, P values$<10^{-06}$ were selected as statistically significant[17] and P values$<10^{-05}$ as nominal association. Multivariable generalized linear analyses adjusting for Principal Components and DNAm potential covariates (age, sex, and current smoking) were also used.

MassARRAY EpiTYPER, Replication Assay

Quantitative DNAm analysis was performed using the MassARRAY EpiTYPER (Sequenom, San Diego, CA) on 3 selected CpGs from the 450-k array discovery study. Selection criteria: have at least a nominal association with vascular recurrence ($P<10^{-05}$), be in a region suitable for MassARRAY EpiTYPER analysis (not all CpGs could be analyzed), and map on a gene previously related to inflammatory processes or cardiovascular events in the literature (bibliographic research performed in PubMed [http://www.ncbi.nlm.nih.gov/pubmed], using the keywords clopidogrel, atherosclerosis, ischemic and vascular). Target-specific primers were designed using the online software Epidesigner [http://www.epidesigner.com], list of primers in the Table III in the online-only Data Supplement). The quantitative methylation data obtained for each CpG site, or aggregates of multiple CpG sites, were analyzed with the EpiTYPER software (Sequenom). Statistical analyses were performed using R (3.1.3 version). P values <0.05 were considered as statistically significant, after the Mann–Whitney *U* test.

Table 1. Descriptive Characteristics of the Study Population

	Vascular Recurrence	Nonvascular Recurrence	Vascular Recurrence	Nonvascular Recurrence
	Discovery cohort (n=42)		Replication cohort (n=191)	
n	21 (50%)	21 (50%)	29 (15%)	162 (85%)
Age, y	70.25±8.7	71.68±8.3	69±10	66±10
Male	18 (85.7%)	18 (85.7%)	16 (55.2%)	140 (86.8%)
Female	3 (14.3%)	3 (14.3%)	13 (44.9%)	42 (26%)
TOAST				
Aterothrombotic	11 (52.4%)	11 (52.4.2%)	3 (10.4%)	21 (13%)
Lacunar	5 (23.8%)	5 (23.8%)	1 (3.5%)	10 (6.2%)
Undetermined	4 (19%)	4 (19%)	3 (10.4%)	35 (21.7%)
Other	1 (4.8%)	1 (4.8%)	13 (44.9%)	12 (7.4%)
Presence of dyslipidemia	12 (57.1%)	8 (38.1%)	5 (17.3%)	52 (32.2%)
Presence of diabetes mellitus	8 (38.1%)	8 (38.1%)	6 (20.4%)	33 (20.5%)
Presence of hypertension	13 (29.9%)	17 (39.1%)	22 (75.9%)	102 (63.2%)
Current smoker	15 (71.4%)	18 (85.7%)	3 (10.4%)	23 (14.3%)
Alcohol	11 (52.4%)	19 (90.4%)	…	…
Previous myocardial infarction	2 (9.5%)	1 (4.76%)	4 (13.8%)	98 (60.8%)
Previous coronary intervention	…	…	5 (17.3%)	28 (17.4%)
Previous angina	2 (9.5%)	…	2 (6.9%)	8 (5%)
Previous tumor	2 (9.5%)	1 (4.76%)	5 (17.3%)	5 (3.1%)
Statin	11 (52.4%)	14 (66.6%)	13 (44.9%)	88 (54.6%)
Atrovastatine	8 (38.1%)	7 (33.3%)	3 (10.4%)	35 (21.7%)
Provastatine	…	…	1 (3.45%)	17 (10.5%)
Rosuvastatine	…	…	…	13 (8.1%)
Sinvastatine	3 (14.3%)	4 (19%)	4 (2.1%)	8 (5%)
Unknown	…	3 (14.3%)	5 (17.3%)	13 (8.1%)

Discovery cohort n=42, replication cohort n=191. TOAST indicates Trial of Org 10172 in Acute Stroke Treatment.

CYP2C19*2 Analysis

We checked the CYP2C19*2 polymorphism (rs4244285) that has been strongly associated with clopidogrel responsiveness[6,7] to know whether it could be a confounding factor in the EWAS analysis (complete Methods in the online-only Data Supplement).

Platelet Aggregation Assay

Spearman rank correlation was used to estimate the correlation between methylation levels of replicated CpGs and platelet aggregation. Values from a subgroup of 90 subjects from the replication study were used. Platelet aggregation values were obtained using Light Transmission Aggregometry with ADP as the agonist at a concentration of 5 μmol/L.[18]

TRAF3 in Aspirin-Treated Patients

A group of patients with stroke treated only with aspirin (n=38) were selected from the Vall d'Hebron University Hospital's cohort following the same inclusion and exclusion criteria used for clopidogrel-treated patients. Of these, 19 patients presented a new vascular event during the first year of follow-up and 19 patients, matched for age, sex, and TOAST, did not present a vascular recurrence. Methylation levels of 37 CpGs located in tumor necrosis factor receptor–associated factor 3 (*TRAF3*) gene were analyzed in this group. See online-only Data Supplement.

Statistical Analysis

A sample size of 21 subjects per condition was needed to achieve a 10^{-05} significance level and 80% statistical power, considering a Cohen effect size=1.8. Sample size calculation was performed using the pwr package (version: 1.1–2) from Bioconductor (http://www.bioconductor.org).

Continuous variables were compared using the Mann–Whitney *U* test, multivariable generalized linear analysis, or Spearman correlation test for nonparametric samples, using the R software (http://www.cran.r-project.org).

Results

Epigenome-Wide Analysis

Epigenome-wide analysis of the 485 577 CpG sites was assessed using the Illumina 450-k BeadChip across the whole genome in each of the 42 individual samples. After preprocessing and QC analysis, 34 059 CpG sites and 1 sample were removed from further analysis: 1848 CpGs with detection P >0.05, 20 935 CpGs that overlap with single-nucleotide polymorphisms, and 11 276 CpGs located on sexual chromosomes, and 1 sample with sex discrepancies.

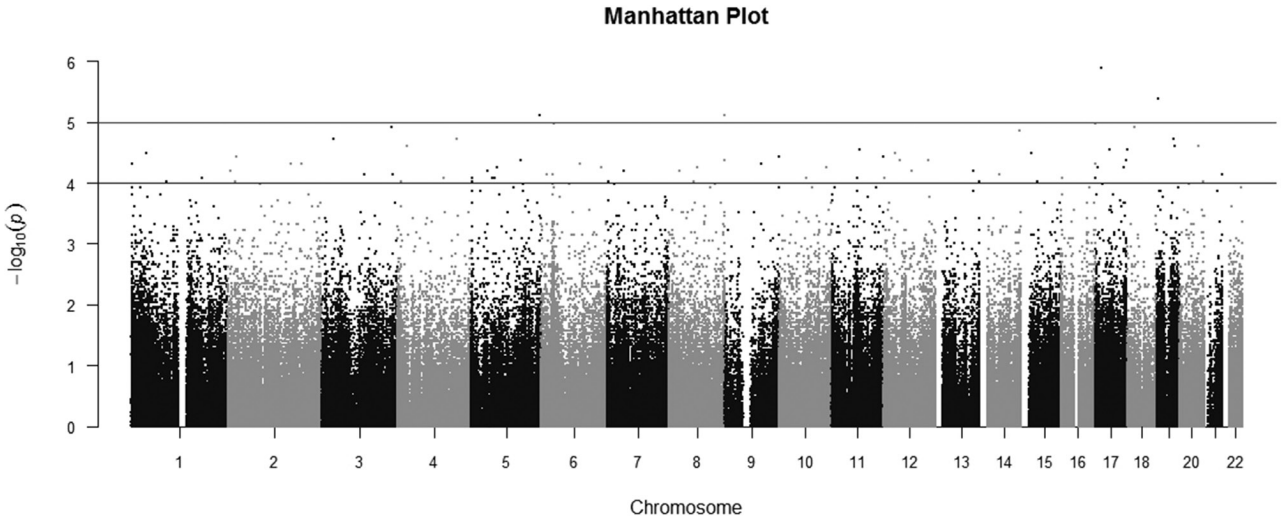


Figure 1. Epigenome-wide association between vascular recurrence patients and nonvascular recurrence patients of 451 518 CpGs. Seventy-three CpGs reached statistical significance, trend ($P<10^{-5}$), represented by the horizontal line. *x*-axis indicates chromosome position, and *y*-axis indicates $-\log_{10}$ of *P* values for each CpG site.

