

DNA Methylation of Fluoxetine Response in Child and Adolescence: Preliminary Results

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Purpose: The search for predictors of antidepressant response is gaining increasing attention, with epigenetic markers attracting a great deal of interest. We performed a genome-wide study assessing baseline differences in DNA methylation between Responders and Non-Responders.

Patients and Methods: Twenty-two children and adolescents, receiving fluoxetine treatment for the first time, were classified as Responders or Non-Responders according to CGI-I score after 8 weeks of fluoxetine treatment. Genome-wide DNA methylation was profiled using the Illumina Infinium MethylationEPIC BeadChip Kit and analyzed using the Chip Analysis Methylation Pipeline (ChAMP).

Results: We identified 21 CpG sites significantly ($FDR < 0.05$) associated with fluoxetine response that showed meaningful differences ($\Delta\beta > \pm 0.2$) in methylation level between Responders and Non-Responders. Two genes, *RHOJ* (*Ras Homolog Family Member J*) and *OR2L13* (*Olfactory Receptor family 2 subfamily L member 13*), presented more than one significant CpG sites.

Conclusion: Our findings provide new insights into the molecular mechanisms underlying the complex phenotype of antidepressant response, indicating that methylation at specific genes could be a promising biomarker that needs further replication in large cohorts.

Keywords: epigenomics, epigenetics, DNA methylation, pharmacogenetics, antidepressants

Introduction

Antidepressants are a first-line treatment for major depressive disorder (MDD) and are widely prescribed for other conditions, such as obsessive-compulsive disorder (OCD). However, between 40% and 50% of patients on antidepressants do not respond to treatment or relapse.^{1,2} This individual variability could be due to the complexity of antidepressant response that involves the interplay of both environmental and genetic factors.³ There are currently no specific sociodemographic or clinical markers to predict the response to antidepressants.⁴

Pharmacogenetic studies have shown that genetic variation influences antidepressant response, but have not fully explained individual variability.⁵ Recent reports have indicated that the estimates of heritability due to common genetic variants are lower than expected and that significant associations are poorly replicated.^{6,7} Thus, the search for biomarkers other than genetic factors that predict antidepressant response is gaining increasing attention,³ with epigenetic markers, especially DNA methylation, attracting a lot of interest.⁸

DNA methylation involves the addition of a methyl group at position 5 of the cytosine pyrimidine ring, a reaction catalyzed by members of the DNA

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methyltransferase (DNMT) family that usually occurs in cytosine bases that are immediately followed by a guanine (CpG). Large clusters of CpGs, known as CpG islands, occur in promoter regions. With some exceptions, active promoters are generally unmethylated, while inactive promoters tend to be methylated.

Several studies strongly indicate that antidepressants can induce the epigenetic modification of DNMTs, thus altering methylation levels and, subsequently, gene expression. This could explain how antidepressants modulate several molecular mechanisms and significantly affect synaptic plasticity.^{3,5}

A number of studies have identified epigenetic biomarkers of antidepressant response, with the majority of these studies using a targeted approach to examine a limited number of CpG sites within a specific gene locus. These gene loci include: the brain-derived neurotrophic factor (BDNF);^{9,10} the sodium-dependent serotonin transporter (*SLC6A4*);^{11–13} the serotonin receptor 1B (*HTR1B*);^{14,15} and the interleukin 11 gene (*IL11*).¹⁶ Recently, a genome-wide methylation study identified a set of CpG sites in specific genes such as *PPFIA4* and *HS3ST1* that accurately predicted paroxetine response.¹⁷

In the present study, we performed a genome-wide study assessing differences in DNA methylation that were characterized at baseline after 8 weeks of fluoxetine treatment in a homogenous sample of child and adolescent patients receiving fluoxetine for the first time.

Patients and Methods

Subjects

Twenty-two children and adolescents aged between 13 and 17 years, receiving fluoxetine treatment for the first time participated in the present study. None of the participants had been treated previously with antidepressants or other psychotropic drugs. Patients were diagnosed using the Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V).¹⁸ The study was carried out at the Child and Adolescent Psychiatry and Psychology Service of the Institute of Neuroscience in Barcelona. Exclusion criteria were comorbidity with other psychiatric disorders, Tourette's syndrome, autism, somatic or neurological diseases, an intelligence quotient <70, and a non-Caucasian ethnicity. All procedures were approved by the Hospital Clinic ethics committee. Written informed consent was obtained from all the parents and verbal informed consent was given by all the participants following explanation of

the procedures involved. All experiments were performed in accordance with relevant guidelines and regulations. This study was conducted in accordance with the Declaration of Helsinki.

