DNA methylation description of hippocampus, cortex, amygdala, and blood of Drug Resistant-Temporal Lobe Epilepsy

Running title: Refractory epilepsy methylation markers

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ABSTRACT

Background: Epigenetic changes such as DNA methylation were observed in drug-resistant temporal lobe epilepsy (DR-DR-TLE), a disease that affects 25-30% of epilepsy patients. The main objective is to simultaneously describe DNA methylation patterns associated with DR-TLE in hippocampus, amygdala, surrounding cortex to the epileptogenic zone (SCEZ), and peripheral blood. **Methods:** An Illumina Infinium MethylationEPIC BeadChip array was performed in 19 DR-TLE patients and 10 postmortem non-epileptic controls. **Results**: Overall, 32, 59, and 3210 differentially methylated probes (DMPs) were associated with DR-TLE in the hippocampus, amygdala,

and SCEZ, respectively. These DMPs affected genes were involved in neurotrophic and calcium signaling in the hippocampus, and voltage-gated channels in SCEZ, among others. One of the hippocampus DMPs (cg26834418 (*CHORDC1*)) showed a strong blood-brain correlation with BECon and IMAGE-CpG, suggesting that it could be a potential surrogate peripheral biomarker of DR-TLE. Moreover, in three of the top SCEZ's DMPs (*SHANK3, SBF1* and *MCF2L*) methylation status was verified with methylation-specific qPCR. The differentially methylated CpGs were classified in DMRs: 2 in the hippocampus, 12 in the amygdala, and 531 in the SCEZ. **Conclusion:** We identified genes that had not been associated to DR-TLE so far such as *TBX5, EXOC7* and *WRHN*. The area with more DMPs associated with DR-TLE was the SCEZ, some of them related to voltage-gated channels. The DMPs found in the amygdala were involved in inflammatory processes. We also found a potential surrogate peripheral biomarker of DR-TLE. Thus, these results provide new insights into epigenetic modifications involved in DR-TLE.

INTRODUCTION

New anticonvulsant drugs (ACs) have been approved for epilepsy in previous years. Nevertheless, 25-30% of epileptic patients are drug-resistant, and this percentage is difficult to reduce [1]. Thus, drug-resistant epilepsy (DRE) is common, costly, and disabling [1] and poses a therapeutic challenge, decreasing patients' quality of life and increasing mortality [2]. Temporal lobe epilepsy (TLE) is often associated with drug resistance (drug resistant temporal lobe epilepsy (DR-TLE), and can progress with hippocampal sclerosis that involves massive neuronal loss and mossy fiber sprouting.[3] Neurosurgical resection of the epileptogenic zone is an effective therapeutic intervention

to achieve seizure freedom. Indeed, two-thirds of the patients achieve Engel I (seizure freedom) and II (patients rarely present incapacitating seizures) response after neurosurgery [4]. Conceivably early detection of DR-TLE using biomarkers that can be detected in peripheral blood, may further improve the success rate.

Diverse mechanisms have been proposed to explain the processes occurring in the epileptogenic zone of drug-resistant epilepsy patients, such as genetic, disease-related, and drug-related mechanisms, which may be interlinked [2]. In patients with DR-TLE, drug resistance appears, on average, nine years after starting pharmacological treatment [5]. This late onset may be explained by the involvement of environmental factors and epigenetic mechanisms in the drug resistance development [2].

Epigenetic factors are stable but reversible modifications that may alter gene expression without affecting DNA sequence. DNA methylation is the most stable epigenetic mark that has been and plays an important role in DR-TLE and DRE [6–23]. Initially, methylation analysis followed a candidate-genes approach [8, 10, 12, 15, 18]. Subsequently, methylation marks across the whole genome were associated with DRE [21, 22] or focal cortical dysplasia [9]. Other studies focused on methylation differences specific to DR-TLE [13, 16], or even explored the methylation differences between DR-TLE with and without hippocampal sclerosis [14, 20]. Hence, previous studies have found methylation differences associated with epilepsy in buccal samples [19], blood [11], and brain samples [14, 20].

The main objective of this study is to analyze DNA methylation differences associated with DR-TLE in different brain regions and peripheral blood samples to understand

distinct processes underlying drug-resistant DR-TLE and to search for putative peripheral biomarkers of this disease.

MATERIALS AND METHODS

Study population

The protocol and the Informed Consent Form were approved by the Independent Clinical Research Ethics Committee of Hospital Universitario de La Princesa. The study was conducted following the Revised Declaration of Helsinki and STROBE guidelines. Resected fresh brain tissue was obtained from DR-TLE patients who had been subjected to surgical treatment (selective amygdalohippocampectomy or anterior temporal lobectomy) following the modified Spencer technique at National Reference Drug-Resistant Epilepsy Unit Center at Hospital Universitario de La Princesa (CSUR-HUP). A multidisciplinary epilepsy team of neurologists, neurosurgeons, neuroradiologists, and neurophysiologists at the CSUR-HUP determined patients' suitability for surgery, considering clinical evaluation, long-term video-EEG monitoring (LTM), ictal and interictal SPECT (single photon emission computed tomography), neuropsychological assessment, MRI, and foramen ovale electrodes (FOEs). All DR-TLE patients included in the study showed anatomical characteristics compatible with hippocampal sclerosis.

The recruitment period started in September 2018 and lasted two years. Blood and brain samples were obtained from DR-TLE patients subjected to neurosurgical resection of the epileptogenic zone who signed the informed consent. The epileptogenic zone and surrounding cortex to the epileptogenic zone (SCEZ) were collected from each patient. Fresh brain tissue samples were frozen in dry ice immediately after neurosurgical resection. Outcomes of neurosurgical resection were evaluated using Engel classification

[24] at 6, 12, and 24 months after surgery. Patients were classified as responders (R) if they achieved Engel I and II and non-responders (NR) if they had Engel III and IV.

Fresh-frozen postmortem tissue of non-epileptic subjects served as control brain samples. Twenty-four samples from cortex, hippocampus, and amygdala were provided by the biobank "Biobanco en Red de la Región de Murcia" BIOBANC-MUR. Due to the lack of available blood samples from brain donors, ten blood control samples were obtained from "Biobanco del Hospital Universitario de La Princesa". All controls did not suffer any neurological, neurodegenerative, or psychiatric diseases. Moreover, only the samples that did not present any confounding pathology or pathological features assessed by an independent pathologist were included in the present study. Control samples were processed following standard operating procedures with the approval of the Ethics and Scientific Committees.

