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Lithium response in bipolar disorder: Epigenome-wide DNA methylation signatures and epigenetic aging

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ABSTRACT

Lithium (Li) is the first-line treatment for bipolar disorder (BD) even though only 30 % of BD patients are considered excellent responders. The mechanisms by which Li exerts its action are not clearly understood, but it has been suggested that specific epigenetic mechanisms, such as methylation processes, may play a role. In this regard, DNA methylation patterns can be used to estimate epigenetic age (EpiAge), which is accelerated in BD patients and reversed by Li treatment. Our first aim was to compare the DNA methylation profile in peripheral blood between BD patients categorized as excellent responders to Li (Ex-Rp) and non-responders (N-Rp). Secondly, EpiAge was estimated to detect differential age acceleration between the two groups.

A total of 130 differentially methylated positions (DMPs) and 16 differentially methylated regions (DMRs) between Ex-Rp ($n = 26$) and N-Rp ($n = 37$) were identified (FDR adjusted p-value < 0.05). We found 122 genes mapping the DMPs and DMRs, nine of which (*HOXB6, HOXB3, HOXB-AS3, TENM2, CACNA1B, ANK3, EEF2K, CYP1A1,* and *SORCS2*) had previously been linked to Li response. We found genes related to the GSK3β pathway to be highly represented. Using FUMA, we found enrichment in Gene Ontology Cell Component for the synapse. Gene network analysis highlighted functions related to the cell cycle, nervous system development and function, and gene expression. No significant differences in age acceleration were found between Ex-Rp and N-Rp for any of the epigenetic clocks analysed.

Our findings indicate that a specific methylation pattern could determine the response to Li in BD patients. We also found that a significant portion of the differentially methylated genes are closely associated with the GSK3β

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pathway, reinforcing the role of this system in Li response. Future longitudinal studies with larger samples will help to elucidate the epigenetic mechanisms underlying Li response.

1. Introduction

Bipolar disorder (BD) is a psychiatric disorder with a prevalence of 1–5 % in the adult population. It presents a complex inheritance model with an estimated heritability ranging between 70 % and 90 %. Although Lithium (Li) is considered the gold standard for long-term management in BD patients, only 20 % to 30 % of patients are considered excellent responders to treatment. Moreover, between 47 % and 60 % of patients present a partial long-term response, and more than 40 % have no clinical response to Li (Papiol et al., 2022; Rybakowski, 2022).

Although Li response (LR) is a multifactorial phenotype, from a molecular perspective, evidence shows that Li exerts multiple effects on neurotransmitter/receptor-mediated signaling, ion transport, signal transduction cascades, and hormonal and circadian regulation (Marie-Claire et al., 2021). Li treatment also profoundly alters gene expression patterns with the final effect of stabilizing neuronal activities, supporting neural plasticity, and providing neuroprotection (Marie-Claire et al., 2021). In this respect, epigenetic mechanisms represent adaptive gene expression patterns that might result from and/or drive the effects of medications. One of the most frequently studied mechanisms is DNA methylation, which dynamically regulates gene expression by adjusting DNA accessibility to transcriptional machinery.

Alterations in DNA methylation have been associated with the pathophysiology of BD (Legrand et al., 2021), and recent studies have shown that Li may modulate epigenetic mechanisms at several levels of regulation. In this regard, decreased global methylation was found on transformed lymphoblasts from BD subjects and their relatives compared to controls (Huzayyin et al., 2014). Furthermore, a decrease in DNA methylation levels was found in the leukocytes of patients on Li monotherapy compared to untreated controls or patients receiving a combination of Li and valproate (Backlund et al., 2015). Many of the candidate gene methylation studies in LR focus on classical pathways, such as the neurotrophic system, mostly concentrating on the Brain-Derived Neurotrophic Factor (*BDNF)* gene (Dell'Osso et al., 2014). For instance, patients treated with Li and valproate showed significant hypomethylation in *BDNF* promoter compared to other drugs (Dell'Osso et al., 2014).

Only one study investigating genome-wide DNA methylation patterns has been conducted on LR, analysing blood samples of 15 responders and 11 non-responders to Li BD patients type I. This study found 111 genomic regions presenting different DNA methylation patterns between the groups (Marie-Claire et al., 2020). Some of the genes identified, such as the Eukaryotic Translation Initiation Factor 2B (*EIF2B)* and the Ral GTPase Activating Protein Catalytic Subunit Alpha 1 (*RALGAPA1),* have been related to Li treatment in rat animal models (Bosetti et al., 2002; Marie-Claire et al., 2020).