The methylation intensities showed bimodal distribution when displayed across all probes (Figure IA in the online-only Data Supplement), but approximately normal distribution for most CpGs when they were considered individually (Figure IB in the online-only Data Supplement). The −log10 (*P* value) values from DMCs site analysis were plotted for 451 518 CpGs across the genome (Figure 1). Seventy-three candidate DMCs were associated with vascular recurrence in stroke patients treated with clopidogrel with nominal associations *P* values ($P<10^{-05}$; Table 2).[17] Figure 2 shows a hierarchical cluster analysis for a panel of the top 73 significant DMC sites ($P<10^{-05}$), this is able to distinguish vascular recurrence patients from nonvascular patients (with only 1 sample misclassified). A heatmap of the 73 DMCs is shown in the Figure II in the online-only Data Supplement. Among the 73 DMCs, 48 had higher and 25 had lower DNAm levels in patients with a recurrent vascular event compared with patients with nonrecurrence (Figure III in the online-only Data Supplement).

Replication Analysis

The replication analysis of 3 CpG sites was performed for 191 new samples by MassARRAY EpiTyper. The selected CpGs mapped to 3 genes known to be involved in atherosclerosis and vascular processes and could be analyzed by MassARRAY

Table 2. Differentially Methylated CpG (DMC) Sites Associated With Vascular Recurrence in Patients With Stroke Treated With Clopidogrel

CpG ID	Chr	Position	Gene	Mapping to Gene	*P* Value
cg06726262	17	15602873	*ZNF286A*	TSS200	1.29E-06
cg09332091	19	3180708			4.18E-06
cg09533145	5*	178563145*	*ADAMTS2**	Body*	7.81E-06*
cg18002896	8	144465845	*RHPN1*	3'UTR	7.81E-06
cg07925064	16	90114301	*LOC100130015*	TSS200; TSS1500	1.06E-05
cg14630099	6	32975702	*HLA-DOA*	Body	1.06E-05
cg01348374	3	181313591			1.23E-05
ch,18,400468R	18	20395250			1.23E-05
cg03548645	14*	103369816	*TRAF3**	Body*	1.42E-05*
cg10318528	4	154702461	*SFRP2*	3'UTR	1.89E-05
cg15107336	19*	44079778*	*XRCC1**	TSS200*	1.89E-05*
cg27093242	3	32927448	*TRIM71*	Body	1.89E-05
cg16126516	19	46850186	*PPP5C*	TSS200	2.51E-05
cg18997433	4	24585879	*DHX15*	Body	2.51E-05
cg22849543	20	46997755	*LOC284749*	Body	2.51E-05

Top 15 most significant CpGs ($P<10^{-5}$), see Table V in the online-only Data Supplement for full 73 DMCs.
*Selected CpGs for replications.

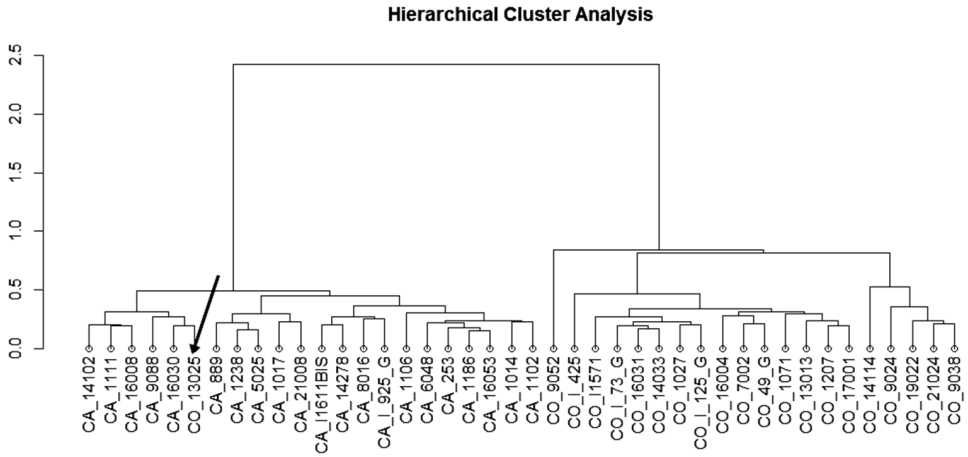


Figure 2. Hierarchical cluster analysis of most significant DMC associated with vascular recurrence in clopidogrel patients. Notice the nearly perfect segregation between the vascular recurrence samples (CA) and the nonvascular recurrence samples (CO).

EpiTyper (*ADAMTS2* cg09533145, $P=7.81\times10^{-06}$; *XRCC1* cg15107336, $P=1.89\times10^{-05}$; and *TRAF3* cg03548645, $P=1.42\times10^{-05}$). Three 400-pb regions containing each one of the 3 selected CpG sites, including other CpG sites and aggregates, were sequenced. For all analyzed regions, the averaged directions of methylation changes were consistent with the methylation changes observed in the 450-k BeadChip array.

The CpG site mapped to *TRAF3* gene cg03548645 was associated with vascular recurrence ($P=0.034$), in the replication cohort. In addition, 3 new aggregated CpGs were associated with vascular recurrence de novo (Table 3). The cg03548645 CpG site was independently analyzed for each individual cohort from the validation stage (Spanish, $P=0.83$; Italian, $P=0.2$; and Swiss, $P=0.15$) and showed the same methylation pattern in each one of them (Figure IV in the online-only Data Supplement). Lower methylation levels were associated with vascular recurrence. These results were consistent with the ones observed when we analyzed the 3 cohorts together, which reaches statistical significance because of the increased statistical power as a result of the larger sample size.

Cardiovascular risk factors (age, sex, and current smoking) were analyzed as possible confounding factors in the discovery cohort, *TRAF3* was independently associated with vascular recurrence ($P=1.33\times10^{-3}$). In addition, *TRAF3* methylation levels were not influenced by cell-type proportions. When excluding patients with previous tumors, as possible confounding factor because of an increased activation of the inflammation pathway, *TRAF3* was still associated with vascular recurrence ($P<3.33\times10^{-05}$). The frequency distribution of the CYP2C19*2 (rs4244285) polymorphism, previously associated with clopidogrel responsiveness,[6,7] was also checked. The risk allele was equally distributed between patients (n=5) and controls (n=5), $P=0.87$. We also checked the methylated status of 5 CpGs of *CYP2C19* gene, none of them presented statistically significant results (Table IV in the online-only Data Supplement).