Nineteen DR-TLE patients subjected to neurosurgical resection of the epileptogenic zone were recruited (Table 1, Fig. 1). The epileptogenic zone was located in the hippocampus but in four of the patients also included the amygdala. Most of the patients (95%) achieved Engel I/II 6 months after neurosurgery and thus were considered responders (R) (Table 1). Ten controls were recruited for peripheral blood samples and another ten for brain postmortem tissue (Table 1).

Sample processing

DNA was extracted from peripheral blood samples using the MagNa Pure LC 2.0 System (Roche Applied Science, Germany). DNA was extracted from brain tissue samples (SCEZ, hippocampus, and amygdala) using the Allprep DNA/RNA Mini Kit according

to the manufacturer's protocol (Qiagen, Germany). Genomic DNA was quantified with Qubit dsDNA BR (Broad-Range) Assay Kit for Qubit™ 4 Fluorometer (Thermo Fisher Scientific, USA).

Genome-wide DNA methylation analysis with high-density arrays

The EZ DNA Methylation Kit (Zymo Research, USA) was applied for bisulfite conversion of 600ng of genomic DNA samples. A genome-wide DNA methylation assay was carried out with the Infinium MethylationEPIC BeadChip Kit (Illumina Inc, USA) by CEGEN (Centro Nacional de Genotipado, Spain) according to the manufacturer's instructions [25]. Samples were randomized to avoid the batch effect. DNA extracted from some brain tissue samples was not enough to perform this methylation analysis. Therefore, certain results could not be obtained for some samples. The number of samples included in the array was shown in Fig. 1.

Methylation preprocessing and analysis

The IDAT files from the Infinium MethylationEPIC BeadChip array were analyzed using the R statistical language (version 4.0.2) following a pipeline built on the minfi packages [26]. Initially, methylation data were normalized by the Noob method [27]. Extracted β-values were subsequently normalized using the BMIQ method [28], implemented in ChAMP [29], to correct for probe design. Quality control and correction of genetic variation were strict. They were conducted by removing the following filtering probes from the initial dataset of 850K CpGs: those with detection p-value>0.01 in any sample, all sex-chromosome probes, those which were cross-reactive and multimapping probes [30, 31] and those with SNPs with MAF≥0.01 at their CpG site or their single-base extension (SBE) site (dbSNP v147). The filtered final dataset comprised 772,002 CpG

sites with good hybridization quality (Fig. 1). M-values were obtained by the logarithmic transformation of β-values. Methylation datasets were adjusted for blood cell heterogeneity using Refbase EWAS.[29]

Differentially methylated probes (DMPs, DR-TLE vs. controls) were calculated by the limma package [32], which was used to compute a moderated t-test adjusted by the batch effect (the ratio of the m-value to its standard error). Raw p-values were corrected using the Benjamini–Hochberg multiple comparison procedure for false discovery rate (FDR). FDR adj. p-value<0.05 was considered significant. Probes were annotated to genes and genomic locations by applying the IlluminaHumanMethylationEPICanno.ilm10b4.hg19 package (v0.6.0).

Enrichment analysis

Enrichr (https://amp.pharm.mssm.edu/Enrichr/) [33] and FUMA GWAS (https://fuma.ctglab.nl/) were applied to identify signaling pathways and biological functions associated with drug-resistant epilepsy or the neurosurgical response. The selected databases were related to Pathways, Ontologies, and Diseases/Drugs. Only significant results with a FDR adjusted p-value<0.05 were considered.

String db (https://string-db.org/) and Cytoscape software were used to perform protein network analysis, and to determine groups of nodes within the network, the Markov Clustering Algorithm, was applied.

Plots

Miami plots were made with qqman (version 0.1.8) [34], and volcano plots were made with the R package ggplot2 (version 3.3.6).

Differentially methylated regions (DMRs)

DMRs were analyzed with the R package DMRcate as previously described [26]. DMRs with a Fisher p-value<0.05 were considered significant. An example of one representative plot for each tissue: hippocampus (Fig. 4), amygdala (Supplementary Fig. 3), and SCEZ (Supplementary Fig. 4), were included.

Blood-brain correlations

To explore the correlation between methylation in the brain and peripheral blood and to search for potential surrogate biomarkers, two different bioinformatic tools were used: BECon (https://redgar598.shinyapps.io/BECon/) [35] and IMAGE-CpG (https://han-lab.org/methylation/default/imageCpG) [36].

DNA Methylation Age Measures

Predicted methylation age (DNAmAge) was calculated using the sesame R package [37]. Linear regression models were performed to analyze the association of chronological age with the predicted epigenetic age.

Methylation-specific qPCR (MS-qPCR)

The EWAS findings were validated with an alternative technique. A subset of three genes, two hypermethylated (*SHANK3, SBF1*) and one hypomethylated (*MCF2L*) were selected from methylation array analysis. All of them presented an increment of β-values>10% between DR-TLE patients and controls, and the CpG was in the gene's body. Actin B

(*ACTB)* was used as a reference gene in the qPCR. A region without CpGs in the *ACTB* gene was selected for amplification. Thus, just one probe was designed for *ACTB*.

Primers and probes were designed using the Methyl Primer Express® software (Thermo Fisher Scientific). Bisulfite conversion of 1000ng of cortex genomic DNA samples obtained from DR-TLE patients and controls. Bisulfite-Converted Human Methylated & Non-methylated DNA Set (Zymo Research, California, USA) was employed as conversion reaction control, and the Non-methylated DNA control of the set for standard curves of unmethylated primers. Bisulfite Converted Universal Methylated Human DNA Standard (Zymo Research, California, USA) was used as positive controls and standard curve of methylated primers.