Epigenomic data allow epigenetic age (EA) estimation using epigenetic clocks (Levine et al., 2018). In this context, BD has been consistently associated with accelerated aging, including shortened telomeres, increased oxidative stress, DNA and RNA damage, and accelerated epigenetic aging compared to controls (Coello et al., 2023; Fries et al., 2020; Okazaki et al., 2020). Two studies have shown controversial effects in epigenetic aging. The first showed an age-deceleration effect in BD patients treated with Li, potentially reversing the effects induced by the disorder (Okazaki et al., 2020). However, the second study, which focused on the *in vitro* effect of Li did not show this effect when comparing BD patients to controls (Fries et al., 2020).

To better understand the biological processes underlying LR in BD, we aimed to compare epigenome-wide DNA methylation patterns between excellent responders to Li and non-responders. In addition, epigenetic age was estimated using different epigenetic clocks to investigate differences in age acceleration between these groups.

2. Experimental procedures

2.1. Participants

A sample of 63 patients with BD type I/II was selected from a previously published study (Mitjans et al., 2015). These BD patients were collected at the Hospital Clinic in Barcelona and Mental Health Services in Oviedo. All patients signed informed consent before their inclusion in the studies and approval was obtained from the institutions' ethics committees.

All patients were assessed following DSM-IV-TR criteria (APA, 2000) with a semi-structured interview based on the Structured Clinical Interview for DSM-IV (SCID) (First and Gibbon, 2004). The main clinical and sociodemographic variables were also collected from this semi-structured interview. The presence of depressive and manic features was evaluated using the Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960) and the Young Mania Rating Scale (YMRS) (Young et al., 1978) respectively.

Inclusion criteria comprised: (i) meeting criteria for BD-I or BD-II DSM-IV-TR diagnosis (APA, 2000); (ii) age older than 18; (iii) European origin; (iv) meeting criteria for euthymia defined as the presence of clinical remission (a score of $\leq$8 and $\leq$6 during the three months before study inclusion on the HDRS and YMRS, respectively); and (v) at least 1 year on Li as maintenance treatment with dose adjusted according to plasma levels. Exclusion criteria were: (i) <70 IQ; (ii) severe organic disease; and (iii) no tolerability or partial response to Li.

Based on a previous study by our group, only patients classified as excellent responders (Ex-Rp) or non-responders (N-Rp) to Li treatment were selected and included for analysis of their methylation status (Mitjans et al., 2015). As stated, at the time of sample collection, patients who presented a 50 % reduction in episodes after the introduction of Li in monotherapy were classified as Ex-Rp (n = 26; female=42.3 %; mean age = 44.88 (SD = 11.95)), and patients who did not present this reduction or those who required electroconvulsive therapy were classified as N-Rp (n = 37; female = 40.5 %; mean age = 47.97 (SD = 12.13)) (Rybakowski et al., 2005).

2.2. Methylation assay

Genomic DNA was extracted from whole peripheral blood samples according to standard protocols, and genome-wide DNA methylation was assessed using the Infinium HumanMethylationEPIC BeadChip Kit (Illumina) at the genotyping service CEGEN-FPGMX, Spain.

The raw Illumina microarray data were processed with R package ChAMP (Tian et al., 2017) using R program 4.1.3 (https://www.R-project.org/). Quality-control (QC) of all samples was carried out to reduce the variability induced during the experimental process and to determine the biological variation between Ex-Rp and N-Rp. Firstly, we filtered: (i) probes with low detection p-value ($p > 0.01$); (ii) probes with <3 beads in at least 5 % of samples per probe; (iii) non-CpGs probes contained in the dataset; (iv) SNP-related probes; (v) multi-hit probes; and (vi) probes located in chromosome X and Y. All 63 samples passed the QC for subsequent analyses. Then, beta mixture quantile normalization (BMIQ) was used to adjust the β-values of type II probes into a type I probe statistical distribution, and Combat was used for the batch effects correction (Johnson et al., 2007). Additionally, the epismoking score was calculated using the EpiSmoker R package (Bollepalli et al., 2019), and blood-cell type proportions using the refbase function (Houseman et al., 2012). M-values were calculated using the Lumi R

package (Du et al., 2008), and QC resulting M-value matrix (742,902 probes) was used for differential methylation status analyses.

2.3. Statistical analyses

As age and sex are important variables in methylation status, firstly the normality of the distribution of age was tested using Shapiro-Wilk split by phenotype groups (Ex-Rp and N-Rp). Levene's test was used to verify equal variances between the two groups. Consequently, a Student's *t*-test for independent samples was employed. Secondly, differences in sex proportions between the sample groups were checked using χ^2. For every statistical test mentioned, a 5 % significance level was considered. These statistical analyses were performed using the IBM SPSS 27.0 (https://www.ibm.com/es-es/spss).