Table 3. Differentially Methylated CpG Sites Identified De Novo, and the One Replicated in the MassARRAY Replication Study

CpG ID	Identification	Chr	Gene	*P* Value
TRAF3_1,2	Replicated	14	*TRAF3*	0.03441
TRAF3_6,7,8	De novo	14	*TRAF3*	0.04089
XRCC1_19,20	De novo	19	*XRCC1*	0.04195
XRCC1_36	De novo	19	*XRCC1*	0.04261

In addition, an inverse statistically significant correlation ($\rho=-0.29$, $P=0.0075$) was observed between DNAm levels (cg03548645 and *TRAF3*) and platelet aggregation (Figure 3), indicating that *TRAF3* methylation was associated with a biochemical resistance to clopidogrel activity. Moreover, we observed that the methylation differences in cg03548645 were not stroke subtype dependent because we observed the same differences between recurrent and nonrecurrent stroke patients within the group of atherothrombotic strokes ($P=0.011$), and between the group of patients with nonatherothrombotic stroke ($P=0.007$; Figure V in the online-only Data Supplement). When the 37 CpG sites of *TRAF3* were assessed in aspirin-treated patients, no association was found between cg03548645 and vascular recurrence ($P=0.48$). However, *TRAF3* cg03548645 methylation was lower in patients with vascular recurrence. Importantly, a different CpG site was associated with vascular recurrence (cg14008679, $P=5.41\times10^{-4}$). Patients treated with aspirin showed the same methylation pattern that clopidogrel-treated patients for these 2 CpG sites, with lower *TRAF3* methylation levels associated with vascular recurrence (Figure VI in the online-only Data Supplement).

Discussion

We performed the first genome-wide DNAm profiling of vascular recurrence in clopidogrel-treated patients with stroke. High-throughput DNAm array-based profiling covering >450 000 CpG sites of the human genome was complemented by MassArray replication of the most physiopathologically relevant results. Quantitative DNAm identified DMCs sites mapped to atherosclerosis and vascular process–related genes.

The EWAS revealed 73 DMCs associated with vascular recurrence in clopidogrel-treated stroke patients. After EpiTYPET analysis, the association with the cg03548645,

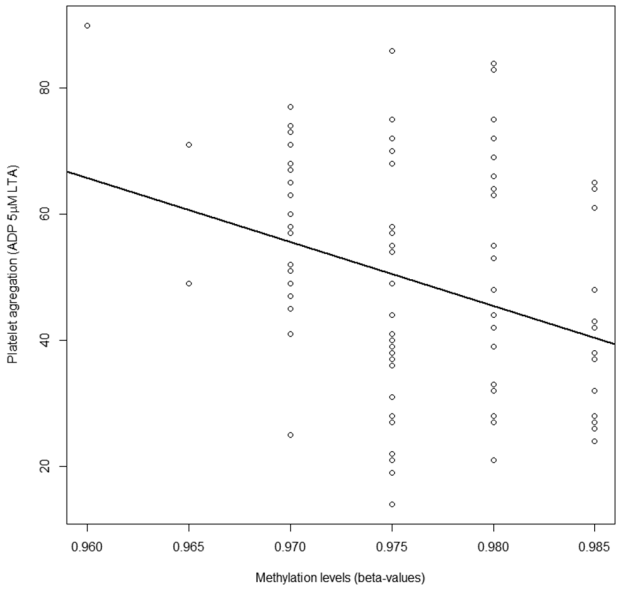


Figure 3. Correlation between cg03548645 DNAm values and platelet aggregation (ρ=−0.29, *P*=0.0075). *x*-axis indicates β-values for CpG 1.2 from EpiTYPER analysis, and *y*-axis indicates ADP 5 µmol/L aggregation percentage by light transmission aggregometry (LTA).

mapping to *TRAF3*, was replicated. The results of the validation stage were not affected by the heterogeneity of the sample. There were no differences between patients with ischemic stroke and patients with cardiovascular disease. Furthermore, the methylation pattern of the *TRAF3* was similar in the 3 cohorts tested, being consistently lower in the patients with vascular recurrence. The combined analysis of the 3 cohorts reached sufficient statistical power to detect significant associations.

As further support of our findings, cg03548645 platelet aggregation levels were correlated with the DNAm levels. The differences between cases and controls were independent of stroke subtype. Furthermore, the CYP2C19*2 polymorphism was not a confounding factor because it was equally distributed between our groups (*P*=0.872). Furthermore, CYP2C19 was not differentially methylated between recurrent and nonrecurrent patients.

The *TRAF3* encodes a protein member of the TRAF family. These proteins participate in the signal transduction of CD40 and tumor necrosis factor receptor, important to immune response activation. It has been shown that patients having cardiovascular disease exhibit increased levels of circulating and soluble CD40 ligand (DC40L).[19,20] Song et al[21] described association between *TRAF3* gene expression and CD40 levels in arterial injury, also Pluvinet et al[22] highlight the anti-inflammatory potential of RNAi-mediated CD40 inhibition, and the relevance of CD40 signaling for therapeutic intervention. del Río-Espínola et al[23] also found an association between CD40 polymorphisms and reocclusion risk after fibrinolysis during the acute phase of ischemic stroke.

Zirlik et al[24] investigated *TRAF* expression in murine and human atherosclerotic plaques. They found increased expression levels of TRAF2 and TRAF3 protein in atherosclerotic tissues compared with nondiseased tissue. The results also establish the functional relevance of these *TRAF* families for proinflammatory signaling events in endothelial cells, and hence in inflammatory vascular diseases such as atherosclerosis. A recent study has also found *TRAF3* upregulation associated with hypertrophied mice hearts and failing human hearts. Transgenic mice overexpressing *TRAF3* in the heart developed exaggerated cardiac hypertrophy.[25]

Our results indicate significantly lower DNAm levels of CpG cg03548645 in patients who had vascular recurrence during clopidogrel treatment, compared with those who did not have vascular recurrence during the first year of follow-up. These results were not influenced by common cardiovascular risk factors ($P=1.33\times10^{-03}$) or previous presence of tumors ($P=3.33\times10^{-05}$). A replication study was conducted on randomly selected samples from 3 international cohorts. cg03548645 remained significantly associated (*P*=0.034) and a new CpG aggregate, located ≈150-bp downstream from the main site, was also significantly associated with vascular recurrence (*P*=0.040). All results were consistent in the discovery and the replication study; in both studies, recurrent patients show lower methylation levels than nonrecurrent patients. When cg03548645 DNAm levels were analyzed on aspirin-treated patients, no association with vascular recurrence was found. However, lower cg03548645 methylation levels were observed in vascular recurrent patients, this trend was the same observed in clopidogrel-treated patients. In addition, a second *TRAF3* CpG site (cg14008679) was found associated with vascular recurrence during aspirin treatment, with recurrent patients showing lower methylation levels in comparison wth nonrecurrent patients. We hypothesize that *TRAF3* lower methylation may be directly related to vascular recurrence regardless aspirin or clopidogrel administration. However, the sample size for aspirin-treated patients is too small to validate this hypothesis. This hypothesis should be further investigated in future studies with patients on other antiplatelet or anticoagulants drugs.

The cg03548645 CpG is located in the *TRAF3* gene body. Lower DNAm levels in gene bodies have been associated with higher levels of gene expression.[26] Therefore, we hypothesize that *TRAF3* expression may be higher in the vascular recurrence patients, which might increase the CD40 ligand signal transduction, thereby enhancing platelet–platelet interactions, secretion, and thrombus growth under artherogenic conditions.[27]

In addition to the association with vascular recurrence in on-clopidogrel treatment patients, we also found that lower cg03548645 DNAm levels were correlated with higher platelet aggregation (ADP-induced platelet aggregation; *P*=0.0075). This association indicates that epigenetics might be involved in the vascular recurrence of stroke and in the pharmacodynamics of clopidogrel. Further studies will be needed to discern if the *TRAF3* (cg03548645) association with vascular recurrence in stroke is because of an increased risk of atherosclerosis, to the inhibition of clopidogrel activity or to a combination of both processes.