Reactions were performed with 10ng of DNA in a final volume of 20µl, adjusted to 400nM of each primer Custom DNA Oligos, Merck, Darmstadt, Germany) and 200nM of methylated and unmethylated probes (Dual-Label Probe, Merck, Darmstadt, Germany) and 1X TaqMan® Genotyping Master Mix (Thermo Fisher Scientific, Massachusetts, USA). Primer sequences appear in Supplementary Table 1. The qPCR cycling conditions were 60ºC for 30s, 95ºC for 10min, followed by 40 cycles for 15s at 95ºC, 1min at 60ºC with fluorescence measurement, and 30s at 60ºC. qPCR was analyzed using QuantStudio^{TM} 12K Flex (Thermo Fisher Scientific, Massachusetts, USA) as previously described [38]. Differentially methylation rate was calculated between patients and controls fold-change.

RESULTS

Epigenome-wide associated study (EWAS) in DR-TLE

Hippocampus

Hippocampal samples containing the epileptogenic zone of DR-TLE patients (N=16) were compared in the methylation analysis with the control hippocampus (N=6) to study changes occurring in the epileptogenic zone (Fig. 1). A total of 32 significant DMPs: 14 hypomethylated and 18 hypermethylated in patients with respect to controls. These DMPs were harbored in 18 different genes (Fig. 2A-B, Table 2, Supplementary Table 2A). An enrichment analysis revealed that the main functions of the genes harboring DMPs were sympathetic and central nervous system development, among others (Fig 3A and Supplementary Table 3A).

Amygdala

In several DR-TLE patients, the epileptogenic zone covered both the hippocampus and the amygdala. In this case, DMPs were also analyzed in the amygdala finding 59 DMPs: 27 hypomethylated and 32 hypermethylated in DR-TLE (N=4) with respect to controls (N=9) (Fig 2C-D, Table 2, Supplementary Table 2B). These DMPs were annotated to 28 genes which were involved in diverse processes such as neuroinflammatory cascades or neurotrophic factor signaling (Supplementary Table 3B).

Cortex (SCEZ)

To access the hippocampal epileptogenic zone the anterolateral temporal lobe was partially resected. To explore potential specific epigenetic modifications in that SCEZ, we analyzed cortical samples from DR-TLE patients (N=16) and controls (N=9). We found 3210 significant DMPs associated with DR-TLE: 1828 hypomethylated and 1383 hypermethylated, in patients with respect to controls (Fig 2E-F, Table 2, Supplementary Table 2C), which harbor 1574 different genes involved in diverse biologic functions (Supplementary Table 3C and Supplementary Fig 1A-B). To understand better the

functions of these genes harbored DMPs, we performed a clustering analysis of the proteins encoded by those genes. They were grouped in clusters of proteins related to specific functions (Supplementary Table 4). Figure 3 shows the 4 largest clusters: Cluster-1 includes 81 proteins involved in different functions, such as inflammation and actin filament organization (Fig 3B); Cluster-2 is composed of 23 voltage-gated channel proteins and transient receptor potential channels (Fig. 3C); Cluster-3 comprises 20 proteins related to the spliceosome (Fig 3D); Cluster-4 contains 16 proteins implicated in DNA repair (Fig 3E).

Peripheral blood

Peripheral blood samples were obtained from DR-TLE patients (N=17) and controls (N=10) to detect putative surrogate biomarkers of DR-TLE that could help predict drug-resistant epilepsy in a non-invasive manner. After applying the Houseman correction [29] to reduce possible bias due to cell heterogeneity, there were not significant DMPs associated with DR-TLE (data not shown).

Brain-Blood correlation

Then, we followed a complementary approach to searched for surrogate biomarkers of DR-TLE that could be rapidly and easily obtained in a minimally invasive manner from peripheral blood samples. For that purpose, we explored the DMPs significant in the hippocampus and we searched for correlations between brain and blood with BECon [35] and IMAGE-CpG [36]. From the 32 DMPs significantly associated with DR-TLE in hippocampal samples (Supplementary Fig. 2A), only cg26834418 showed a strong correlation between blood and brain with BECon [35] (r=0.56) (Supplementary Fig 2A-

B) and IMAGE-CpG[36] (p=0.004, r=0.61) (Supplementary Fig 2C). This DMP, located on *CHORDC1* gene could be a potential biomarker of DR-TLE.

Validation by MS-qPCR

The EWAS findings were validated with an alternative technique. We confirmed with methylation-specific qPCR (MS-qPCR) that *SHANK3* and *SBF1* were hypermethylated in patients compared to controls (Table 3). Moreover, we observed that *MCF2L* presented a ratio<1, verifying that it was hypomethylated in patients with respect to controls (Table 3). These results are consistent with those obtained in the methylation array (Supplementary Table 2C).

DMRs associated with DR-TLE

We also analyzed whether several proximal CpGs were concordantly differentially methylated to identify DMRs, that are correlated with gene transcription [39].

For that, DMRs were obtained from significant DMPs between DR-TLE patients with respect to controls. In the hippocampus, there were two DMRs hypomethylated, harboring 3 genes (Fig. 4, Supplementary Table 5A). In the amygdala, 12 DMRs harboring 11 genes were found (Supplementary Fig. 3). Eight of them were hypermethylated, and 4 were hypomethylated (Supplementary Table 5B). Moreover, 531 significant DMRs associated with DR-TLE, harboring 500 genes, were found in the SCEZ (Supplementary Table 5C, Supplementary Fig 4). Most DMRs (81.7%, N=434) were hypermethylated in patients regarding controls. Genes harboring DMRs perform functions such as regulation of Rho activity, NGF, tight junctions, and PI3K/AKT activation (Supplementary Table 5D).

Epigenetic clock

The epigenetic clock was calculated to determine if the epileptogenic zone is more aged than its chronological age would suggest. Chronological age was highly correlated with predicted age in the hippocampus (ρ=0.779, p=0.000) and cortex (ρ=0.875, p=0.000) (Supplementary Fig 5A, B). Nevertheless, no significant correlation was found between predicted and chronological age in the amygdala (ρ=0.875, p=0.125) and blood (ρ=0.067, p=0.828) (Supplementary Fig 5B, D).