Limma was used to obtain differentially methylated positions (DMPs) between Ex-Rp and N-Rp BD patients (Ritchie et al., 2015). DMRcate was used to test differentially methylated regions (DMRs) between groups; these are the regions differentially methylated across the whole epigenome that accumulate the differences in methylation of consecutive probes (Peters et al., 2015). Sex, age, blood cell estimate, and epismoking score were used as covariates. For both DMPs and DMRs, Benjamini-Hochberg multiple-testing correction was used to correct the false discovery rate (FDR), and a p-value of 0.05 after correction was considered significant.

2.4. Gene network analyses

To gain an understanding of the biological processes in which DMPs and DMRs may be involved, gene network analyses were conducted by Ingenuity Pathway Analysis (IPA) by Qiagen. The standard setup for network analysis provided by the IPA core analysis, with 35 genes per network, was used with one exception; the species used were restricted to "human". All genes annotated to DMPs and DMRs were included in the IPA analyses ($n = 120$). Gene names, gene methylation FDR adjusted p-values, and methylation status coded as −1 (hypomethylated in Ex-Rp) and +1 (hypermethylated in Ex-Rp) were used as input data.

2.5. Enrichment analyses

We also used the GENE2FUNC function from Functional Mapping and Annotation (FUMA) (https://fuma.ctglab.nl/) with the genes related to DMPs and DMRs to annotate them in biological context. The gene expression data sets used were GTEx v8:54 tissue types and GTEx v8 general tissue types.

2.6. Blood-brain DNA correlation

To strengthen the mechanistic insights and interpretation of the significant methylation findings, the BECon web application (https://redgar598.shinyapps.io/BECon) was used to assess the blood-brain correlation of the significant CpGs.

2.7. Epigenetic aging analyses

EA in each patient was estimated using Horvath's calculator for different epigenetic clocks (https://dnamage.genetics.ucla.edu/home) (Horvath, Skin and Blood, Hannum, PhenoAge, GrimAge, and GrimAge2). Additionally, DunedinPACE was also calculated (Belsky et al., 2022). Several EA measures were estimated for all patients including age acceleration for all clocks, Hannum's Intrinsic EA acceleration (IEAA) and extrinsic (EEAA).

Normality was tested using Shapiro-Wilk, and Pearson's correlation coefficients between chronological age and EA estimates. The calculation applied FDR multiple-testing correction, and a p-value < 0.05 after correction was considered for significance. Student's *t*-test or Mann-Whitney's test was used accordingly to evaluate differences in age acceleration measures between groups corrected by age, sex, epismoking score, and cell counts. A p-value < 0.05 after correction was considered significant. All statistical analyses were performed using IBM SPSS 27.0 (https://www.ibm.com/es-es/spss).

3. Results

3.1. Sample descriptives

In our sample, no significant differences were found for age between Ex-Rp and N-Rp (T_{61} d.f.= 1.00, p-value = 0.321), or for sex distribution (χ^2_1d.f. = 0.02, p-value = 0.888). Sociodemographic and clinical data are described in Table 1.

3.2. Differentially methylated positions (DMP)

A total of 130 CpG sites reached epigenome-wide significance between Ex-Rp and N-Rp after multiple testing corrections (p-value$<8.72 \times 10^{-6}$; FDR adjusted p-value<0.05) (Fig. 1A, Supplementary Table 1). Ninety-three DMPs out of the 130 were mapped to genes; some of them were mapped to more than one gene, resulting in 106 genes associated.

Seventy-four DMPs (57 %) were significantly hypermethylated (range log FC = from 0.17 to 0.86) and 56 DMPs (43 %) were significantly hypomethylated (range logFC = from −0.94 to −0.20) in Ex-Rp compared to N-Rp (Fig. 1B, Supplementary Table 1). These CpG sites were located as follows: 50 % in open sea regions, 25 % in islands, 20 % on island shores, and 5 % on shelves.

3.3. Differential methylated regions (DMRs)

A total of 16 DMRs were found between Ex-Rp and N-Rp (FDR adjusted p-value $< 1.48 \times 10^{-10}$) (Table 2). All of them but three were mapped to at least one gene. Seven DMRs (43 %) were hypermethylated in Ex-Rp compared to N-Rp.

3.4. Gene network analyses

Gene networks were constructed for all the genes associated with the DMPs and the DMRs, and IPA clustered 77 of the 120 genes into 10 gene networks, by generating direct and indirect relationships between our input genes, and other genes that IPA adds to complete the network (Ingenuity Pathway Analysis, 2005). Since many of these genes resided in networks 1–6, and were the ones with the highest score, we focused

Table 1
Socio-demographic and clinical data were split by excellent responders (Ex-Rp) and non-responders (N-Rp).