Information on illness severity was obtained during the initial phase of the study using the following questionnaires: the Children's Depression Inventory (CDI) for MDD patients (Kovacs, 1992) and the Children's Yale-Brown Obsessive Compulsive Scale (CYBOCS) for OCD patients.^{19,20} The same scales, as well as the CGI-Improvement scale (CGI-I), were administered after 8 weeks of fluoxetine treatment. The clinical response after 8 weeks of fluoxetine treatment was evaluated using the percentage of improvement: $((CDI_{8weeks} - CDI_{basal}) / CDI_{basal}) * 100$ or $((CYBOCS_{8weeks} - CYBOCS_{basal}) / CYBOCS_{basal}) * 100$. Patients were classified as Responders or Non-Responders according to CGI-I score after 8 weeks of fluoxetine treatment. The CGI-I scale assesses the adequacy of clinical response since the start of treatment and is rated on a 7-point scale, as follows: 1=very much improved, 2=much improved, 3=minimally improved, 4=no change from baseline, 5=minimally worse, 6=much worse and 7=very much worse. According to this rating, and according to the literature: Responders were patients with CGI-I<2 (Very much improved or much improved) and Non-Responders were patients with CGI-I>3 (from minimally improved to very much worse).

DNA Isolation

A blood sample from each participant was collected in EDTA (BD Vacutainer K2EDTA tubes; Becton Dickinson, Franklin Lakes, New Jersey, USA) before the start of fluoxetine treatment. Genomic DNA was extracted using the MagNA Pure LC DNA Isolation Kit III and a MagNA Pure LC system (Roche Diagnostics GmbH, Mannheim, Germany). DNA concentration and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Surrey, UK).

Preprocessing DNA Methylation

Genome-wide DNA methylation was profiled using the Illumina Infinium MethylationEPIC BeadChip Kit carried out at CEGEN-PRB3-ISCIH. Raw.IDAT files were received and bioinformatics processes were conducted in house using the Chip Analysis Methylation Pipeline (*ChAMP*) Bioconductor package.²¹ Raw intensity data

files were used to load the data into the R environment with the `champ.load` function, which also allows for probe QC and removal steps to occur simultaneously. Probes with low detected signals ($p < 0.01$) ($n = 3302$), cross reactive probes ($n = 11$), non-CpG probes ($n = 2954$), probes with < 3 beads in at least 5% of samples per probe ($n = 6891$), probes that bound to SNP sites ($n = 96,621$), and sex chromosome probes ($n = 61,734$) are all considered problematic for accurate downstream methylation detection. After removing these probes, 739,405 probes remained for downstream analysis. Beta values were then normalized using the `champ.norm` function, specifically with the beta mixture quartile method (BMIQ function). Cell counts were measured using the `champ.refbase` function. The following cells were counted: CD8+ T cells, CD4+ T cells, natural killer (NK) cells, B cells, monocytes, and granulocytes. Next, the singular value decomposition (SVD) method was performed by `champ.SVD` in order to assess the amount and significance of technical batch components, along with any potential confounding variables (sex, age, diagnosis, cell count, fluoxetine dosage), in our dataset. Using the `champ.runCombat` function, Combat algorithms were applied in order to correct for slide and array as significant components detected by SVD. No effect of sex, age, diagnosis, cell count, or fluoxetine dosage was detected.

Differentially Methylated Positions (DMPs)

After filtering, normalization, and detection of batches and covariates, differentially methylated positions (DMPs) were identified using the function `champ.DMP`, which implements the `limma` package to calculate the p-value for differential methylation using a linear model. The absolute value of the difference between β -value medians ($\Delta\beta$) of Responders and Non-Responders higher than 0.2 was set as a cut-off value to decrease the number of significant CpGs and identify sites with more biologically relevant methylation differences. Hierarchical cluster analysis of significant DMP was plotted as a heatmap and a dendrogram using the `gplot` and `d3heatmap` R packages.