DMPs associated with clinical and demographic characteristics

Moreover, we wondered if there were DMPs associated with clinical and demographic characteristics of the patients based on Table 1 and Supplementary Table 6A. We did not find any DMP associated with the presence of aura in any of the tissues analyzed. Moreover, we failed to observe any significant DMPs between ever smokers and non-smokers. Besides, no significant differences in methylation were associated with alcohol consumption. Regarding the etiology we compared patients whose epilepsy was structural (N=5) with patients whose etiology is unknown (N=15). Surprisingly we found one DMP (cg17466567 (*ETV5, DGKG*)) associated with structural etiology both in the hippocampus and in the SCEZ (Supplementary Table 6B). This CpG is the same in the hippocampus and the SCEZ, thus increasing the robustness of this result.

DISCUSSION

In the present study, we explored differential methylation patterns associated with DR-TLE. To our knowledge, and unlike previous studies [6–12, 14–18, 20, 22, 23], we simultaneously analyzed different brain regions (hippocampus, amygdala, SCEZ) and peripheral blood and searched for surrogate biomarkers, validated part of the results with

an alternative technique and studied the epigenetic clock. These types of studies could act as hypothesis generators to identify new genes and molecular mechanisms involved in DR-TLE and DRE and find potential therapeutic targets for this disease.

In the hippocampus, a total of 32 DMPs harboring 18 different genes were associated with DR-TLE. These genes were involved in processes affected in epilepsy, such as autonomic and nervous system development, and positive regulation of axon guidance, among others (Table 2, Supplementary Table 3A, Fig 3A). Previous studies conducted in the hippocampus found 146 [14] or 5523 [13] DMPs. The difference in the number of differentially methylated genes between previous publications [13, 14] and our study may be partially explained by the methodology applied, the population ethnicity, and the number of patients. We also observed predominant hypermethylation status in the hippocampus, which agrees with another study conducted in a rat model of DR-TLE [40]. Nevertheless, SCEZ and peripheral blood showed higher hypomethylation. If we focus on the top hypermethylated and hypomethylated genes, we will identify genes that had previously been associated with the disease, such as *GATA-3* [41], *NRP1* [42], and *PRDM8* [43]. However, there are other genes, such as *TBX5, EXOC7* and *WRHN* that play roles, such as transcription factor, cytoskeletal assembly, or vesicle trafficking. To our knowledge, these genes had not been related to epilepsy so far (Table 2). Methylation studies following a candidate-genes approach showed increased expression of DNA methyltransferases 1 and 3a (*DMT1* and *DMT3a*) in DR-TLE patients [18]. According to previous studies [8, 10, 12], we did not observe differences in methylation of Reelin, *EPHX1,* or *CPA6* in DRE. However, *ACAP3* was hypomethylated in the SCEZ, as described previously [15].

To our knowledge, this is the first study to analyze amygdala methylation of DR-TLE patients. Methylation analysis in the amygdala identified 59 DMPs harboring 28 genes. These genes are involved in signaling pathways previously associated with DRE, such as PKC activation through G-Proteins, Trk and $p75^{NTR}$ neurotrophic signaling cascades [44] (Table 2, Supplementary Table 3B). Besides, these genes participate in IL-1 and TNF signaling cascades, which suggest that neuroinflammation also affects the amygdala, providing further evidence for the involvement of inflammation in drug-resistant epilepsy [2, 13, 22, 45]. However, due to the reduced sample size of the amygdala samples (N=4), these results should be interpreted with caution.

In the SCEZ, a total of 3210 DMPS harboring 1574 genes showed methylation differences in patients compared to control tissue. These genes are involved in processes priorly associated with DRE, such as calcium signaling, neurotrophic signaling [44], axon guidance, and long-term potentiation (Table 2, Supplementary Table 2C, Supplementary Fig. 1A-B). The proteins encoded by those genes harboring DMPs, were grouped in clusters of genes related to specific functions. Interestingly, Cluster-1 was associated with growth factors and kinases, and inflammation, consistent with a previous publication that suggests that SCEZ presents neuroinflammation [13] and with our results obtained in the amygdala. Cluster-2 consisted of voltage-gated channels, the main targets of ACs (Fig. 3B-E, Supplementary Table 4). These results suggest that genes encoding ACs targets may be altered even in the SCEZ, which has often classified as “unaffected” tissue. The high implication of epigenetics in this area reinforces the hypothesis of a neural network disease [3], independently of the location of the epileptogenic zone. Our results are in accordance with a preceding publication that observed a higher implication of epigenetics in SCEZ than in the epileptogenic zone [13]. These results, may partially be explained by

the cell death observed in the epileptogenic zone in DR-TLE [3], thereby suggesting that part of the neurons subjected to methylation changes may die by apoptosis.

Moreover, the SCEZ DMPs were grouped into 531 DMRs (Supplementary Table 5C, Supplementary Fig 4), which could be correlated with transcription [39]. Surprisingly, most of them were hypermethylated in patients concerning controls, thus suggesting that genes located in these regions, may be repressed. These genes participate in signaling cascades key for epilepsy, such as Ionotropic glutamate receptor, cadherin [46], mTor-PI3K, Akt pathway [47], Rho signaling pathways [48], and NGF [49] (Supplementary Table 5D). Tight junction proteins are downregulated in the hippocampus of epilepsy patients [50, 51]. Our results suggest that genes involved in tight junctions may be regulated by epigenetics, even in the SCEZ (Supplementary Table 5D). We also observed that the Rho pathway, which is involved in epilepsy [48], is regulated by methylation. Accordingly, these methylation-regulated genes are located at the synapse and post-synapse (Supplementary Table 5D). Moreover, enrichment showed that several rare pathologies are associated with these genes harboring DMRs, such as recurrent encephalopathy of childhood or juvenile myoclonic epilepsy (Supplementary Table 2C and 3C).

To confirm the array's DMPs reliability subset of 3 genes with body DMPs in the SCEZ was validated by an alternative technique MS-qPCR. We verified that *SHANK3* and *SBF1* were hypermethylated and *MCF2L* was hypomethylated in DR-TLE patients compared to controls in both techniques. *SHANK3* is a multidomain scaffold protein of the postsynaptic density that connects neurotransmitter receptors, ion channels, and other membrane proteins to the actin cytoskeleton and G-protein-coupled signaling pathways.

On the contrary, previous studies have shown that *SHANK3* is overexpressed in drug-resistant epilepsy [52]. *MCFL2* is involved in Rho/Rac signaling pathways and is upregulated in epilepsy [48]. On the other hand, *SBF1* protein levels seem to be impaired in a rat model of epilepsy [53].