	Excellent responders (n = 26)	Non-responders (n = 37)	Comparison between groups
Age, mean (SD)	44.88 (11.95)	47.97 (12.13)	$T_{61\ d.f.} = 1.00$; p-value = 0.321
Female, n (%)	11(42.3)	15 (40.5)	$\chi^2_{1\ d.f.} = 0.02$; p-value = 0.888
Age at onset, mean (SD)	26.50 (10.35)	28.08 (12.01)	$T_{60\ d.f.} = -0.54$; p-value = 0.590
BD type I, n (%)	18 (69.2)	27 (73.0)	$\chi^2_{1\ d.f.} = 0.11$; p-value = 0.746
Duration of illness (years), mean (SD)	18.77 (2.45)	20.22 (1.83)	$U = 437{,}500$; p-value = 0.663
Duration of Li treatment (years), mean (SD)	8.82 (1.68)	9.86 (1.25)	$U = 120{,}500$; p-value = 0.275
Number of episodes, mean (SD)	9.42 (2.85)	12.85 (1.56)	$U = 226{,}500$; **p-value =0.002**
Number of episodes/ Duration of illness (years), mean (SD)	0.53 (0.11)	0.77 (0.09)	$U = 276{,}000$; **p-value = 0.019**

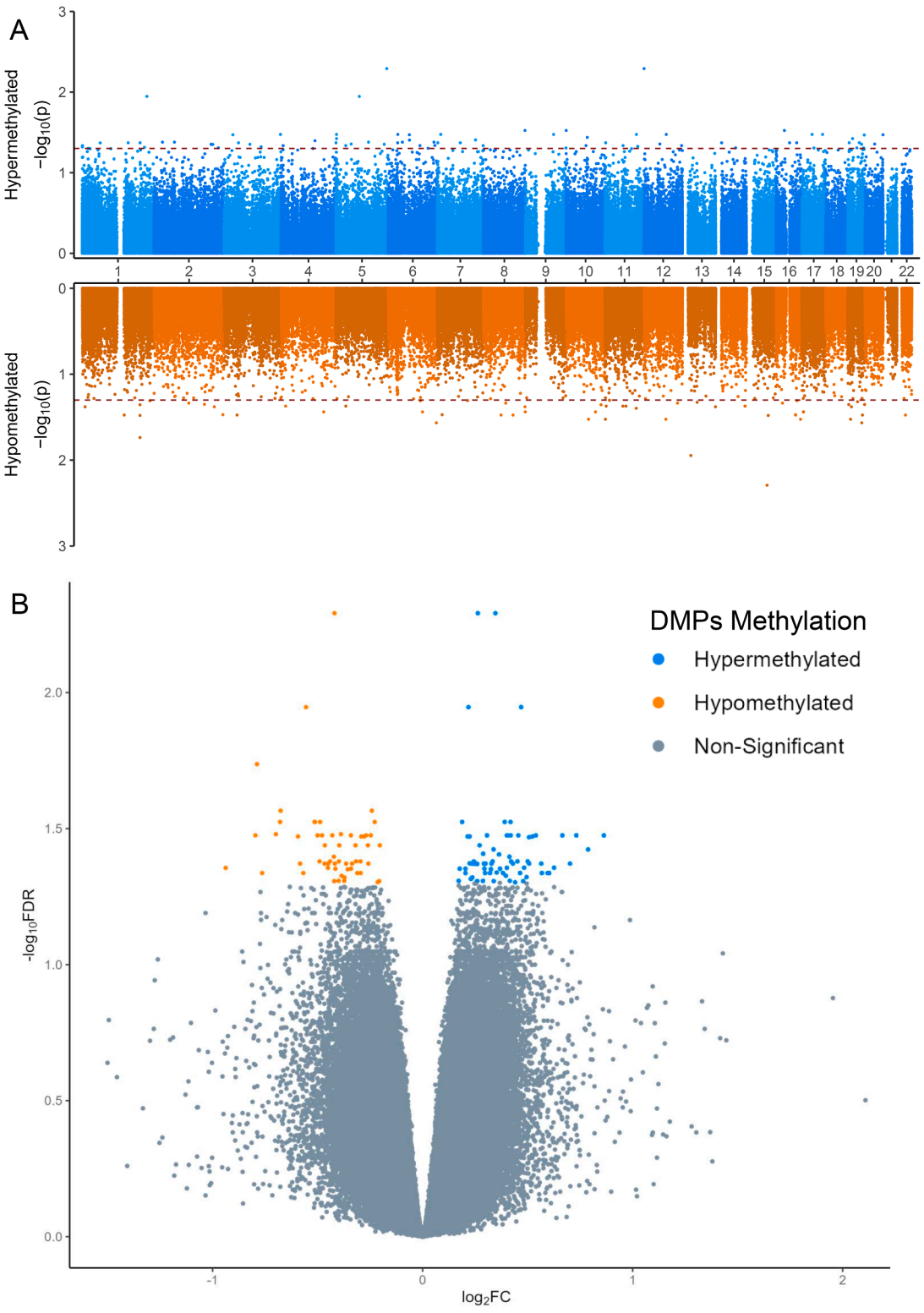

Fig. 1. DMPs between Ex-Rp and N-Rp. **(A) Miami plot.** Association between methylation probes and lithium response across chromosomes. Hypermethylated DMPs are in blue and hypomethylated DMPs in orange. Significantly DMPs are located above the signification lines (FDR adjusted p-value < 0.05). **(B) Volcano Plot.** Significance against log2 fold change, where positive and negative log2 fold changes indicate hypermethylation and hypomethylation in the Ex-Rp group, respectively. Supplementary Table 1 presents all the significant DMPs. .

our results on these six networks (Supplementary Figure 1).