The next steps will include the analyses of *TRAF3* transcriptional levels in patients treated with clopidogrel and, it could also be interesting to study these findings in animal models to determine the role of TRAF3 and the reason of the association

with vascular recurrence. Prospective studies analyzing the methylation levels previous to secondary prevention treatment and post recurrence will also be helpful to determine whether *TRAF3* methylation levels can act as a predictive tool in the clinical practice. In summary, the measurement of methylation levels could be, in the future, useful to predict a higher risk of recurrent stroke in patients treated with clopidogrel and to pave the way for an improved personalized management.

Limitations

Analysis of mRNA expression levels was not possible in our study cohorts. Further analysis are needed to confirm the biological meaning of the results. Sample size of discovery phase (clopidogrel and aspirin) was relatively small, although the study had enough statistical power to reach epigenome-wide significance.

Acknowledgments

The Laboratory of Stroke Pharmacogenomics and Genetics is part of the International Stroke Genetics Consortium and coordinates the Spanish Stroke Genetics Consortium.

Sources of Funding

This study was funded by the Miguel Servet grant (CP12/03298), by Instituto de Salud Carlos III and Fondo Europeo de Deasarrollo Regional (ISCIII-FEDER). Joanna Pera is supported by the JUMC No. K/ZDS/003844 grant.

Disclosures

None.

References

1. Hillen T, Coshall C, Tilling K, Rudd AG, McGovern R, Wolfe CD; South London Stroke Register. Cause of stroke recurrence is multifactorial: patterns, risk factors, and outcomes of stroke recurrence in the South London Stroke Register. *Stroke*. 2003;34:1457–1463. doi: 10.1161/01.STR.0000072985.24967.7F.
2. Born G, Patrono C. Antiplatelet drugs. *Br J Pharmacol*. 2006;147(suppl 1):S241–S251. doi: 10.1038/sj.bjp.0706401.
3. Greer DM. Aspirin and antiplatelet agent resistance: implications for prevention of secondary stroke. *CNS Drugs*. 2010;24:1027–1040. doi: 10.2165/11539160-000000000-00000.
4. Weber R, Diener HC. Controversies and future perspectives of antiplatelet therapy in secondary stroke prevention. *J Cell Mol Med*. 2010;14:2371–2380. doi: 10.1111/j.1582-4934.2010.01162.x.
5. Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. *N Engl J Med*. 2009;360:354–362. doi: 10.1056/NEJMoa0809171.
6. Shuldiner AR, O'Connell JR, Bliden KP, Gandhi A, Ryan K, Horenstein RB, et al. Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. *JAMA*. 2009;302:849–857. doi: 10.1001/jama.2009.1232.
7. Holmes MV, Perel P, Shah T, Hingorani AD, Casas JP. CYP2C19 genotype, clopidogrel metabolism, platelet function, and cardiovascular events: a systematic review and meta-analysis. *JAMA*. 2011;306:2704–2714. doi: 10.1001/jama.2011.1880.
8. Feinberg AP. Genome-scale approaches to the epigenetics of common human disease. *Virchows Arch*. 2010;456:13–21. doi: 10.1007/s00428-009-0847-2.
9. Zhang BK, Lai X, Jia SJ. Epigenetics in atherosclerosis: a clinical perspective. *Discov Med*. 2015;19:73–80.
10. Zaina S, Heyn H, Carmona FJ, Varol N, Sayols S, Condom E, et al. DNA methylation map of human atherosclerosis. *Circ Cardiovasc Genet*. 2014;7:692–700. doi: 10.1161/CIRCGENETICS.113.000441.
11. Su J, Li X, Yu Q, Liu Y, Wang Y, Song H, et al. Association of P2Y12 gene promoter DNA methylation with the risk of clopidogrel resistance in coronary artery disease patients. *Biomed Res Int*. 2014;2014:450814. doi: 10.1155/2014/450814.
12. Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke*. 1993;24:35–41.
13. Morisky DE, Green LW, Levine DM. Concurrent and predictive validity of a self-reported measure of medication adherence. *Med Care*. 1986;24:67–74.
14. Touleimat N, Tost J. Complete pipeline for Infinium(®) Human Methylation 450K BeadChip data processing using subset quantile normalization for accurate DNA methylation estimation. *Epigenomics*. 2012;4:325–341. doi: 10.2217/epi.12.21.
15. Pidsley R, Y Wong CC, Volta M, Lunnon K, Mill J, Schalkwyk LC. A data-driven approach to preprocessing Illumina 450K methylation array data. *BMC Genomics*. 2013;14:293. doi: 10.1186/1471-2164-14-293.
16. Price ME, Cotton AM, Lam LL, Farré P, Emberly E, Brown CJ, et al. Additional annotation enhances potential for biologically-relevant analysis of the Illumina Infinium HumanMethylation450 BeadChip array. *Epigenetics Chromatin*. 2013;6:4. doi: 10.1186/1756-8935-6-4.
17. Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. *Nat Rev Genet*. 2011;12:529–541. doi: 10.1038/nrg3000.
18. Paniccia R, Antonucci E, Maggini N, Miranda M, Romano E, Gori AM, et al. Light transmittance aggregometry induced by different concentrations of adenosine diphosphate to monitor clopidogrel therapy: a methodological study. *Ther Drug Monit*. 2011;33:94–98. doi: 10.1097/FTD.0b013e3182052ff4.
19. Antoniades C, Bakogiannis C, Tousoulis D, Antonopoulos AS, Stefanadis C. The CD40/CD40 ligand system: linking inflammation with atherothrombosis. *J Am Coll Cardiol*. 2009;54:669–677. doi: 10.1016/j.jacc.2009.03.076.
20. Hassan GS, Merhi Y, Mourad W. CD40 ligand: a neo-inflammatory molecule in vascular diseases. *Immunobiology*. 2012;217:521–532. doi: 10.1016/j.imbio.2011.03.010.
21. Song Z, Jin R, Yu S, Rivet JJ, Smyth SS, Nanda A, et al. CD40 is essential in the upregulation of TRAF proteins and NF-kappaB-dependent proinflammatory gene expression after arterial injury. *PLoS One*. 2011;6:e23239. doi: 10.1371/journal.pone.0023239.
22. Pluvinet R, Olivar R, Krupinski J, Herrero-Fresneda I, Luque A, Torras J, et al. CD40: an upstream master switch for endothelial cell activation uncovered by RNAi-coupled transcriptional profiling. *Blood*. 2008;112:3624–3637. doi: 10.1182/blood-2008-03-143305.
23. del Río-Espínola A, Fernández-Cadenas I, Rubiera M, Quintana M, Domingues-Montanari S, Mendióroz M, et al. CD40 -1C>T polymorphism (rs1883832) is associated with brain vessel reocclusion after fibrinolysis in ischemic stroke. *Pharmacogenomics*. 2010;11:763–772.
24. Zirlik A, Bavendiek U, Libby P, MacFarlane L, Gerdes N, Jagielska J, et al. TRAF-1, -2, -3, -5, and -6 are induced in atherosclerotic plaques and differentially mediate proinflammatory functions of CD40L in endothelial cells. *Arterioscler Thromb Vasc Biol*. 2007;27:1101–1107. doi: 10.1161/ATVBAHA.107.140566.
25. Jiang X, Deng KQ, Luo Y, Jiang DS, Gao L, Zhang XF, et al. Tumor necrosis factor receptor-associated factor 3 is a positive regulator of pathological cardiac hypertrophy. *Hypertension*. 2015;66:356–367. doi: 10.1161/HYPERTENSIONAHA.115.05469.
26. Jjingo D, Conley AB, Yi SV, Lunyak VV, Jordan IK. On the presence and role of human gene-body DNA methylation. *Oncotarget*. 2012;3:462–474. doi: 10.18632/oncotarget.497.
27. Kuijpers MJ, Mattheij NJ, Cipolla L, van Geffen JP, Lawrence T, Donners MM, et al. Platelet CD40L modulates thrombus growth via phosphatidylinositol 3-kinase β, and not via CD40 and IκB kinase α. *Arterioscler Thromb Vasc Biol*. 2015;35:1374–1381. doi: 10.1161/ATVBAHA.114.305127.

