DNA Methylation of *POMC* and *NR3C1-1F* and Its Implication in Major Depressive Disorder and Electroconvulsive Therapy

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Key words

electroconvulsive therapy, epigenetics, DNA methylation, proopiomelanocortin, NR3C1, hypothalamic-pituitary-adrenal axis

received 13.06.2022
revised 27.12.2022
accepted 06.02.2023

Bibliography

Pharmacopsychiatry 2023; 56: 64–72
DOI 10.1055/a-2034-6536
ISSN 0176-3679

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Supplementary Material is available under https://doi.org/10.1055/a-2034-6536

ABSTRACT

Introduction Precision medicine in psychiatry is still in its infancy. To establish patient-tailored treatment, adequate indicators predicting treatment response are required. Electroconvulsive therapy (ECT) is considered one of the most effective options for pharmacoresistant major depressive disorder (MDD), yet remission rates were reported to be below 50 %.

Methods Since epigenetics of the stress response system seem to play a role in MDD, we analyzed the DNA methylation (DNAm) of genes encoding the glucocorticoid receptor (*NR3C1*) and proopiomelanocortin (*POMC*) through Sanger Sequencing. For analysis, blood was taken before and after the first and last ECT from MDD patients (n = 31), unmedicated depressed controls (UDC; n = 19, baseline), and healthy controls (HC; n = 20, baseline).

Results Baseline DNAm in *NR3C1* was significantly lower in UDCs compared to both other groups (UDC: 0.014(± 0.002), ECT: 0.031(± 0.001), HC: 0.024(± 0.002); p < 0.001), whereas regarding *POMC*, ECT patients had the highest DNAm levels (ECT: 0.252(± 0.013), UDC: 0.156(± 0.015), HC: 0.162(± 0.014); p < 0.001). *NR3C1*m and *POMC*m decreased after the first ECT (*NR3C1*: p < 0.001; *POMC*: p = 0.001), and responders were less methylated compared to non-responders in *NR3C1*(p < 0.001).

Discussion Our findings indicate that both genes might play a role in the chronification of depression and *NR3C1* may be relevant for ECT response prediction.

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Introduction

Electroconvulsive therapy (ECT) is considered one of the most effective therapy options for difficult-to-treat major depressive disorder (MDD). Nonetheless, large-scale population-based studies show remission rates below 50 % [1, 2]. A few clinical patient characteristics, such as psychotic features and older age, seem to play a role in ECT outcome, suggesting that a subgroup of depressed individuals is particularly sensitive to ECT. However, considering the heterogeneity of MDD, a full range of markers (incorporating clinical and biological data) is needed to provide a reliable basis for treatment decisions.

Traumatic or stressful life events may trigger the onset of MDD and, in a subtype of MDD, may cause dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis [3–5]. During a single ECT, the HPA axis was shown to be activated, and abberantly increased hormones such as catecholamines or cortisol normalize within one hour post-ECT [6, 7]. Epigenetic mechanisms, e. g., DNA methylation (DNAm), might play a role in this phenomenon, as they likely regulate gene expression and thus, protein production [8]. The nuclear receptor subfamily 3 group C member 1 (*NR3C1*) gene encodes the human glucocorticoid receptor and is thus a fundamental component of our stress response system, i. e., the HPA axis [9]. The epigenetic regulation of *NR3C1*, particularly its exon 1 F (*NR3C1-1F*), has been extensively studied and repeatedly linked to depression, childhood-maltreatment, and other stress-related processes [10, 11]. Yehuda et al. [12, 13], for example, found initially lowered mean *NR3C1-1F* promoter DNAm in veterans diagnosed with posttraumatic stress disorder (PTSD), which correlated negatively with PTSD symptom severity after psychotherapeutic treatment. In a group of female adolescents, DNAm of four stress-reguating genes (including *NR3C1*) was reported to be predictive for the development of MDD, and, thus, may be therefore interpreted as a direct link between stress sensitivity, depression, and epigenetics [14].