Although most DMPs were simultaneously not found in the SCEZ and hippocampus, one gene that plays a role in transcriptional regulation, *RP11-73O6.4 LINC02157* (Long Intergenic Non-Protein Coding RNA 2157), showed DR-TLE-associated differential methylation in both brain regions. Furthermore, *RNF40, MPPED1, RP11-560I19.4,* and *PRKCZ,* were also subjected to epigenetic regulation in both the cortex and amygdala. *RNF40* (Ring Finger Protein 40) encodes an E3 ubiquitin-protein ligase that facilitates the degradation of syntaxin 1, involved in the neurotransmitter release machinery. This result is agree with a study showing that ubiquitin ligases are upregulated in hippocampal sclerosis epilepsy samples [54]. Three more genes were hypomethylated in the SCEZ and amygdala: *MPPED1, RP11-560I19.4* and *PRKCZ. MPPED1* (metallophosphoesterase domain-containing protein) regulates the development and the function of cortical and hippocampal neurons through its metallophosphodiesterase activity [55]. *RP11-560I19.4* (FEZF1 antisense RNA 1), with an a yet unknown function, seems to be related to schizophrenia. *PRKCZ* encodes a kinase involved in long-term potentiation. Consistently, a previous study reported a deletion in this gene in epilepsy patients [56]. Moreover, *LINC02157* was also hypermethylated in the cortex and amygdala. However, it has not been associated with DRE yet.

Preceding studies have also investigated DMPs in peripheral blood, finding 216 DMPs associated with epilepsy in white blood cells. These DMPs harbor 130 genes [11].

Nevertheless, after applying the Houseman correction [29] to avoid spurious associations, no significant DMPs were associated with DR-TLE in the present study.

DNA methylation reflecting epilepsy-associated changes detected in the different brain regions analyzed (hippocampus, cortex, or amygdala) were not found in peripheral blood samples. This fact may be explained by the tissue and cell specificity of DNA methylation [35].

We found a potential peripheral biomarker cg26834418 (*CHORDC1*) that could reflect the methylation state found in the hippocampus. We observed a strong correlation between blood and brain of this cg26834418 using two independent bioinformatics tools (BECon [35] and IMAGE-CpG [36], Supplementary Fig 2). Cg26834418 is located on *CHORDC1* gene. *CHORDC1* (Cysteine and Histidine Rich Domain Containing 1) is involved in centrosome duplication, chaperone-mediated protein folding, and regulation of cellular response to heat. Surprisingly, this gene has not been related to epilepsy so far. Thus, this CpG could be proposed as a potential surrogate biomarker of DR-TLE. However, further research is needed to confirm this result in a larger cohort of patients.

Epigenetic biomarkers of aging were measured in the different brain tissue samples from DRE patients to determine the effects of epilepsy on brain aging. The predicted epigenetic age was like the chronological age for the hippocampus and cortex, thus ruling out a potential acceleration of aging in the epileptogenic zone. Although the mean age predicted of the amygdala and peripheral blood was higher than its chronological age, the difference between them was not significant.

Then, we explored DMP associated with clinical and demographic factors. We did not find any DMP associated with the presence of aura in any of the tissues analyzed. Moreover, we failed to observe any significant DMPs associated to smoke or alcohol consumption. Regarding the etiology we compared patients whose epilepsy was structural (N=5) with patients whose etiology is unknown (N=15). We found one DMP (cg17466567 (*ETV5, DGKG*)) associated with structural etiology both in the hippocampus and in the SCEZ. The same CpG was observed in two different tissues thus increasing the robustness of this result. *ETV5* (ETS Variant Transcription Factor 5) encodes a transcription factor involved in the generation of glial cells and in the expression of neuronal subtype-specific genes in newly differentiated neurons [57]. Interestingly, *ETV5* is downregulated in TLE [58].

The study presents several limitations. First, differential methylation results may be affected by the reduced sample size of some tissues such as the amygdala, due to the low frequency of these events. However, in this study, the brain samples have a high quality because they were frozen immediately after neurosurgical resection, and thus they were not subjected to fixation in formalin and the bias associated. Second, as postmortem brain donors' blood samples were not available, a different control cohort was included for blood samples. Third, we could not analyze the effect of ACs [16] or drug-sensitive epilepsy in the methylation due to the impossibility of finding brain donors with epilepsy not treated with ACs or sensitive to these treatments in the biobanks. Forth, the correction for cell type-specific effects on methylation could not be applied to brain samples because the different cell subpopulation could not be properly assessed in brain samples. Since the biomarker available for this determination (NeuN) is expressed only in a subpopulation of cells in the hippocampus this adjustment could introduce a new bias in

the study [59, 60] This may be the reason why it was also not applied in the previous EWAS in DRE [13]. Taken all together, although our results provide interesting insights into the role of methylation epilepsy, further analysis should be performed to validate the results.

In conclusion, we have unveiled different epigenetic patterns associated with DR-TLE in diverse brain regions (hippocampus, SCEZ, amygdala) and peripheral blood. Thus, we identified genes that had not been associated to DR-TLE so far such as *TBX5, EXOC7* and *WRHN*. For the first time, we analyzed the differential methylation in the amygdala, detecting an involvement of inflammation. We observed that genes encoding voltage-gated channels present epigenetic modifications even in the surrounding cortex to the epileptogenic zone. Moreover, we characterized a potential surrogate biomarker of DR-TLE in peripheral blood (*CHORDC1*). These results provide new insights into epigenetic modifications involved in drug-resistant DR-TLE that could be candidate therapeutic targets.

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DECLARATIONS

FUNDING

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ETHICAL DECLARATION

The protocol and the Informed Consent Form were approved by the Independent Clinical Research Ethics Committee of the Hospital Universitario de La Princesa. The study followed the STROBE guidelines and the Revised Declaration of Helsinki.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

CONSENT TO PUBLISH

This manuscript does not contain any individual person's data in any form (including any individual details, images, or videos).

DATA AVAILABILITY

All data produced in the present study are available upon reasonable request to the authors.