Results

Table 1 shows the sociodemographic and clinical data of the 22 participants of this study classified as Responders or Non-Responders according to the CGI-I scale after 8 weeks of fluoxetine treatment. No significant differences in age, sex, BMI, fluoxetine dose or basal clinical scores were observed between the two groups.

We classified 47,690 probes as significant DMPs (adjusted p-values $FDR < 0.05$): however, this included DMPs with very small differences in methylation between Responders and Non-Responders. Therefore, a $\Delta\beta > \pm 0.2$

Table 1 Sociodemographic, Clinical and Pharmacological Data of the 22 Study Participants

	All	Responders	Non-Responders	Statistic
N	22	11	11	
Age (years, mean \pm SD)	15.1 \pm 1.3	15.2 \pm 1.2	14.9 \pm 1.4	$t_{20} = -0.47$, $p = 0.641$
Age at onset (years, mean \pm SD)	14.9 \pm 1.2	15.0 \pm 1.2	14.7 \pm 1.3	$t_{20} = -0.50$, $p = 0.620$
Gender (Female, %)	19 (86.3)	9 (81.8)	10 (90.9)	$\chi^2 = 0.38$, $p = 0.534$
BMI (mean \pm SD)	21.9 \pm 4.6	21.6 \pm 5.7	22.25 \pm 3.4	$t_{20} = -0.30$, $p = 0.765$
Diagnosis				$\chi^2 = 1.88$, $p = 0.171$
Major Depression (N, %)	15 (68.1)	6 (54.5)	9 (81.8)	
OCD (N, %)	7 (31.8)	5 (45.5)	2 (18.2)	
Clinical scales, baseline				
CDI (mean \pm SD)	30.1 \pm 12.2	26.5 \pm 7.9	32.4 \pm 12.2	$t_{13} = 1.0$, $p = 0.314$
CYBOCS (mean \pm SD)	24.1 \pm 13.9	24.0 \pm 2.9	24.0 \pm 2.9	$t_5 = 0.87$, $p = 0.422$
Clinical scales, 8 weeks				
CDI (mean \pm SD)	24.1 \pm 13.9	15.5 \pm 7.8	29.8 \pm 14.4	$t_{13} = 2.21$, $p = 0.04$
CYBOCS (mean \pm SD)	14.0 \pm 6.6	12.2 \pm 6.4	18.5 \pm 6.4	$t_5 = 1.18$, $p = 0.291$
Fluoxetine dose (mean \pm SD)	23.3 \pm 6.2	21.6 \pm 4.1	24.4 \pm 7.3	$t_{20} = -0.84$, $p = 0.413$

cutoff was applied to identify 21 DMPs with methylation changes that are more likely to be biologically relevant (Table 2).

We assessed the distribution of these 21 DMPs and the other probes in the array in relation to genomic regulatory elements and CpG islands. The genomic regulatory elements considered were the first exon, 3'UTR, 5'UTR, the gene body, and promoter-proximal regions (TSS1500 and TSS200). Hypermethylated probes in Responders were enriched in the first exon (27% vs 0.025% of all probes) and hypomethylated probes were enriched in the 5'UTR (30% vs 0.08% of all probes) (Figure 1A). Regarding the CpG islands, we differentiated between CpG islands, shores (2 kbp from a CpG site), shelves (2 to 4 kbp from a CpG site) and open sea CpGs (isolated CpG in the genome). Hypermethylated probes in Responders were

enriched in CpG islands (45% vs 18%) and hypomethylated probes were enriched in open sea CPGs (90% vs 58%) (Figure 1B).