TRAF3 Epigenetic Regulation Is Associated With Vascular Recurrence in Patients With Ischemic Stroke

Cristina Gallego-Fabrega, Caty Carrera, Jean-Luc Reny, Pierre Fontana, Agnieszka Slowik, Joanna Pera, Alessandro Pezzini, Gemma Serrano-Heras, Tomás Segura, Joan Martí-Fàbregas, Elena Muiño, Natalia Cullell, Joan Montaner, Jerzy Krupinski and Israel Fernandez-Cadenas

Stroke. 2016;47:1180-1186; originally published online March 29, 2016;
doi: 10.1161/STROKEAHA.115.012237
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0039-2499. Online ISSN: 1524-4628

Supplemental Data

Supplemental methods.

*CYP2C19*2 analysis:*

OmniQuadHuman 1M (Illumina). was used to genotype the samples. Data was analyzed following WTCC recommendation and the specific methodological guidelines from the Broad Institute and Harvard University. Quality controls (QC) were perform before the analyses.: Missings (-0.3) and checksex QCs were perform for each individual. Only individuals with Caucasian ethnicity were included. We also tested for population stratifications by analyzing the individual IBS, performing MDS plots and adjusting for principal components (PCs). Hardy-Weinberg (p-value>10^{-08}), missingness (0.01), Mishap (p-value>10^{-09}), were analyzed for each SNP.

After QC process, the genotypic results were analyzed by Plink, Haploview, STATA, SNPtest and GTOOL software solutions.

Replication cohorts

The Italian and Spanish cohorts consist of consecutively recruited patients that started clopidogrel treatment after the first ischemic stroke. Vascular recurrence information was available from all patients.

The Geneva cohort consists of consecutively recruited patients with symptomatic documented ischemic atherothrombotic disease (coronary artery disease [CAD], ischemic cerebrovascular disease and/or peripheral artery disease) treated with aspirin and/or clopidogrel for < 5 years. Information about clinical ischemic events over an ongoing 3-years follow-up was available from all patients. Only clopidogrel treated patients were selected from this cohort.

All samples with enough DNA concentration for Sequenom EpiTYPER analysis available, were selected from each cohort (Supplemental Table I).

TRAF3 in aspirin treated patients assay:

Thirty eight subjects from a cohort of 1.900 ischemic stroke patients recruited prospectively at Vall d'Hebron Hospital (Barcelona, Spain), who started aspirin treatment after the first ischemic stroke were analyzed. Nineteen participants presented a new vascular event during the first year of follow up, and were matched one-to-one with 19 participants without a new vascular event. Ethical approval was obtained from the ethical

committee of the Vall d'Hebron Hospital (PR(AG) 03/2007). Each participant signed the informed consent for the study.

Total genomic DNA was extracted from whole blood samples using Gentra Puregen Blood Kid (Quiagen, Hilden, Germany) following the manufacturer's instructions. Samples were obtained during the first 24h after stroke onset, before aspirin initial administration. Quality control (QC) of all samples was performed to check the DNA integrity before bisulfite conversion.

Genome-wide DNA methylation was assessed using the Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego Ca) that analyze methylation levels of 485.577 CpGs sites. All samples were processed in a single working batch. All pre-processing, correction and normalization steps as well as plots, were implemented using the computing environment R (3.1.3 version) with Bioconductor packages. QC and pre-processing steps were the same as described in the main manuscript for the clopidogrel analysis. Differentially methylated CpG sites (DMC) between vascular recurrent patients and non vascular recurrent patients were analyzed using the non parametric Mann-Whitney U-test for independent samples, only in 37 CpG sites located in the *TRAF3* genes.

Supplemental Table I: Descriptive characteristics of the 3 replication cohorts.

	Switzerland's Cohort		Italy's Cohort		Spain's Cohort	
	Rec	**Non-Rec**	**Rec**	**Non-Rec**	**Rec**	**Non-Rec**
N	8 (8.9%)	82 (91%)	17 (19%)	72 (80.6%)	4 (33.4%)	8 (66.6%)
Age (years)	68±10	71±11	70±12	68±14	69±7	65±8
Male	6 (75%)	69 (84.2%)	7 (41.2%)	45 (62.1%)	3 (75%)	6 (75%)
Female	2 (25%)	13 (15.9%)	10 (58.8%)	27 (37.3%)	1 (25%)	2 (25%)
Presence Dyslipidemia	4 (50%)	50 (61%)	-	-	1 (25%)	2 (25%)
Presence Diabetes Mellitus	2 (25%)	20 (%)	3 (17.6%)	9 (12.4%)	0	4 (50%)
Presence Hypertension	7 (87.5%)	54 (24.4%)	11 (64.7%)	44 (60.72%)	4 (100%)	4 (50%)
Current Smoker	1 (12.5%)	22 (26.8%)	2 (11.8%)	-	0	1 (12.5%)
TOAST						
Atherothrombotic			3 (17.6%)	18 (24.4%)	0	3 (37.5%)
Lacunar			1 (5.9%)	8 (11%)	0	2 (50%)
Undetermined			1 (5.9%)	35 (48.3%)	3 (75%)	0
Other			12 (70.6%)	11 (15.2%)	1 (25%)	1 (12.5%)
Previous Myocardial Infarction	1 (12.5%)	23 (28.1%)	3 (17.6%)	25 34.7(%)	-	-
Previous Coronary Intervention	4 (50%)	28 (34.2%)	-	-	1 (25%)	-
Previous Angina	-	8 (9.8%)	1 (5.9%)	-	1 (25%)	-
Previous Tumor	-	-	4 (23.52%)	5 (6.9%)	1 (25%)	-
Statin	6 (75%)	71 (86.6%)	3 (17.6%)	11 (15.2%)	4 (100%)	6 (75%)
Atrovastatine	3 (37.5%)	35 (42.7%)	-	-	-	-
Provastatine	1 (12.5%)	17 (20.7%)	-	-	-	-
Rosuvastatine	-	13 (15.9%)	-	-	-	-
Sinvastatine	2 (25%)	8 (9.8%)	-	-	2 (50%)	-
Unknown	-	2 (2.4%)	3 (17.6%)	11 (15.2%)	2 (50%)	-

Supplemental Table II: Bioconductor packages for the processing and analysis of array-based DNAm data.

DNAm processing/analysis step	**Bioconductor packages**
Methylation data loading	MethyLumi
Quality control sample/probe	wateRmelon, minfi
Normalization and background correction	wateRmelon

Supplemental Table III: Sequences of primers used in MassARRAY EpiTYPER replication study.