CONFLICT OF INTEREST

F Abad-Santos has been a consultant or investigator in clinical trials sponsored by the following pharmaceutical companies: Abbott, Alter, Chemo, Farmalíder, Ferrer, GlaxoSmithKline, Gilead, Janssen-Cilag, Kern, Normon, Novartis, Servier, Teva, and Zambon. AB Gago-Veiga has received honoraria as a consultant and speaker for: AbbVie-Allergan, Chiesi, Exeltis, Novartis, Eli Lilly, and Teva. MC Ovejero-Benito has potential conflicts of interest (honoraria for speaking and research support) with Janssen-Cilag and Leo Pharma. The rest of the authors have no relevant financial or non-financial interests to disclose.

CRedIT Author contributions

PSJ: Data curation, Formal analysis, Methodology, Validation, and Visualization; MEH and ASC: Data curation, Formal analysis, Methodology, and Visualization; IGC: Investigation and Resources; MdT, PP, MN, ABGV, MCAC, DNC, and FAS: Resources; LAG: Visualization and Methodology; CVTD: Resources, Project administration, Funding acquisition; MCOB: Conceptualization, Formal analysis, Data curation, Methodology, Investigation, Visualization, Project administration, Funding acquisition,

Supervision, and Writing - Original Draft. All authors have read, reviewed, and approved the final manuscript.

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FIGURES AND TABLES

FIGURES

Fig. 1: Workflow of the experiments and analysis carried out.

Abbreviations: DMPs: differentially methylated probes, DMR: differentially methylated regions; SCEZ: Surrounding cortex to the epileptogenic zone; * Due to the lack of blood samples available from control brain donors, these blood samples were obtained from another cohort of non-epileptic controls.

Fig. 2. Miami and volcano plots of differentially methylated probes (DMPs) associated with DR-TLE (drug resistant-temporal lobe epilepsy). Miami plot of the DMPs associated with DR-TLE in the different tissues analyzed: **A**) Hippocampus, (**C**) Amygdala, (**E**) Surrounding cortex to the epileptogenic zone. Genomic coordinates are displayed along the X-axis. These are a Miami plot version in with the top panel shows the DMPs with positive logarithm of fold change (logFC) and thus hypermethylated in patients with respect to controls, while the bottom panel shows the DMPs with negative fold change and thus hypomethylated in patients with respect to controls. The logarithm of the association p-value for each CpG site displayed along the Y-axis. Green dots represent significant DMPs FDR adjusted p value <0,05 CpGs were highlighted, and their gene names annotated when available. Dots without a gene name denote a CpG in an intergenic region. Volcano plots of the DMPs associated with DRE in the different tissues analyzed: (**B**) Hippocampus, (**C**) Amygdala, (**D**) Surrounding cortex to the epileptogenic zone. The logarithm of the association p-value for each CpG site is displayed along the Y-axis while the logarithm of fold change is shown along the X-axis. Green and red dots show DMPs hypomethylated and hypermethylated in patients with respect to controls, respectively. Blue dots show DMPs that do not have a significant association with DRE.

Fig. 3. A) Enrichment analysis performed with FUMA GWAS analyzing functions of the genes involved in DMPs in the hippocampus according to the GO molecular function library. Red bars represent the proportion of overlapping genes in the gene set. Blue bars show the enrichment p value, represented as the -logarithm of the FDR adjusted p value. Yellow squares show the genes involved in every enrichment term. Abbreviation: FDR: false discovery rate. B-E) Cytoscape networks of the top 4 largest clusters obtained with String. B) Cluster 1 includes 81 proteins involved in diverse functions such as actin filament organization, growth factors and kinases. C) Cluster 2 is composed by 23 proteins related to voltage-gated channel, and Transient receptor potential channels. D) Cluster 3 is made up of 20 proteins related to the spliceosome. E) Cluster 4 has 16 proteins involved in DNA repair.

Fig. 4. An example of a differentially methylated region of the hippocampus. Upper panel depicts coordinates in chromosome 3 (hg19). Orange squares represent the genes located in the chromosomic region shown. Green vertical lines show probes in the EPIC array. Differentially methylated region is shown in purple. Then, methylation values are shown for every control (green) or every patient (orange). Methylation values of every sample are shown in red or blue. Bottom panel shows methylation beta values, smoothed lines denote mean methylation levels for controls (C, forest green) and patients (T, orange). Each point represents the methylation level of a particular individual at a specific genomic location.

Table 1: Summary of the clinical and demographic characteristics of the study population and the controls.

		DR-TLE	Controls (brain)	Controls (blood)
Women (%)		8 (42%)	3 (30%)	3 (30%)
Age (years)		45 ± 9	60 ± 12	57 ± 9
Age at onset (years)		18± 15	NA	NA
Febrile seizures in childhood		2 (10%)	NA	NA
Number of seizures/month		7.6 ± 9.1	NA	NA
Aura		8 (42%)	NA	NA
nº anticonvulsant drugs		4.4 ± 1.9	NA	NA
Left dominant hand		3 (15%)	NA	NA
Stress		5 (26%)	NA	NA
Traumatism		2 (11%)	NA	NA
Sensory, gait, or developmental disturbances		3 (16%)	NA	NA
Neurosurgical hemisphere	Left	11 (58%)	NA	NA
	Right	8 (42%)	NA	NA
Seizure etiology	Structural	4 (21%)	NA	NA
	Infectious	2 (10%)	NA	NA
	Unknown	13 (69%)	NA	NA
Ever smoker		7 (37%)	NA	NA
Alcohol		7 (37%)	NA	NA
Engel (12 months)	R	16 (84%)	NA	NA
	NR	3 (16%)	NA	NA
Severe adverse effects (12m)		3 (16%)	NA	NA
Engel (24 months)	R	17 (89%)	NA	NA
	NR	2 (10%)	NA	NA
Severe adverse effects (24m)		4 (21%)	NA	NA

Data are shown as mean ± SD or number and percentage. As blood samples were not available from the subjects who donated their brains, control blood samples were taken from a different group of control subjects. Accordingly, two different cohorts of controls were used: control (blood) and control (brains)
Abbreviations: DRE, drug resistant epilepsy; NA, not applicable; NR, non-responders; R, responders.

Table 2: Summary of the top 5 hypomethylated and hypermethylated CpGs in different brain areas.