The 21 significant CpGs mapped to 11 genes (*RHOJ*, *RPTOR*, *ADAP1*, *SPAG1*, *GPR1-AS*, *SLC15A5*, *OR2L13*, *NDUFAF1*, *PPP5D1*, *LOX2* and *ZNF697*) and five intergenic regions. Two genes showed more than two significant DMPs (FDR<0.05, $\Delta\beta > \pm 0.2$) (Figure 2A). *RHOJ* (*Ras Homolog Family Member J*) presented four CpGs that were significantly hypermethylated in Non-Responders. These CpGs were in the 5'-UTR and first exon of the gene, a region that, according to the UCSF browser, includes a promoter region enriched with H3K27AC marks in all cell lines considered by ENCODE (Figure 2B). Two of these CpGs (cg18771300 and cg07157030) were included in The

Table 2 21 Significant (FDR<0.05, $\Delta\beta > \pm 0.2$) Differentially Methylated Probes (DMPs) Between Responders and Non-Responders

Probe ID	Chr	Position	Gene	Responder (β)	Non-Responder (β)	$\Delta\beta$	FDR
cg16322792	1	120,165,303	<i>ZNF697</i>	0.43	0.21	-0.23	0.0330
cg03748376	1	248,100,585	<i>OR2L13</i>	0.34	0.13	-0.22	0.0156
cg20507276	1	248,100,600	<i>OR2L13</i>	0.40	0.15	-0.25	0.0227
cg08944170	1	248,100,614	<i>OR2L13</i>	0.39	0.14	-0.25	0.0228
cg19522960	2	42,287,377		0.62	0.33	-0.29	0.0021
cg22444562	2	207,090,465	<i>GPR1-AS</i>	0.24	0.63	0.40	0.0174
cg00086247	5	29,568,333		0.29	0.49	0.20	0.0109
cg25181997	6	44,788,336		0.87	0.63	-0.24	0.0091
cg19665696	7	949,154	<i>ADAP1</i>	0.32	0.11	-0.20	0.0015
cg03126799	8	23,178,986	<i>LOXL2</i>	0.79	0.57	-0.22	0.0284
cg00191853	8	101,177,733	<i>SPAG1</i>	0.36	0.59	0.23	0.0032
cg03447554	11	43,094,025		0.47	0.67	0.20	0.0153
cg10618621	12	16,342,991	<i>SLC15A5</i>	0.49	0.72	0.23	0.0177
cg11079896	14	63,671,314	<i>RHOJ</i>	0.08	0.33	0.25	7.4×10^{-7}
cg07157030	14	63,671,356	<i>RHOJ</i>	0.46	0.73	0.27	0.0002
cg07189587	14	63,671,517	<i>RHOJ</i>	0.36	0.66	0.30	0.0002
cg18771300	14	63,671,737	<i>RHOJ</i>	0.38	0.64	0.26	0.0002
cg18705301	15	41,695,430	<i>NDUFAF1</i>	0.59	0.37	-0.22	0.0268
cg11949518	17	78,912,765	<i>RPTOR</i>	0.65	0.29	-0.35	0.0006
cg06675417	18	77,292,443		0.39	0.69	0.30	0.0465
cg06489993	19	47,082,996	<i>PPP5D1</i>	0.60	0.39	-0.21	0.0276