Table 3 summarizes the main findings of the IPA analyses. The highest-scored networks by IPA were Networks 1 and 2, with 16 and 14 input genes, respectively, out of the 35 total genes in each network. Network 1 is related to "Cellular Development, Cellular Growth, and Proliferation, Haematological System Development and Function" and Network 2 is related to "Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair".

Table 2
Differentially methylated regions (DMRs) between Ex-Rp and N-Rp bipolar patients. All the DMRs listed are ordered by p-value from most to least significant. EPIC Annotation information and statistical results are presented for each region.

DMRs	Chromosome: Position (GRCh37/hg19)	No. of CpGs	Gene name	Mean difference	FDR Adjusted p-value
DMR1	Chr6:31,650,735–31,651,676	18	*LY6G5C*	−0.061	3.73×10^{-37}
DMR2	Chr6: 30,853,014–30,854,551	16	*DDR1*	−0.035	8.47×10^{-25}
DMR3	Chr1: 200,271,670–200,272,215	5	–	−0.073	1.30×10^{-19}
DMR4	Chr16: 58,533,743–58,534,708	9	*NDRG4*	−0.096	3.68×10^{-19}
DMR5	Chr12: 4918,337–4919,591	7	*KCNA6*	−0.047	2.76×10^{-16}
DMR6	Chr2:113,992,694–113,993,313	8	*PAX8; PAX8-AS1*	0.108	8.35×10^{-16}
DMR7	Chr17: 46,681,316–46,682,413	11	*HOXB-AS3; HOXB6; HOXB3*	−0.063	2.56×10^{-14}
DMR8	Chr10: 61,900,413–61,900,940	5	*ANK3*	−0.040	3.49×10^{-14}
DMR9	Chr15: 78,631,878–78,632,184	7	–	0.030	1.47×10^{-13}
DMR10	Chr17: 80,407,379–80,407,779	7	*CYBC1 (C17orf62)*	0.020	2.31×10^{-13}
DMR11	Chr10: 73,848,615–73,849,167	9	*SPOCK2*	0.016	6.16×10^{-13}
DMR12	Chr7: 53,879,210–53,879,789	7	*GS1–179L18.1*	0.026	7.62×10^{-13}
DMR13	Chr6: 29,520,698–29,521,162	11	*OR2I1P*	−0.033	1.54×10^{-12}
DMR14	Chr6: 48,037,180–48,037,415	4	–	0.055	1.63×10^{-11}
DMR15	Chr19: 58,715,577–58,715,677	2	*ZNF274*	−0.036	6.50×10^{-11}
DMR16	Chr16: 1014,765–1015,103	5	*LMF1*	0.022	1.48×10^{-10}

Table 3
Summary of the IPA network analyses with the 6 most significant networks.

Top Diseases and functions	Characteristics	Input genes involved	Suppl. Fig. 1, Panels
Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function	Score: 28 Input genes: 16	*ACTN1, ADAMTS12, CACNA1B, CYBC1, DDR1, FAM104, HOXB3, HOXB6, HP1BP3, IL4I1, NDRG4, PPP2R5C, PSME3, RGS10, RLTPR,* and *SSBP2*	A
Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair	Score: 23 Input genes: 14	*ABI2, CEP85L, CNIH3, COL11A2, FAAP24, NCALD, PAX8, PAX8-AS1, PDE2A, PRKCG, PXMP3, RNF168, SORCS2,* and *TMEM201*	B
Molecular Transport, Protein Synthesis, Protein Trafficking	Score: 19 Input genes: 12	*B3GNT9, CD19, FANCC, FLT1, LRMDA, MIR200B, NUP62, PAK4, PPP2R5C, PARK2, PSMB6,* and *UACA*	C
Cell Death and Survival, Gene Expression, Organismal Injury and Abnormalities	Score: 17 Input genes: 11	*DLEU2, FADS1, FBXL5, H1FX, MIR1908, NEUROD4, POLR3C, RPS6KA2, SPOCK2, THSD7A,* and *ZNF274*	D
Embryonic Development, Nervous System Development and Function, Organismal Development	Score: 17 Input genes: 11	*ACAT1, ALK, ANK3, CACNA2D2, EEF2K, EXOSC7, KCNJ10, KCNQ5, SEMA6B, TEP1,* and *ZW10*	E
Cell Cycle, Cell Death and Survival, Gene Expression	Score: 15 Input genes: 10	*AP2A2, CTNNA3, CYP1A1, EIF6, HNRNPUL2, PAK4, SYTL3, TMEM177, VPS35,* and *ZNHIT3*	F

3.5. Enrichment analyses

Using FUMA, we found enrichment for the genes associated with the DMPs and the DMRs in Gene Ontology Cell Component for the synapse (Fig. 2).