Gene	Primer*	Size	Sequence	Product Size	Nº of CpGs	Coverage
TRAF3	LP	25	GGGTTAGTAGTGTGTATTTGGGTTT	327	12	9
	RP	25	ACCAACAAATCCTAACCTCTACCAT			
XRCC1	LP	25	GTTTGGTTAGAAGGATGAGGTAGAG	476	37	26
	RP	25	TCCATCCTAAATAAAAAAACAAAACC			
ADAMTS2	LP	25	GGAGTTTTGATGGTTTTTTTATGTG	408	13	11
	RP	25	CCTCAACCTCCCAAATATCTAAAAT			

* LP, Left Primer; RP, Right Primer.

Supplemental Table IV: Differentially methylated analysis of the 5 CpG sites located in *CYP2C19* gene, present in the Illumina BeadChip array.

CpG ID	Chr	Position	Gene	p-value
cg00051662	10	96521086	CYP2C19	0,672
cg04189838	10	96523347	CYP2C19	0,901
cg18564458	10	96522433	CYP2C19	0,901
cg20031717	10	96523248	CYP2C19	0,126
cg24857560	10	96521152	CYP2C19	0,369

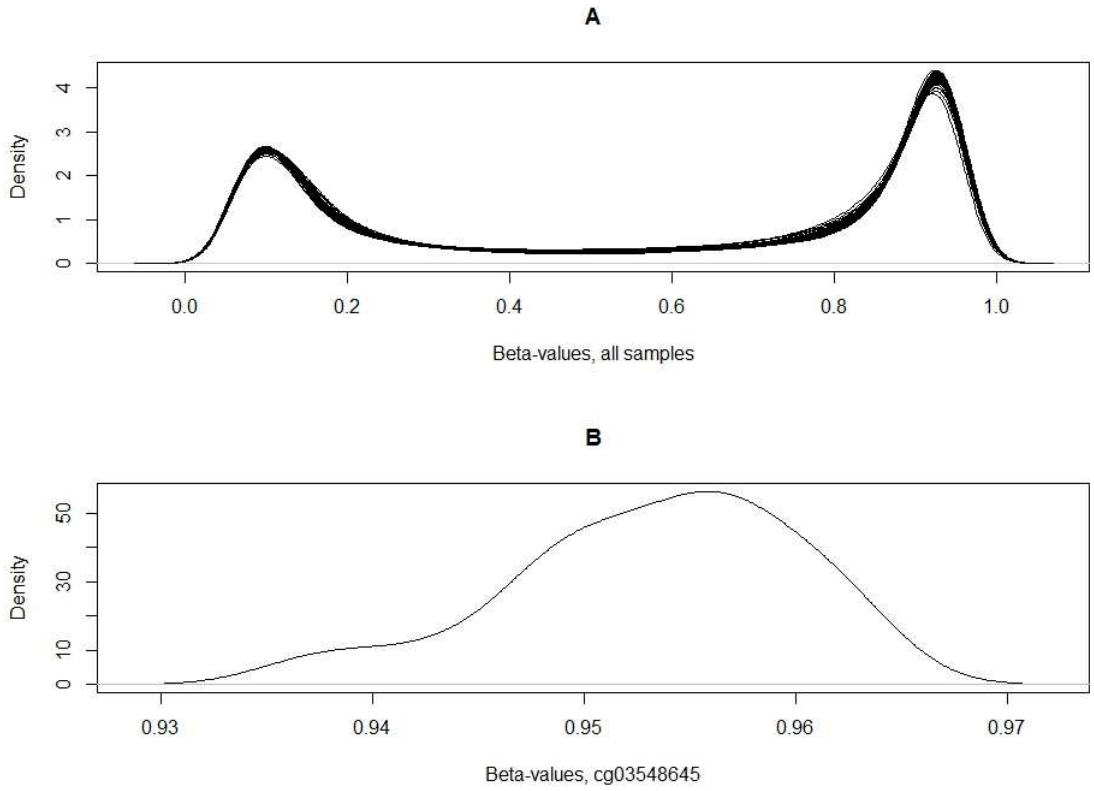
Supplemental Table V: Top 73 differentially methilated CpG sites (p-value<10^{-5}), associated with vascular recurrence in stroke patients treated with clopidogrel.

CpG ID	Chr	Position	Gene	Mapping to gene	p-value
cg06726262	17	15602873	*ZNF286A*	TSS200	1,29E-06
cg09332091	19	3180708			4,18E-06
cg09533145	**5**	**178563145**	***ADAMTS2***	**Body**	**7,81E-06**
cg18002896	8	144465845	*RHPN1*	3'UTR	7,81E-06
cg07925064	16	90114301	*LOC100130015*	TSS200; TSS1500	1,06E-05
cg14630099	6	32975702	*HLA-DOA*	Body	1,06E-05
cg01348374	3	181313591			1,23E-05
ch,18,400468R	18	20395250			1,23E-05
cg03548645	**14**	**103369816**	***TRAF3***	**Body**	**1,42E-05**
cg10318528	4	154702461	*SFRP2*	3'UTR	1,89E-05
cg15107336	**19**	**44079778**	***XRCC1***	**TSS200**	**1,89E-05**
cg27093242	3	32927448	*TRIM71*	Body	1,89E-05
cg16126516	19	46850186	*PPP5C*	TSS200	2,51E-05