Altered HPA axis hormones such as corticotropin-releasing hormone, adrenocorticotropic hormone (ACTH), and cortisol are indicators pointing towards lowered stress resilience in depressed individuals [15–19]. Transcription of the gene coding for proopiomelanocortin (POMC) – a pituitary prohormone of ACTH – plays a role in various somatic (e. g., tumors [20], metabolic syndrome [21]) as well as mental illnesses (e. g., alcohol dependence [22, 23], heroin addiction [24], or eating disorders [25]). In rodents, early life stress led to HPA axis hyperactivity and depression-like behavior associated with persistent hypomethylation in the distal promoter region of *Pomc* [26]. Further, DNAm of *POMC* (*POMC*m) was increased in depressed adolescent patients with non-suicidal self-injury behaviors compared to healthy controls [27].

The aim of our study was to (a) investigate putative differences in DNAm of *POMC* and *NR3C1-1F* between patients undergoing ECT (ECT group), severely depressed patients without antidepressant treatment at baseline (unmedicated depressed controls; UDC), and healthy controls (HC), (b) assess differences in DNAm of ECT responders (ECT-R) and non-responders (ECT-NR), and (c) examine whether DNAm of *POMC* and *NR3C1-1F* changes during a course of ECT to evaluate differences in outcome groups in terms of acute and long-term mechanisms.

Materials and Methods

Study Design

In this exploratory study, we set out to analyze a cohort from the Northern German Electroconvulsive Therapy Outcome Registry (Norddeutsches Elektrokonvulsionstherapie Outcome Register [NEKTOR]; ECT, n = 31). For group comparisons, we additionally recruited MDD patients without current antidepressant treatment (UDC, n = 19) and a cohort of healthy controls (HC, n = 20). All patients were inpatients at the Department of Psychiatry, Social Psychiatry, and Psychotherapy at Hannover Medical School (Germany) at the time of study inclusion. The study was approved by the Ethics Committee of Hannover Medical School (2842–2015) and followed the ethical principles of the Declaration of Helsinki (1964) and its later amendments. All participants provided written informed consent prior to study inclusion.

Patients

Patients with a diagnosis of MDD (according to the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM IV)) and a scheduled ECT series were enrolled in NEKTOR. Depression severity was assessed at baseline and after the last ECT, using the Beck Depression Inventory-II (BDI-II) and the Montgomery Åsberg Depression Rating Scale (MADRS). The response was defined as a ≥ 50 % reduction in MADRS score.

UDC subjects not undergoing any sort of antidepressant therapy were examined shortly after hospitalization. Depression severity was likewise assessed at baseline.

HC and UDC were matched with the ECT group for gender, body mass index (BMI), and smoking status.

Application of ECT

NEKTOR patients received ECT treatment three times weekly for up to 10 (± 4) treatments. The brief-pulse Thymatron System IV (Somatics) was used for stimulation, and the intensity was determined by the age-based method and right unilateral electrode placement. Methohexital or propofol, remifentanil, and succinylcholine or mivacurium were used for anesthesia and muscle relaxation. ECT seizure duration was monitored with an electroencephalogram with two frontomastoidal channels. A blood pressure cuff was inflated on the right lower leg before muscle relaxation to record motor seizure activity.

Sample Collection and Processing

Fasting blood was withdrawn directly before administration of anesthesia (08:00 am) and 15 min after the first and last ECT (between 09:00–10:00 am). For UDC and HC, fasting blood samples were collected at baseline between 8 and 9 am. After collection, blood monovettes were kept at 4°C for a maximum of 3 h until peripheral blood mononuclear cells (PBMCs) were isolated.

Isolation of peripheral blood mononuclear cells

PBMCs were isolated by gradient centrifugation, as described in an earlier study [28]. After isolation, cells were stored directly in liquid nitrogen until further processing.