Tissue	Illumina CpG ID	Genomic elements included in the CpGs*	Genes associated to CpG	%Δβ	Name	Function and relevance
Hp	cg03935183	Body	*GATA3*	-3.55	GATA Binding Protein 3	Transcription factors involved in the differentiation of serotonergic neurons preventing epilepsy [41].
Hp	cg23820885	Body	*TBX5*	-2.33	T-Box Protein 5	Transcription factors involved in the regulation of developmental processes.
Hp	cg00552168		*EXOC7*	-1.75	Exocyst Complex Component 7	Involved in vesicular trafficking and the secretory pathway by targeting post-Golgi vesicles to the plasma membrane.
Hp	cg26834418	TSS1500	*CHORDC1*	-1.63	Cysteine And Histidine Rich Domain Containing 1	Involved in centrosome duplication; chaperone-mediated protein folding; and regulation of cellular response to heat.
Hp	cg06338245	Body	*NRP1*	-1.42	Neuropilin 1	Control cell migration. A mutation in this gene is associated with epilepsy [42].
Hp	cg16873016	5'UTR	*WHRN*	0.34	Whirlin	Stabilization of stereocilia elongation and actin cytoskeletal assembly.
Hp	cg08900101	Body	*TBX5*	0.51	T-Box Protein 5	Transcription factors involved in the regulation of developmental processes.
Hp	cg09623338	5'UTR	*TBX5*	0.64	T-Box Protein 5	Transcription factors involved in the regulation of developmental processes.
Hp	cg14321837	TSS1500	*UXS1*	0.66	UDP-Glucuronate Decarboxylase 1	Synthesis of UDP-xylose used in glycosaminoglycan (GAG) synthesis on proteoglycan.
Hp	cg20817902	Body	*PRDM8*	0.67	PR Domain Zinc Finger Protein 8	Histone methyltransferases that predominantly act as negative regulators of transcription. Associated with epilepsy [43].
Am	cg24742912	TSS1500	*MYBPH*	-5.78	Myosin Binding Protein H	Involved in regulation of striated muscle contraction. Downregulated in the hippocampus of an animal model of epilepsy [61].
Am	cg08217163		*AC007392.3, LINC01798*	-3.58	long intergenic non-protein coding RNA 1798	Remodeling chromatin and genome architecture.
Am	cg22884656		*RP11-89K21.1*	-3.51	long noncoding RNA	Remodeling chromatin and genome architecture.
Am	cg02442509		*RP11-89K21.1*	-1.76	long noncoding RNA	Remodeling chromatin and genome architecture.
Am	cg01847380	Body	*MYCL1*	0.94	MYCL Proto-Oncogene, BHLH Transcription Factor	DNA binding transcription factor.
Am	cg20528127	TSS1500; Body	*MRGPRG-AS1, C11orf36*	1.74	long noncoding RNA	Remodeling chromatin and genome architecture.
Am	cg06330169		*CXCL12*	3.09	C-X-C Motif Chemokine Ligand 12	Involved in immune surveillance, inflammation response and autoimmune epilepsy [45].
Am	cg17424452		*RP11-89K21.1*	3.26	long noncoding RNA	Remodeling chromatin and genome architecture.
Am	cg25806347		*RP11-89K21.1*	5.84	long noncoding RNA	Remodeling chromatin and genome architecture.

Tissue	Illumina CpG ID	Genomic elements included in the CpGs*	Genes associated to CpG	%Δβ	Name	Function and relevance
SCEZ	cg10957528	TSS1500	*TRA2A*	-12.40	Transformer 2 Alpha Homolog	Regulation of pre-mRNA splicing.
SCEZ	cg04958157	Body	*MCF2L*	-11.16	MCF.2 Cell Line Derived Transforming Sequence Like	Involved in Rho/Rac signaling pathways and upregulated in epilepsy [20]
SCEZ	cg01794805	5'UTR	*RARB*	-10.49	Retinoic Acid Receptor Beta	Nuclear transcriptional regulator.
SCEZ	cg04657768	Body	*JAKMIP3*	-9.21	Janus Kinase and Microtubule Interacting Protein 3	Enables kinase binding activity and microtubule binding activity.
SCEZ	cg00769112	Body	*EEF2*	-9.08	Eukaryotic Translation Elongation Factor 2	Encodes a member of the GTP-binding translation elongation factor family.
SCEZ	cg14585083			13.69		
SCEZ	cg01441865	Body	*ZFYVE28*	13.88	Zinc Finger FYVE-Type Containing 28	Enables phosphatidylinositol-3-phosphate binding activity. Involved in negative regulation of epidermal growth factor-activated receptor activity.
SCEZ	cg08505222	Body	*SHANK3*	14.43	Shank 3 And Multiple Ankyrin Repeat Domains 3	Multidomain scaffold proteins of the postsynaptic density that connect neurotransmitter receptors, ion channels, and other membrane proteins to the actin cytoskeleton and G-protein-coupled signaling pathways. Overexpressed in DRE [52].
SCEZ	cg14166863		*CEP250*	16.68	Centrosomal Protein 250	Involved in centriole-centriole cohesion during interphase of the cell cycle
SCEZ	cg16973535		*RP11-170M17.1*	17.01	long noncoding RNA	Remodeling chromatin and genome architecture

Probes hypomethylated in patients with respect to controls are shown in green. Probes hypermethylated in patients with respect to controls are shown in red.

* Body: Gene body; TSS1500: 1500 bp upstream of transcriptional start site (TSS): TSS200, 200bp upstream of TSS; UTR: untranslated region. %Δβ: Percentage of methylation differences between the drug resistant epilepsy patients and controls. Abbreviations: Hp: hippocampus, Am: amygdala; SCEZ: surrounding cortex to the epileptogenic zone. Gene functions were obtained from the dataset GeneCards (https://www.genecards.org/).

Table 3: Summary of hypomethylated and hypermethylated genes validation by MS-qPCR in the surrounding cortex to the epileptogenic zone.