3.6. Blood-brain DNA correlation

To identify the correlation of DNAm in blood and brain tissue for all the differentially methylated CpGs, the BECon web application was used (https://redgar598.shinyapps.io/BECon). Of the 130 CpGs, 73 exhibited blood-brain correlation data, all of them relatively weak, between −0.5 and 0.5, except for six CpGs, which were positively correlated (>0.5) (Supplementary Table 4). In summary, these findings suggest that some of the significant CpGs identified in our study likely reflect DNAm levels in the brain.

3.7. Epigenetic aging analyses

We found significant positive correlations between chronological and epigenetic age for all the clocks used ($r \geq 0.881$; FDR-adjusted p-value $\leq 2.36 \times 10^{-21}$), except for DunedinPACE ($r = 1.10$; FDR-adjusted p-value $= 3.69 \times 10^{-02}$) (Supplementary Table 2).

When we evaluated differences in epigenetic age acceleration between Ex-Rp and N-Rp, we found no significant association for any of the clocks analysed (Supplementary Table 3).

4. Discussion

This study aimed to i) identify differential methylation patterns between excellent responders and non-responders to Li treatment and ii) estimate differences in age acceleration between the two groups. In summary, we found 130 DMPs and 16 DMRs between the two groups, with 106 genes annotated to the DMPs and 16 to the DMRs. Gene network analysis by IPA highlighted networks related to "Cellular Development, Cellular Growth, and Proliferation, Hematological System Development and Function" (16 genes) and "Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair" (14 genes). FUMA analysis showed an enrichment of genes related to "Cell Component for the Synapse". When blood-brain correlations were tested, 6 DMPs presented a significantly positive correlation. Finally, we found no differences in age acceleration when we compared excellent responders and non-responders to Li treatment.

Four of the genes annotated to the DMPs and DMRs have been reported in previous methylation studies of lithium response (*HOXB6, HOXB3, HOXB-AS3*, and *TENM2)* (Marie-Claire et al., 2020). In addition, five more genes (*CACNA1B, ANK3, EEF2K, CYP1A1*, and *SORCS2)* have been related to LR using non-methylation approaches (Garza et al., 2018; Karyo et al., 2010; Pedrosa et al., 2010; Piguel et al., 2023; Shawahna et al., 2017; Wakita et al., 2015).

Lithium is thought to exert its therapeutic effect by acting on cellular targets and modulating neural pathways (Malhi et al., 2013). The

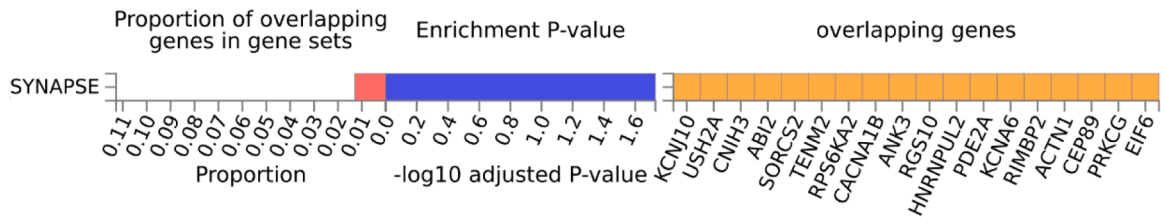

Fig. 2. Gene Ontology Cellular Components results. Enrichment of the genes annotated to the DMPs and DMRs conducted with FUMA.

evidence suggest action at all levels of brain function including functional and structural neural changes or neurochemical and molecular processes (Malhi et al., 2013), among them, effects on transcription factors such as the homeobox genes (Duverger and Morasso, 2008). In this respect, and according to our results, the specific role of differential methylation in *HOXB6, HOXB3, HOXB-AS3* genes and its relation to LR remains to be elucidated. Additionally, the *TENM2* gene, encoding for the Teneurin Transmembrane protein 2, plays a role in synaptogenesis, neurite outgrowth, axon guidance, and neuronal connectivity (Silva et al., 2011) which may be related to the effects of Li in neuroprotection and neurotransmission (Malhi et al., 2013).