Genomic DNA isolation

After thawing blood samples (see [28]), genomic DNA (gDNA) was isolated using the Qiagen AllPrep 96 DNA/RNA Kit. Minor modifications were made to the manufacturer's protocol. Briefly, PBMCs stored in 500 µL Qiagen RNAprotect Cell reagent for a maximum of one week at 4°C were centrifuged at 5000 xg at 4°C for 5 min prior to gDNA isolation. The supernatant was discarded, and 300 µL of RT buffer (containing 1 % β-mercaptoethanol) was added to each sample. After vortexing (2 × 30 s, 4°C), PBMCs were stored on ice for 15 min to obtain full cell lysis. The lysates were pipetted into an AllPrep DNAplate, which was centrifuged (5600 xg, 4 min, room temperature) and washed three times with AW buffer (1 x AW1, 2 x AW2). To elute the gDNA, samples were incubated twice with 50 µL of heated EB buffer (70°C) for 5 min. The eluates were kept at − 80°C until further processing.

Bisulfite Sequencing

Bisulfite conversion

Bisulfite conversion of gDNA was performed using the QiagenEpiTect 96 Bisulfite Kit. Bisulfite-converted DNA (bisDNA) was stored at − 20°C or used directly for amplification.

DNA amplification

Primers for the regions of interest were designed using the following software and online tools: Methyl Primer Express V1.0, Geneious Pro5.6.7., Ensembl†, SNPCheck V3‡, NetPrimer§, Metabion Biocalculator**, and Match 1.0††. The sequences of interest were selected based on current literature ([12, 13, 26, 29–35]; **Fig. S1, S2**). Primer sequences are listed in **Table S1** in the **supplemental material**. For amplification, 1 µL of bisDNA was mixed with 5 µL HotStarTaq Master Mix, 3.2 µL RNase free water, and 0.4 µL of the forward and reverse primer (manufactured by MetabionGmbH), respectively. For the polymerase chain reaction (PCR), the temperature profiles were used according to **Tables S2A** and **S4A ,B** in the supplements. The resulting amplification products were cleaned by a Biomek NXp pipetting robot using CleanPCR magnetic beads according to the instructions of the Agencourt Beckman Coulter AMPureXPKit. Fragment sizes were determined by 1 % agarose gel electrophoresis. Amplified PCR products were stored at 4°C for up to two weeks until Sanger sequencing.

Sanger sequencing

Sanger sequencing was performed as described elsewhere [36]. PCR for sequencing was performed according to **Tables S2B, S4B**. The same quality measures (> 20 quality value (QV) and continuous read length close to expected fragment size) as in [36] were applied.

† www.ensembl.org last accessed:02.10.2019, 6.29 p.m.

‡ https://genetools.org/SNPCheck/snpcheck.htm last accessed: 02.10.2019, 6.31 p.m.

§ http://www.premierbiosoft.com/netprimer/ last accessed:02.10.2019, 6.33 p.m.

** http://www.metabion.com/support-and-solution/biocalculator/ last accessed:02.10.2019, 6.34 p.m.

†† http://gene-regulation.com/ last accessed:02.10.2019, 6.54 p.m.

Data Processing

Statistical analysis

For baseline demographics, Fisher's exact test (FET) was used for nominal scaled variables (e. g., smoking behavior). In addition, baseline differences between the three groups (ECT, UDC, and HC) and between ECT-R and ECT-NR were analyzed using FET. Metric data (e. g., BMI) were analyzed using the Mann-Whitney-U-Test or the Kruskal-Wallis-Test with Dunn's post hoc correction (KWT), as appropriate. Results are reported as mean ± standard deviation for baseline demographic data or ± standard error (SE) for methylation data. In this explorative study, a nominal p-value of ≤ 0.05 (two-tailed) was considered significant.