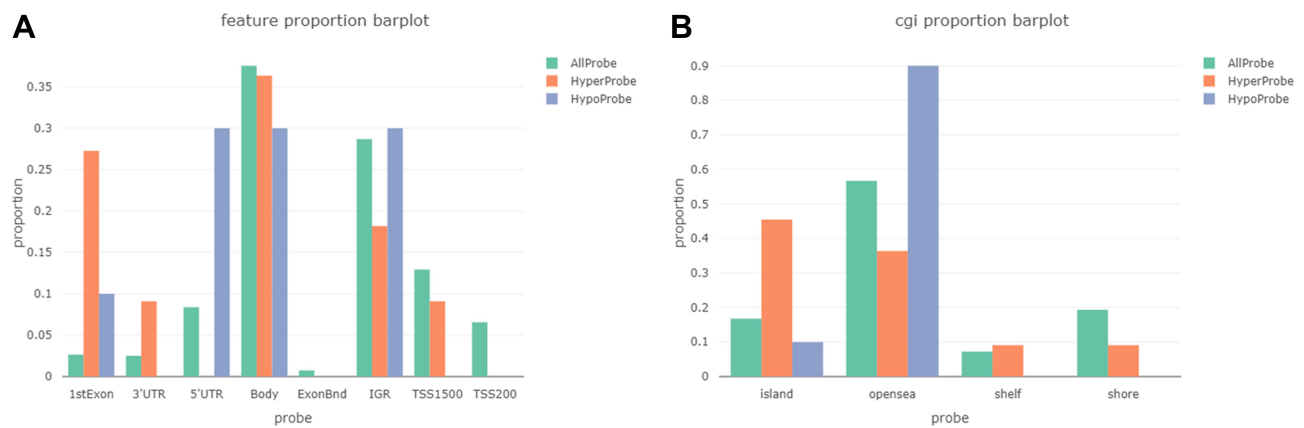


Figure 1 (A) Distribution of 21 significant ($FDR < 0.05$, $\Delta\beta > \pm 0.2$) DMPs and the rest of the probes of the array relative to regulatory elements including transcription start sites (TSS1500, and TSS200), gene body, untranscribed regions (3'UTR and 5'UTR) and first exon. **(B)** Distribution of DMPs and the rest of the probes of the array relative to CpG islands, shores, shelves, and sea.

Blood-Brain Epigenetic Concordance database (BECon; <https://redgar598.shinyapps.io/BECon/>)²² and showed significant correlation between methylation levels in blood and Brodmann Area 10 (BA10) and Brodmann

Area 20 (BA20) ($r > 0.66$). Both CpGs were highly variable in the blood (reference range > 0.1) and fitted with the definition of a blood-brain informative CpG in the BECon.