Of the different mechanisms by which Li exerts its mood-stabilizing effects, the inhibition of the glycogen synthase kinase 3 beta (GSK3β) clearly seems to be fundamental (Jacoby et al., 2016; Jaworski et al., 2019; Kato, 2022). Interestingly, three of the genes associated with LR in our study (*CACNA1B, ANK3, and EEF2K*) are involved in this pathway (Garza et al., 2018; Karyo et al., 2010; Pedrosa et al., 2010; Piguel et al., 2023).

CACNA1B gene encodes for a subunit of a calcium voltage-gated channel (subunit alpha 1B) that has been related to Li response through the GSK3β pathway, targeted by β-catenin. This gene has been considered of interest as a possible target for therapeutic intervention as calcium channel blockers have been used in the past to treat BD (Pedrosa et al., 2010). We found a CpG site in *CACNA1B* gene body to be hypermethylated in the Ex-Rp group compared to N-Rp. Since gene body methylation has been associated with the activation of gene expression and the use of alternative promoters (Jjingo et al., 2012), our findings suggest a putative increase in *CACNA1B* expression in Ex-Rp compared to N-Rp. Evidence from sequencing and Genome-wide Association Study (GWAS) approaches also suggests that rare and common variants of *CACNA1B* could also play a role in BD risk (Ament et al., 2015; Li et al., 2022).

The *ANK3* gene, which encodes for the ankyrin 3 protein, plays a central role in neuronal microtubule dynamics through GSK3 (Garza et al., 2018). Studies on RNA interference have found a relationship between induced anxiety behaviours and its attenuation by chronic Li treatment (Leussis et al., 2013). Changes in ANK3 protein level after Li exposure were found in the rat hippocampal postsynaptic proteome (Nanavati et al., 2011). Moreover, reductions in dendrite complexity and in dendritic spine number have been seen in a knockdown mouse model which disrupts *ANK3* expression in the adult forebrain. This effect, similar to that reported in BD, is corrected by Li treatment (Piguel et al., 2023). Additionally, *ANK3* has been strongly associated with BD by recent GWAS (Maletic and Raison, 2014; Schulze et al., 2009). Moreover, *AKN3* polymorphism rs10994336 is both associated with BD and differentially methylated in BD patients (Ferreira et al., 2008; Tang et al., 2021).

Lastly, we found hypermethylation in the Ex-Rp group of the *EEF2K* (eukaryotic elongation factor-2) promoter region, suggesting a lower expression of the gene. Interestingly, a previous study using animal and *in-vitro* models showed that Li can reduce EEFK2 phosphorylation through GSK3β inhibition (Karyo et al., 2010).

As we believe that the genes involved in the GSK3β pathway warrant special consideration given their potential role in the mechanism of action of Li, Supplementary Table 5 provides a summary of all genes identified in our study that have been previously associated with this pathway.

Other genes previously related to LR were also differentially methylated in our study. In this respect, it is well known that interindividual variability in drug disposition is a major cause of the lack of efficacy and adverse effects of drug therapies. Most hepatically cleared drugs are metabolized by cytochrome P-450 (CYP) enzymes, including CYP1 (Tornio and Backman, 2018). Interestingly, we found hypomethylation of *CYP1A1* gene in the Ex-Rp group. As the hypomethylated CpG is located in the promoter region, it could be hypothesized that this gene is overexpressed in the Ex-Rp group (Klose and Bird, 2006). Although Li is not metabolized by CYP enzymes, a previous study found that the concentration of CYP1A1 in the cytochrome P-450 could be increased by Li *in vitro* (Shawahna et al., 2017). Furthermore, there is a physical interaction between β-catenin, involved in the GSK3 pathway, and aryl hydrocarbon receptor, which controls the expression of *CYP1A1* (Braeuning et al., 2011).

Additionally, *SORCS2* encodes for a VPS10 domain-containing receptor for the precursor of BDNF. The literature shows that Li can exert its action by raising the levels of BDNF after long-term use (Wakita et al., 2015). BDNF has a critical role in the control of neuronal viability and function. Neurons lacking SorCS2 failed to respond to BDNF, impacting neurite outgrowth and spine formation (Glerup et al., 2016). Moreover, *VPS10* has been implicated in BD risk in one GWAS and two candidate gene studies (Baum et al., 2008; Ollila et al., 2009; Takata et al., 2011).

Other genes highlighted in our study have previously been related to BD, such as *PDE2A* (Farmer et al., 2020), *FADS1* (Zhao et al., 2018), *MIR1908* (Banach et al., 2017), *TRAF3IP2-AS1* (Fabbri and Serretti, 2016), *CD19* (Pietruczuk et al., 2019), and *DDR1* (Garcia-Ruiz et al., 2021). More information about these genes and their relation to BD is given in Supplementary Table 6.