CpG methylation

Forty-three single CpG sites in *NR3C1* and 28 in *POMC* were analyzed by bisulfite sequencing. At the outset, quality control was performed to exclude potentially unreliable reads: first, sequencing quality was checked using the ABI sequence scanner (Applied-Biosystems); samples with a QV < 20 were reanalyzed. CpG sites with > 5 % missing values were excluded from the analysis (none in *NR3C1;* 11 out of 28 CpG sites in *POMC*). Five participants had > 5 % missing values for *POMC* and were thus excluded from *POMC* analysis, leaving 65 study participants (*NR3C1*: ECT n = 31, UDC n = 19, HC n = 20; *POMC*: ECT n = 28, UDC n = 17, HC n = 20). Only CpGs with an interindividual variance > 0.01 were included in our analysis, a criterion which all remaining *POMC* CpG sites met, but two *NR3C1* CpG sites were excluded (finally, 41 remained for analysis). After the initial ECT, two patients were eliminated from the longitudinal study in the ECT group for *POMC*, and two other patients were omitted for having > 5 % missing CpG values after baseline (*POMC*: baseline n = 29, after first ECT n = 25, before last ECT n = 18, after last ECT n = 18; *NR3C1*: baseline n = 31, after first ECT n = 29, before last ECT n = 20, after last ECT n = 20).

Model 1 in all groups

Mixed linear modeling (MLM; residual maximum likelihood approach) was used with age, BMI, and smoking status as a covariate. A scaled identity covariance structure was assumed. We analyzed the group difference at baseline: DNAm was calculated as the dependent variable; group (ECT vs. UDC vs. HC), CpG site, and age were included as fixed effects, as were the interaction group and the CpG site. The CpG site was entered as a repeated measure. Additionally, we calculated the baseline difference in ECT-R/ECT-NR and the two control groups. This model was run separately for both genes.

Model 2 only in the ECT group

To test whether ECT has an acute/chronic effect on DNAm and whether ECT-R and ECT-NR differed at different time points, a second model was carried out separately for both genes. DNAm was again set as a dependent variable. Age, BMI, and smoking status were used as covariates. To test the effect of the outcome (ECT-R vs. ECT-NR), acute effect (before and after a single ECT), chronic effect (before the first and before the last ECT), and their interaction, another model was calculated that included the mentioned effects as fixed effects and the CpG site as a repeated measure. As in model 1, a scaled identity covariance was assumed.

▶ **Table 1** Clinical baseline characteristics of patients with major depressive disorder (MDD) treated with electroconvulsive therapy (ECT) (ECT; ECT responders vs. non-responders), of unmedicated depressed controls (UDC) and of healthy controls (HC), presented as mean (± standard deviation, SD) or quantity (absolute and percentage, n (%)). Baseline characteristics did not differ between groups except for suicidality which was higher in UDC when compared to ECT (** Fisher's Exact Test $p < 0.01$) and age (ECT group was significantly older, * Kruskal-Wallis-Test with Dunn-Bonferroni-correction $p < 0.05$). [1]Pharmacoresistance: non-responsiveness to two state-of-the-art antidepressants (after two weeks of treatment with adequate dosages, respectively); BDI-II = Beck's Depression Inventory; MADRS = Montgomery-Åsberg Depression Rating Scale; MMSE = Mini-Mental State Examination.