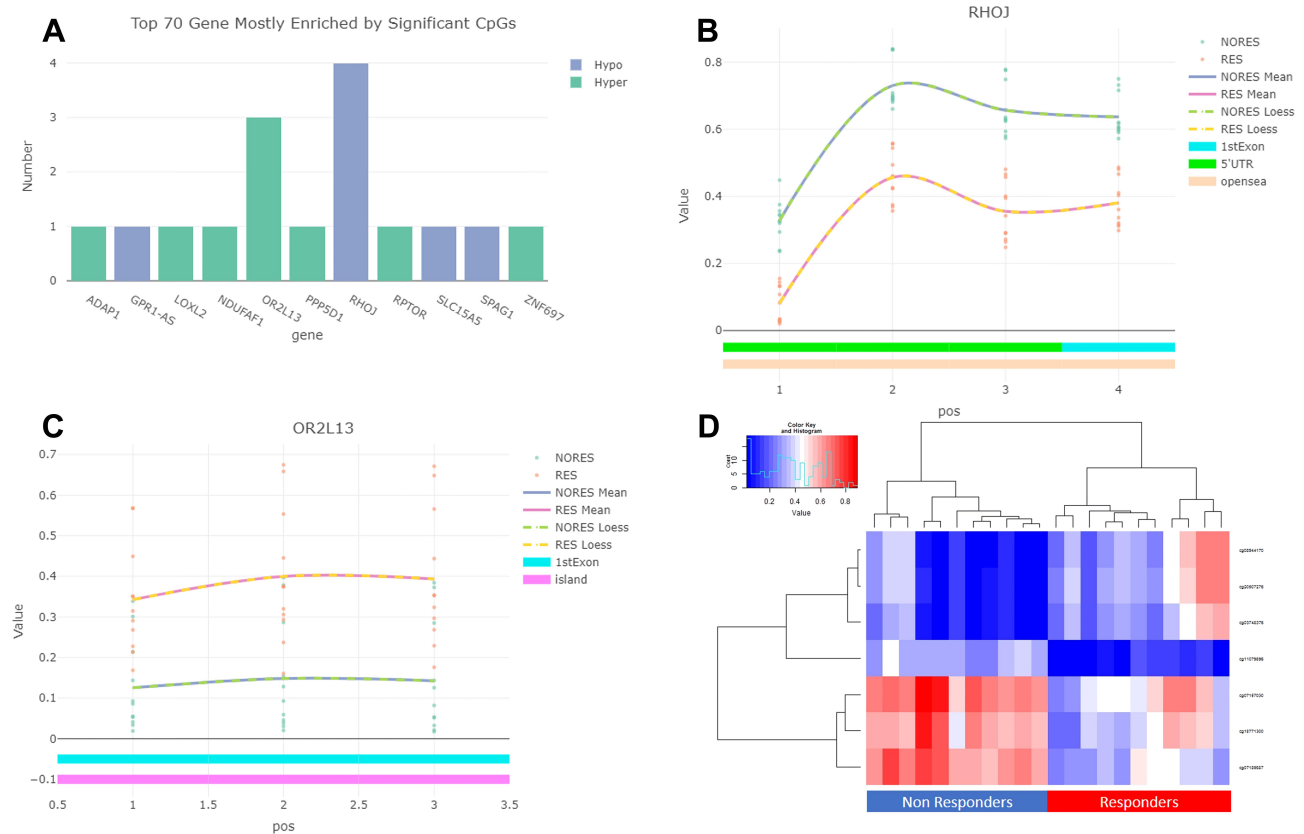


Figure 2 (A) Genes most enriched by the 21 significant DMPs ($FDR < 0.05$, $\Delta\beta > \pm 0.2$). **(B)** Distribution of significant DMPs ($FDR < 0.05$, $\Delta\beta > \pm 0.2$) in the *RHOJ* (*Ras Homolog Family Member J*) gene, and methylation β values in Responders (RES) and Non-Responders (NORES). **(C)** Distribution of significant DMPs ($FDR < 0.05$, $\Delta\beta > \pm 0.2$) in the *OR2L13* (*Olfactory Receptor family 2 subfamily L member 13*) gene and methylation β values in Responders and Non-Responders. **(D)** Hierarchical cluster analysis of the seven CpG sites in the *RHOJ* (*Ras Homolog Family Member J*) and *OR2L13* (*Olfactory Receptor family 2 subfamily L member 13*) genes.

OR2L13 (Olfactory Receptor family 2 subfamily L member 13) presented three CpGs that were significantly hypomethylated in Non-Responders, located on a large CpG island in the first exon of the gene (Figure 2C). According to the BECon database, the three CpGs showed significant correlations between methylation levels in blood and the BA10, BA20 and BA7 areas ($r > 0.5$) and were also highly variable in blood and could be considered blood–brain informative CpGs.

As a sensitivity analysis, we tested the correlations between the methylation level of the seven CpG sites in the *RHOJ* (Ras Homolog Family Member J) and *OR2L13* (Olfactory Receptor family 2 subfamily L member 13) genes and the percentage of improvement scored using the CDI or the CYBOCS. Significant correlations were obtained in all cases: cg03748376 ($r = 0.55$, $p = 0.008$), cg20507276 ($r = 0.54$, $p = 0.010$), cg08944170 ($r = 0.54$, $p = 0.010$), cg11079896 ($r = -0.44$, $p = 0.038$), cg07157030 ($r = -0.49$, $p = 0.021$), cg07189587 ($r = -0.48$, $p = 0.024$) and cg18771300 ($r = -0.43$, $p = 0.045$).

We conducted a hierarchical cluster analysis of the seven sites in these two genes *RHOJ* (Ras Homolog Family Member J) and *OR2L13* (Olfactory Receptor family 2 subfamily L member 13). The results were expressed as a heat map indicating the methylation level at each CpG, and as a dendrogram (Figure 2D). The dendrogram clearly indicated that Responders and Non-Responders differed from each other.

Discussion

To our knowledge, the present study is the first to analyze differences in DNA methylation in association with response to fluoxetine in the peripheral blood of children and adolescents using a genome-wide approach. We identified 21 CpG sites significantly ($FDR < 0.05$) associated with fluoxetine response that showed meaningful differences ($\Delta\beta > \pm 0.2$) in methylation level between Responders and Non-Responders. Two genes, *RHOJ* and *OR2L13*, were enriched in significant CpG sites that showed a strong correlation in DNA methylation between the blood and brain (The Blood-Brain Epigenetic Concordance database BECon; <https://redgar598.shinyapps.io/BECon/>).

RHOJ (Ras Homolog Family Member J) is a member of the Cdc42 subfamily of the Rho family of GTPases, a group of small signaling molecules that are major regulators of cytoskeleton properties.²³ Rho GTPases are involved in various cellular processes, including adhesion, cell

polarization, motility and transformation, gene activation and vesicular trafficking, and have been associated with cytoskeletal organization and the regulation of axon outgrowth.²⁴ Early studies suggested that RhoJ plays a role in modulating the formation of distinct cytoskeletal structures and lamellipodia as well as in actin filaments.²⁵ Also, RhoJ has been shown to regulate the early endocytic pathway, being necessary for the transport of endocytosed receptors.²⁶ Recently, the *crp-1* gene in *Caenorhabditis elegans* that encodes a protein that resembles human RhoJ has been linked to axon guidance and neuronal migration.²⁷

OR2L13 (Olfactory Receptor family 2 subfamily L member 13) is responsible for the initialization of the neuronal response to odorants.²⁸ Differential DNA methylation in a CpG site of this gene has been identified in multiple independent studies examining epigenetic modification in neurodevelopmental disorders.²⁹ The CpG of interest in these studies (cg20507276) was also identified in the current study.