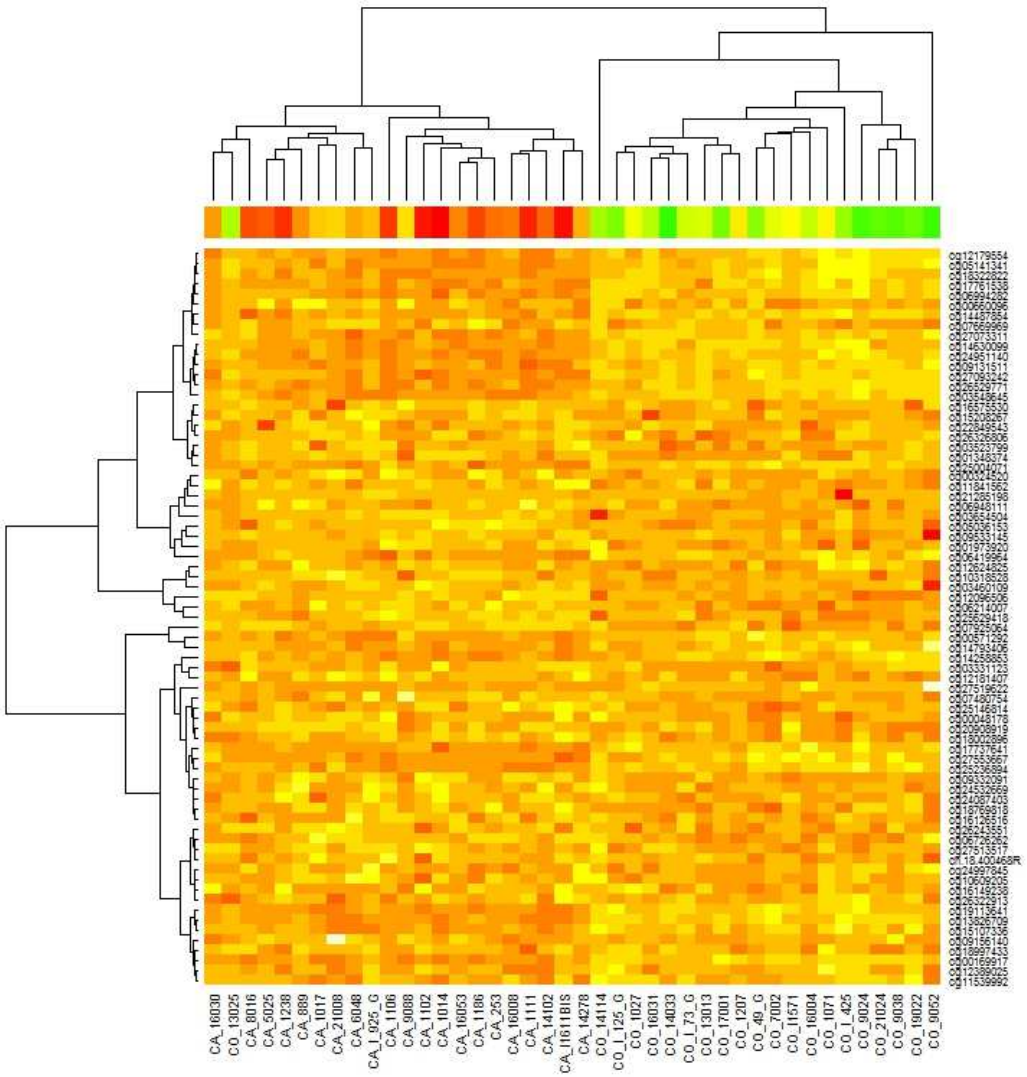
cg18997433	4	24585879	*DHX15*	Body	2,51E-05
cg22849543	20	46997755	*LOC284749*	Body	2,51E-05
cg00660096	17	81023323			2,88E-05
cg06419964	17	34965294	*MRM1*	3'UTR	2,88E-05
cg18769818	11	72433059	*ARAP1*	5'UTR; 1stExon; Body	2,88E-05
cg03654504	1	37495105	*GRIK3*	Body	3,31E-05
cg14258853	12	29935411	*TMTC1*	5'UTR	3,31E-05
cg27553667	15	27216083	*GABRG3*	TSS1500	3,31E-05
cg05036153	9	140128254	*SLC34A3*	Body	3,79E-05
cg12096506	11	134341284			3,79E-05
cg16149238	2	23608001	*KLHL29*	TSS1500	3,79E-05
cg11841562	5	128320387	*SLC27A6*	Body	4,33E-05
cg14793406	12	115135906			4,33E-05
cg17761538	8	145753477	*MGC70857; LRRC24*	Body; TSS1500	4,33E-05
cg20908919	12	42877995	*PRICKLE1*	5'UTR; TSS200; TSS1500	4,33E-05
cg25146814	17	78977970			4,33E-05
cg07480754	16	88298508			4,94E-05
cg09131511	2	163089214	*FAP*	Body	4,94E-05
cg12624825	9	94895189	*LOC100128076*	Body	4,94E-05
cg21285198	1	1360970	*TMEM88B*	TSS1500	4,94E-05
cg24997845	2	191878460	*STAT1*	5'UTR	4,94E-05
cg26243551	6	99873348	*SFRS18*	TSS200	4,94E-05
cg00169917	10	120514287	*C10orf46*	1stExon; 5'UTR	5,64E-05
cg00571292	8	74792031	*UBE2W*	TSS1500	5,64E-05
cg03331123	5	67511540			5,64E-05
cg11539992	17	74497631	*RHBDF2*	TSS200	5,64E-05
cg25236894	17	5323110	*RPAIN; NUP88*	Body; 1stExon; 5'UTR; TSS200	5,64E-05
cg27073311	6	155091036	*RBM16*	Body	5,64E-05
cg03523799	13	98913531	*FARP1*	Body	6,42E-05
cg05141341	7	44796036	*ZMIZ2*	Body	6,42E-05
cg09156140	5	43515218	*C5orf34*	TSS200	6,42E-05
cg16575530	2	8118404	*LOC339788*	TSS1500	6,42E-05
cg17737641	12	71834466	*LGR5*	Body	6,42E-05
cg24951140	8	29156846			6,42E-05
cg00048178	6	13488257	*GFOD1*	TSS1500	7,30E-05
cg10609205	3	112770222			7,30E-05
cg12389025	6	30028738	*ZNRD1;*	TSS1500; Body	7,30E-05

			NCRNA00171		
cg13826709	21	46708041	*POFUT2; LOC642852*	TSS1500; Body	7,30E-05
cg26322913	14	50999702	*MAP4K5; ATL1*	TSS1500; TSS200	7,30E-05
cg26326806	3	186153174			7,30E-05
cg01973920	5	980997			8,28E-05
cg07669969	5	60608527			8,28E-05
cg12181407	5	54052513			8,28E-05
cg14487854	17	714866	*NXN*	Body	8,28E-05
cg15208267	4	119760467			8,28E-05
cg24087403	10	71078535	*HK1*	Body; TSS200	8,28E-05
cg24532669	11	66112147	*BRMS1*	5'UTR	8,28E-05
cg25629418	12	1929265	*LRTM2; CACNA2D4*	TSS200; Body	8,28E-05
cg26529771	16	89260650	*CDH15*	Body	8,28E-05
cg27513517	1	183439365			8,28E-05
cg27519622	16	3079877	*CCDC64B*	Body	8,28E-05
cg00324520	15	41411926			9,39E-05
cg03460109	4	7427313	*SORCS2*	Body	9,39E-05
cg06214007	1	89829308	*GBP6*	TSS200	9,39E-05
cg06948111	8	64093405	*YTHDF3*	Body	9,39E-05
cg06994282	7	2005347	*MAD1L1*	Body	9,39E-05
cg12179554	13	114821832	*RASA3*	Body	9,39E-05
cg18322822	5	1119648			9,39E-05
cg19113641	2	20866242	*GDF7*	TSS200	9,39E-05
cg25004071	20	60639474	*TAF4*	Body	9,39E-05