		ECT (n = 31)			UDC (n = 19)	HC (n = 20)
		Whole cohort	Responders (n = 15)	Non-responders (n = 16)		
Demographics						
Age in years, mean (± SD)		55*(± 16)	60 (± 13)	51 (± 18)	42* (± 14)	44*(± 15)
Gender, n (%)	Female	20 (64.5)	10 (66.7)	10 (62.5)	11 (57.9)	12 (60.0)
	Male	11 (35.5)	5 (33.3)	6 (37.5)	8 (42.10)	8 (40.0)
Body-mass-index, mean (± SD)		22.31 (± 4.56)	22.3 (± 4.56)	22.31 (± 4.73)	22.59 (± 3.63)	22.41 (± 5.69)
Smoking, n (%)	Yes	12 (41.4)	6 (46.2)	6 (37.5)	6 (31.6)	2 (10.0)
Alcohol consumption, n (%)	Yes	2 (6.5)	1 (6.7)	1 (6.3)	3 (15.8)	1 (5.0)
	Occasionally	0 (0.0)	0 (0.0)	0 (0.0)	12 (63.2)	16 (80.0)
Marital status, n (%)	Single	6 (23.1)	3 (27.3)	3 (20.0)	10 (52.6)	6 (30.0)
	Married	15 (57.7)	7 (64.6)	8 (53.3)	7 (36.8)	14 (70.0)
	Divorced	4 (15.4)	1 (9.1)	3 (20.0)	2 (10.5)	0 (0.0)
	Widowed	1 (3.8)	0 (0.0)	1 (6.7)	0 (0.0)	0 (0.0)
Education, n (%)	None	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Secondary School	5 (20.0)	4 (40.0)	8 (53.3)	8 (42.1)	9 (45.0)
	A levels	11 (44.0)	6 (60.0)	7 (46.7)	7 (36.8)	11 (55.0)
Living situation, n (%)	Alone	6 (24.0)	3 (27.3)	3 (21.4)	6 (31.6)	6 (30.0)
	With partner / family	18 (72.0)	7 (63.6)	11 (78.6)	13 (68.4)	14 (70.0)
	Outpatient care	1 (4.0)	1 (9.1)	0 (0.0)	0 (0.0)	0 (0.0)
Age at diagnosis in years, mean (± SD)		33 (± 15)	31 (± 14)	34 (± 17)	40 (± 16)	–
Current episode in weeks, mean (± SD)		42 (± 37)	39 (± 42)	46 (± 32)	27 (± 43)	–
BDI-II, mean (± SD)		36 (± 12)	35 (± 12)	37 (± 12)	27 (± 10)	2 (± 2)
MADRS, mean (± SD)		33 (± 9)	35 (± 11)	31 (± 7)	31 (± 10)	0 (± 0)
MMSE, mean (± SD)		28 (± 4)	26 (± 6)	29 (± 2)	39 (± 2)	30 (± 1)
Psychotic symptoms, n (%)	Yes	9 (± 29.0)	5 (33.4)	4 (25.0)	0 (0.0)	–
Suicidality, n (%)	Yes	8 (25.8)**	3 (20.0)	5 (31.3)	14 (73.7)**	–
Pharmacoresistance, n (%)[1]	Yes	24 (± 77.4)	14 (93.3)	10 (62.5)	–	–
Psychotherapy, n (%)	Yes	18 (± 64.3)	9 (64.3)	9 (64.3)	13 (68.4)	–
Antidepressant drugs, n (%)	Yes	29 (± 93.5)	14 (93.3)	15 (93.8)	–	–
Benzodiazepines, n (%)	Yes	18 (± 58.0)	10 (66.7)	8 (50.0)	–	–
Antipsychotic drugs, n (%)	Yes	22 (71.0)	12 (80.0)	10 (62.5)	–	–
Lithium, n (%)	Yes	5 (16.1)	1 (6.7)	4 (25.0)	–	–
Cortisol (µg/dL)		14.68 (± 6.84)	15.75(± 8.12)	13.68 (± 5.53)	14.24 (± 3.86)	–

In both models, estimated marginal means were compared by Bonferroni-corrected post hoc tests. Parameter estimates were computed for all factors and manually inspected. To compare model fits, the −2log likelihood ratio was used. Type 1 errors were only corrected for the mixed linear models (Bonferroni correction).

For all statistical analyses, Statistical Package for the Social Science (version 26 for Windows, IBM) was used. For data display, Graph Pad Prism 6 (GraphPad Inc.) was used.

Results

Baseline Characteristics

The ECT group was older than the other two groups (KWT: ECT vs. UDC: $z = 2.590$, $p = 0.029$; ECT vs. HC: $z = 2.446$, $p = 0.043$; UDC vs. HC: $z = -0.166$, $p = 1.000$). Suicidal ideation occurred more frequently in UDC patients than in ECT patients. Other baseline parameters showed no significant differences between groups (▶ **Table 1**).