Other genes that are differentially methylated in our sample have previously been associated with other psychiatric disorders besides BD, such as schizophrenia (*RGS10, CNIH3, TRAF3IP2-AS1, CACNA1B, and KCNQ5)* (Baird et al., 2021; Drummond et al., 2012; Fabbri and Serretti, 2016; Hishimoto et al., 2004; Pedrosa et al., 2010), substance abuse *(OPRL1)* (Lutfy and Zaveri, 2016), and anxiety *(OPRL1)* (Andero et al., 2013).

Among the functions of the six most significant networks highlighted by IPA, the Cell Cycle, Nervous System Development and Function, and Gene Expression seem to have special relevance in Li response or BD. In this respect, it has been shown that Li causes sustained G2/M cell cycle arrest without affecting cell viability *in-vitro* via the inactivation of GSK3β and β-catenin (Mao et al., 2001) and the induction of phosphorylation of ERK (Tsui et al., 2012). Moreover, Wnt signaling, which is related to Li response, is required for neurogenesis, the formation of neuronal circuits during development, neuron positioning and polarization, axon and dendrite development, and synaptogenesis (Meffre et al., 2014). In addition, Li-induced gene expression alterations have been seen in cell models of BD (Kittel-Schneider et al., 2019).

FUMA analysis points to the synapse function. It has been shown that Li can exert its action by inhibiting serotonin auto-receptors or upregulating glutamate reuptake (Shaldubina et al., 2001). Moreover, Li reduced cochlear synaptic loss after overexposure by inhibiting NMDA receptor activity in rat models (Choi et al., 2023). Notably, of the five

associated genes in our study that have previously been related to LR, three (*CACNA1B, ANK3*, and *SORCS2*) were overrepresented in the synapse gene set.

Previous results highlight the potential anti-aging effects of Li, but few studies emphasize the DNA methylation changes concerning epigenetic aging (Salarda et al., 2021). In this sense, only one study reported that mood-stabilizers provoked age deceleration, but the exact role of Li is difficult to elucidate (Okazaki et al., 2020). When we used the different epigenetic clocks in our study, we found no differences in age acceleration between Ex-Rp and N-Rp to Li.

Our results should be interpreted with caution given the study's limitations. Despite the relatively small sample size, as far as we know, this is the largest study to date investigating the role of DNA methylation in Li response. Moreover, the groups were comparable in terms of age at onset, BD type, duration of illness and duration of Li treatment. In addition, the measurement of methylation levels was performed in peripheral blood rather than in brain tissue. Although brain DNA methylation may be more related to pathophysiology, access to brain tissues remains difficult and the analysis must account for many confounders (Legrand et al., 2021). This means that peripheral-level approaches like the present one would be useful to achieve a more personalized Li treatment and to define which patients will benefit the most, within the context of a precision psychiatry approach (Salagre and Vieta, 2021). This initiative is supported by recent studies that have reported inter-individual methylation differences correlating significantly between brain and blood (Cheung et al., 2020). Along these lines, the brain-blood correlations tested on our significant CpGs in blood suggest that some of them might reflect DNAm levels in the brain. Thirdly, LR was retrospectively measured without the use of a specific scale. Finally, Li coadministration with other medications, drug abuse, or other environmental factors, which may influence methylation patterns, were not considered (Ilzarbe and Vieta, 2023).

Our results suggest that Li can modulate gene expression by epigenetic mechanisms at different levels, especially genes related to the GSK3β pathway, which is involved in the lithium mechanism of action. In this respect, the use of epigenetic markers as discriminants for clinical response is emerging as an intriguing tool to integrate into routine practice (Marie-Claire et al., 2023, 2022). This approach holds promise in advancing the objectives of personalized medicine and may help to achieve individualized treatment goals.

Author contributions

MZ and MA have participated in the analysis and interpretation of the data and are major contributors to the work drafting. BA, MM and AB contributed to the conception, design and supervision of the work. They also participated in the interpretation of the data and revision of the draft. AB, EJ, PAS, AGP, VR, LGB have actively participated in the sample recruitment and selection. All authors have participated in revising the article critically and approved the final version to be published.

Declaration of competing interest

AGP has received CME-related honoraria, or consulting fees unrelated to the present work from Angelini, Janssen-Cilag, Casen Recordati, Rovi, LCN and Lundbeck. EV has received grants and served as consultant, advisor or CME speaker for the following entities: AB-Biotics, AbbVie, Adamed, Angelini, Biogen, Biohaven, Boehringer-Ingelheim, Celon Pharma, Compass, Dainippon Sumitomo Pharma, Ethypharm, Ferrer, Gedeon Richter, GH Research, Glaxo-Smith Kline, HMNC, Idorsia, Janssen, Lundbeck, Medincell, Merck, Novartis, Orion Corporation, Organon, Otsuka, Roche, Rovi, Sage, Sanofi-Aventis, Sunovion, Takeda, and Viatris, outside the submitted work. The rest of authors have nothing to disclose.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2024.03.010.

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