Our hierarchical cluster analysis indicated that methylation sites in *RHOJ* (Ras Homolog Family Member J) and *OR2L13* (Olfactory Receptor family 2 subfamily L member 13) could be important for explaining interindividual differences in fluoxetine response. However, experimental research is needed to confirm that the methylation of these genes plays an important role in the pharmacological effect of fluoxetine and to elucidate their involvement in the mechanism of action of antidepressant drugs.

The significant CpGs identified in relation to fluoxetine in our analysis also mapped to other genes. There is some connection with neuronal physiology or pathological mechanisms of neuropsychiatric disorders for some of these genes, including *ADAPI* (Stricker and Reiser, 2014), *SPAG1*, *SLC15A5* and *RPTOR*.^{30–33} For the other genes (*GPR1-AS*, *NDUFAF1*, *PPP5D1*, *LOX2* and *ZNF697*) or intergenic regions identified we have little or no information about their physiological connection with the pharmacological effect of fluoxetine or their role in the pathophysiology of neuropsychiatric disorders.

To our knowledge, this study is the first genome-wide DNA methylation study of fluoxetine response in children and adolescents. The major strength of our study was that several potential confounders were controlled for, such as age, smoking status, pharmacological treatment and the course of the disease. Our sample contained children and adolescents of similar ages who had not previously been

treated with antidepressants or other psychotropic drugs and who were at the initial stages of the illness. We also controlled for blood cell composition, as DNA methylation is cell-type specific and different cell compositions between samples could affect the methylation data obtained.

However, the findings of this study should be interpreted by bearing in mind several important limitations. The sample size limited the statistical power of the study and made it difficult to detect small or modest effects on DNA methylation. Given that the study was hypothesis-driven and due to the small sample size, our results should be seen as preliminary and should be considered as exploratory findings that require further confirmation. Our study had several limitations. We used peripheral blood even though DNA methylation is known to be tissue-specific. However, blood is considered to be a useful proxy for detecting changes across tissues and is the most appropriate tissue in which to look for biomarkers. Moreover, there is a moderate correlation between blood and the brain for non-specific regulatory regions across the methylome.²² Third, the observation period was eight weeks, which could not be enough to detect long-term epigenetic changes. Finally, our study included patients with different diagnoses, MDD and OCD. For this reason, in the primary analysis, Responders and Non-Responders were defined according to the CGI-I scale. However, the sensitivity analysis, replacing the dichotomous classification of patients according to the CGI by the symptom's improvement scored using the CDI and the CYBOCS, confirms our significant findings.

In conclusion, our findings provide new insights into the molecular mechanisms underlying the complex phenotype of antidepressant response and suggest that methylation at specific genes, such as (*RHOJ* and *OR2L13*) could become potential biomarkers for predicting antidepressant response. However, the replication of our results in large samples is necessary in order to include the methylation level of these specific genes as biomarkers to develop predictors for clinical applications.

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Author Contributions

Rodríguez N and Martínez-Pinteño A participated carrying out the experimental procedures, performing the

bioinformatic analyses and the interpretation of results and wrote the first draft of the manuscript.

Gassó P helped in performing the statistical analyses and the interpretation of results and helped in drafting the manuscript.

Blázquez A, Varela E and Plana MT participated in the recruitment and assessment of the sample and helped in drafting the manuscript.

Lazaro L participated in the coordination of the recruitment and assessment of the sample, the maintenance of the database, acquisition of funding, and helped in drafting the manuscript.

Lafuente A participated in helping in conceiving, designing and coordinating the whole study, interpreting the results and drafting the manuscript.

Mas S conceived and designed the whole study and participated in performing the statistical analysis, interpretation of results and wrote the first draft of the manuscript.

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval for the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

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