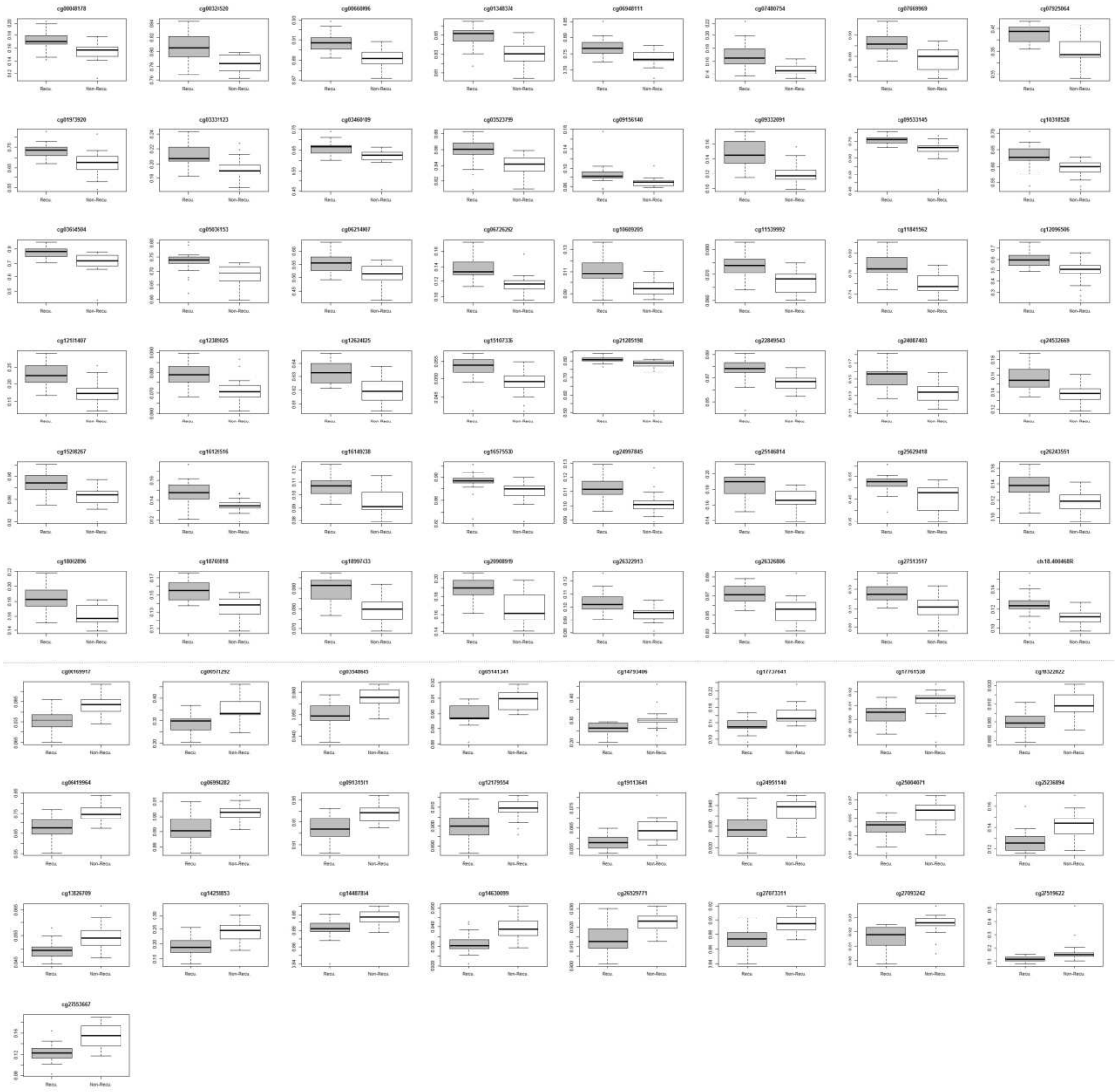
Supplemental Figure I: Density plot showing the distribution of normalized methylation levels in our data. Bimodal distribution was observed when considering all 451,518 CpG sites whereas approximately normal distribution was observed for most individually plotted CpG sites. (a) Beta across all CpGs analyzed; (b) Beta for one representative CpG (cg03548645). X-axis indicates methylation β-values and Y-axis frequency.



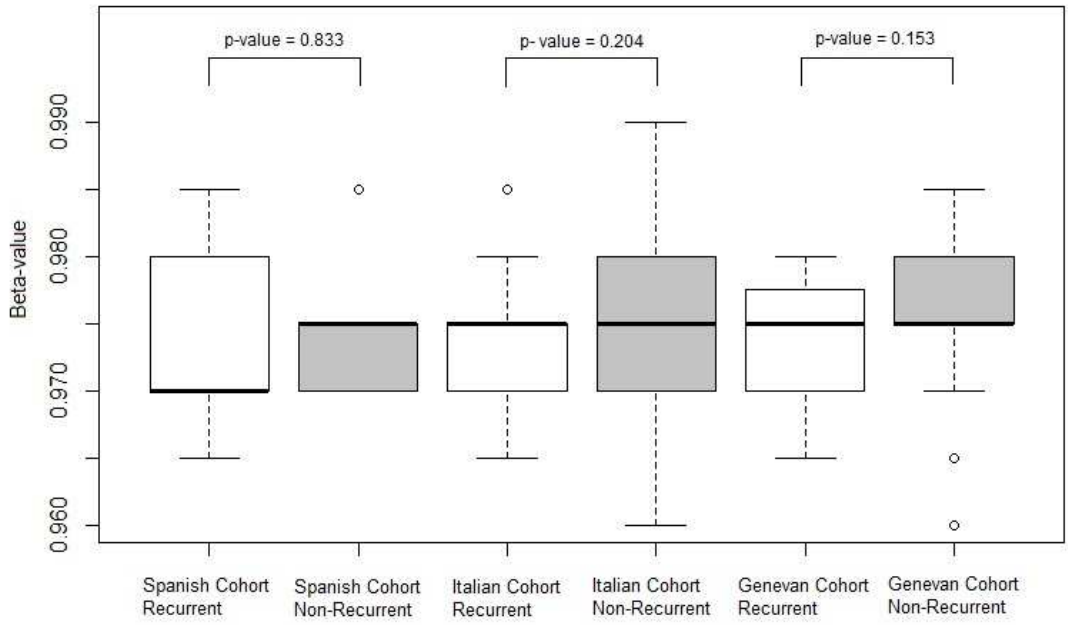
Supplemental Figure II: Clustering heatmap of the 73 differentially methylated CpG sites identified by 450k analysis. The green/red color bar indicates the sample type according to case-control classification in Figure 2. Each column represents a sample and each horizontal line represents the methylation levels of a given CpG across samples. Methylation levels are expressed as 0-1 β-values (green and red, unmethylated and completely methylated, respectively).



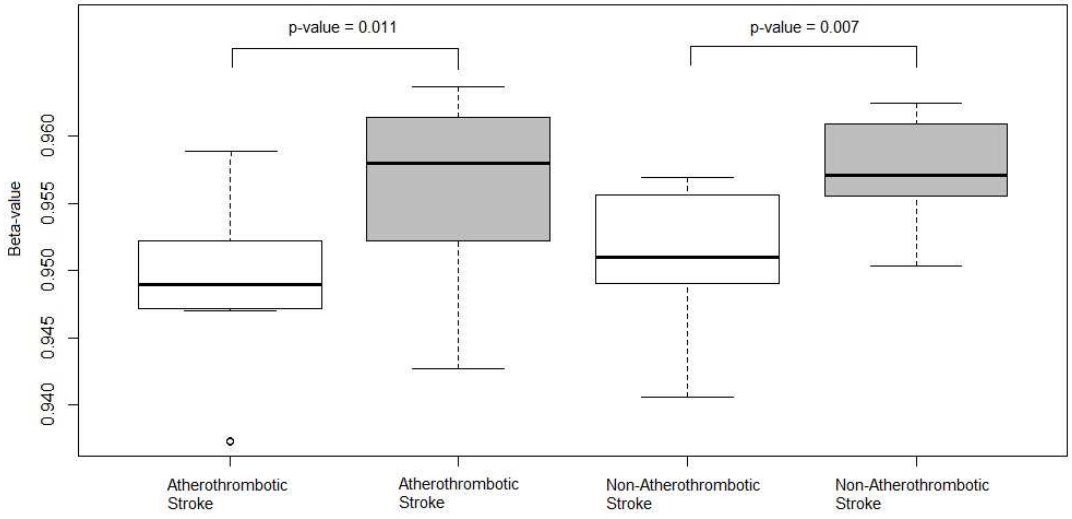
Supplemental Figure III: Box plot representation of 73 DMCs. 48 CpG sites show higher methylation levels in vascular recurrent patients than in non-vascular recurrent patients, above the line. 25 CpG sites show lower methylation levels in vascular recurrent patients than in non-vascular recurrent patients, below the line.



Supplemental Figure IV: Box plot representing the differences between recurrent and non-recurrent patients from each independent cohort of the validation study. X-axe: white box plots represent recurrent patients, and grey box plots represent non-recurrent patients. Y-axe: Methylation levels.



Supplemental Figure V: Box plot representing the cg03548645 methylation levels differences between recurrent and non-recurrent stroke patients within atherothrombotic and non-atherothrombotic patients. White box plots represent recurrent patients, and grey box plots represent non-recurrent patients.



Supplemental Figure VI: Box plot representing the *TRAF3* methylation levels differences between recurrent and non-recurrent stroke patients treated with aspirin. White box plots represent recurrent patients, and grey box plots represent non-recurrent patients.