Gene		**Drug resistant epilepsy Patients**	**Healthy Controls**	**Methylation Rate**
SHANK3	ΔΔCT Average	-33.35 ± 2.10	-32.92 ± 1.76	1.34 (1.06 – 1.71)
	Fold Change	$1.09 \cdot 10^{10}$ ($2.54 \cdot 10^{09} – 4.69 \cdot 10^{10}$)	$8.12 \cdot 10^{09}$ ($2.39 \cdot 10^{09} – 2.75 \cdot 10^{10}$)	
SBF1	ΔΔCT Average	-38.50 ± 1.61	-33.77 ± 1.63	26.71 (27.19 – 26.23)
	Fold Change	$2.90 \cdot 10^{11}$ ($1.28 \cdot 10^{11} – 1.19 \cdot 10^{12}$)	1.46·1010 ($4.71 \cdot 10^{09} – 4.54 \cdot 10^{10}$)	
MCF2L	ΔΔCT Average	-30.01 ± 1.31	-31.09 ± 2.13	0.47 (0.84 – 0.27)
	Fold Change	$1.08 \cdot 10^{09}$ ($4.37 \cdot 10^{08} – 2.68 \cdot 10^{09}$)	$2.29 \cdot 10^{09}$ ($5.22 \cdot 10^{08} – 1.01 \cdot 10^{10}$)	

Data are shown as mean ± SD or fold-change and its range for each gene. The fold-change range was calculated as $2^{-(\Delta\Delta CT - SD)}$ and $2^{-(\Delta\Delta CT + SD)}$. Methylation rates were calculated as a ratio between patients and controls fold-change. The results were expressed as the fold change and 95% confidence interval.
As there was a fold-change range, methylation rate had a confidence range. SD: standard deviation.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLES

Supplementary Table 1. Sequence of primers and probes designed for methylation-specific qPCR.

Supplementary Table 2. Significant differentially methylated probes associated with drug resistant epilepsy in different tissues. A) Hippocampus, B) Amygdala, C) Surrounding cortex to the epileptogenic zone. Probe location and the gene annotation were taken from Illumina reference files.
* Body: Gene body; TSS1500: 1500 bp upstream of transcriptional start site (TSS): TSS200, 200bp upstream of TSS; UTR: untranslated region. %Δβ: Percentage of methylation differences between the drug resistant epilepsy patients and controls. chr: chromosome; FDR: false discovery rate.
Probes hypomethylated in patients with respect to controls are shown in green. Probes hypermethylated in patients with respect to controls are shown in red.

Supplementary Table 3. Enrichr of the significant differentially methylated probes associated with drug resistant-temporal lobe epilepsy in different tissues. A) Hippocampus, B) Amygdala, C) Surrounding cortex to the epileptogenic zone.

Supplementary Table 4. Definition of the main clusters created by Cytoscape. Cluster 1 includes 81 proteins involved in functions such as actin filament organization, growth factors and kinases. Cluster 2 is composed by 23 proteins Voltage-gated channel, and Transient receptor potential channels. Cluster 3 is formed by 20 proteins related to the spliceosome. Cluster 4 has 16 proteins involved in DNA repair. Proteins included in cluster 1 are shown in orange, cluster 2 in blue, cluster 3 in green and cluster 4 in yellow.

Supplementary Table 5. Significant differentially methylated regions found in the different tissues (A-C). A) Hippocampus, B) Amygdala, C) Surrounding cortex to the epileptogenic zone. D) Enrichr of the genes located on the differentially methylated regions located in the surrounding cortex to the epileptogenic zone.
Abbreviations: chr: chromosome; HMFDR: harmonic mean of the individual; meandiff: Mean differences in DNA methylation (%) between patients and controls are shown as a measurement of the effect size. Fisher <0.05 is considered significant.

Supplementary Table 6. A) Number of samples from the different tissues available for the different clinical and demographic factors studied. B) Significant differentially methylated probes associated with structural etiology in different tissues.
* Body: Gene body; TSS1500: 1500 bp upstream of transcriptional start site (TSS): TSS200, 200bp upstream of TSS; UTR: untranslated region. %Δβ: Percentage of methylation differences between the drug resistant epilepsy patients and controls. chr: chromosome; FDR: false discovery rate.

SUPPLEMENTARY FIGURES

Supplementary Fig. 1. Enrichment analysis performed with FUMA GWAS analyzing functions of the genes involved in DMPs in Cortical surrounding zone of patients compared with cortex of healthy controls. A) Biocarta. B) KEGG pathways. Red bars represent the proportion of overlapping genes in gene set. Blue bars show the enrichment p value, represented as the -logarithm of the FDR adjusted p value. Yellow squares show the genes involved in every enrichment term. Abbreviation: FDR: false discovery rate.

Supplementary Fig. 2. A) Metrics of the brain-blood correlation of the 32 differentially methylated probes observed in the hippocampus by BECon. B) Inter-individual variability of cg26834418 identified previously in blood-based studies of psychiatric disorders by BECon. C) Blood brain correlation of cg26834418 observed with IMAGE-CpG.

Supplementary Fig. 3. An example of a differentially methylated region of the amygdala. Upper panel depicts coordinates in chromosome 3 (hg19). Orange squares represent the genes located in the chromosomic region shown. Green vertical lines show probes in the EPIC array. Differentially methylated region is shown in purple. Then, methylation values are shown for every control (green) or every patient (orange). Methylation values of every sample are shown in red or blue. Bottom panel shows methylation beta values, smoothed lines denote mean methylation levels for controls (C, forest green) and patients (T, orange). Each point represents the methylation level of a particular individual at a specific genomic location.

Supplementary Fig. 4. An example of a differentially methylated region of the surrounding cortex to the epileptogenic zone. Upper panel depicts coordinates in chromosome 3 (hg19). Orange squares represent the genes located in the chromosomic region shown. Green vertical lines show probes in the EPIC array. Differentially methylated region is shown in purple. Then, methylation values are shown for every control (green) or every patient (orange). Methylation values of every sample are shown in red or blue. Bottom panel shows methylation beta values, smoothed lines denote mean methylation levels for controls (C, forest green) and patients (T, orange). Each point represents the methylation level of a particular individual at a specific genomic location.

Supplementary Fig. 5. Correlation between drug resistant patients' real age and predicted age by the epigenetic clock in the different tissues. A) Hippocampus, B) Amygdala, C) Surrounding cortex to the epileptogenic zone, D) Peripheral blood after adjusting by the different cell types**.** *$p<0.05$