Baseline differences of demographics and clinical characteristics in ECT-R vs. ECT-NR

In the ECT group, we found no significant difference between ECT-R and ECT-NR in smoking, alcohol use, gender, education, marital status, antidepressant use, treatment resistance, diagnoses leading to ECT, patients receiving ECT for the first time, psychotherapy history, suicidal ideation, substance use, and personality disorder (FET: all $p > 0.05$). There was a trend toward comorbid anxiety disorder in ECT-NR (FET: $p = 0.054$), as previously reported in the literature.

Mean *NR3C1* DNAm at baseline

Baseline DNAm in *NR3C1* showed a significant difference between groups, with the UDC group having the lowest methylation levels compared to the other two groups (corrected for age, BMI, and smoking status; estimated marginal means (EMM)(± SE): UDC: 0.014(± 0.002), ECT: 0.031(± 0.001), HC: 0.024(± 0.002); $p < 0.001$) (▶ **Fig. 1**). We found the same results when comparing ECT-R and ECT-NR separately with the control groups (ECT-R: 0.028(± 0.002), ECT-NR: 0.040(± 0.002); all $p < 0.001$). In the ECT group, the mean *NR3C1* DNAm (*NR3C1*m) rate correlated positively with age at diagnosis ($r = 0.448$, $p = 0.028$). There was a significant difference between ECT-R and ECT-NR ($F(1, 3559) = 28.574$, $p < 0.001$; ▶ **Fig. 2**).

Separate analysis of distinct regions in *NR3C1* at baseline

Applying MLM, we found that the CpG site had an effect on baseline *NR3C1*m. Therefore, distinct regions within the *NR3C1* promoter and exon were analyzed separately. Results are presented in **Tables S3**, **S6**.

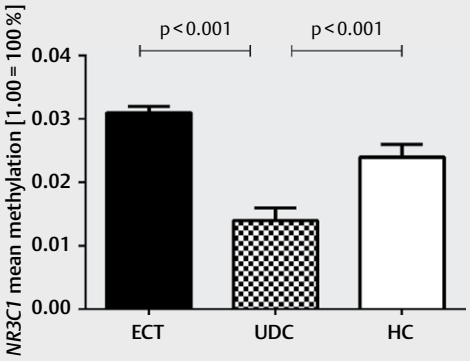
*NR3C1*m changes over time in ECT-R and ECT-NR

MLM revealed an acute effect of ECT (when measured before and directly after a single ECT session; $F(1,4292) = 12.442$, $p < 0.001$) and an effect of ECT number (meaning first or last ECT; ($F(1,3563) = 15.248$, $p < 0.001$)) on *NR3C1* when ECT-R and ECT-NR were analyzed together. There was no interaction between the ECT number and the acute effect ($F(1,3559) = 0.574$, $p = 0.449$). Our analysis revealed that the acute effect was mainly driven by the group of NR who had lower methylation after ECT ($F(1,3559) = 16.328$, $p < 0.001$) as well as over the entire ECT course compared to baseline (response x ECT number: ($F(1,3559) = 46.664$, $p < 0.001$; ▶ **Fig. 2**).

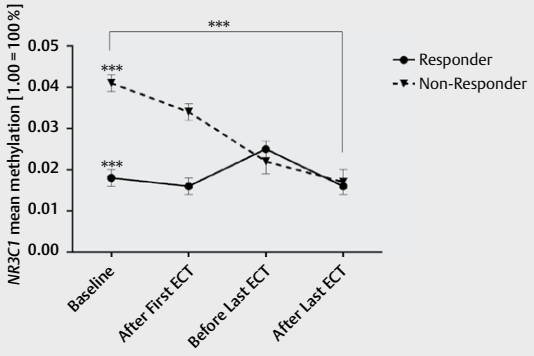
POMC DNAm at baseline

At baseline, ECT patients showed higher DNAm levels compared with UDC and HC (EMM(± SE): ECT: 0.252(± 0.013), UDC: 0.156 (± 0.015), HC: 0.162(± 0.014); ▶ **Fig. 3**).

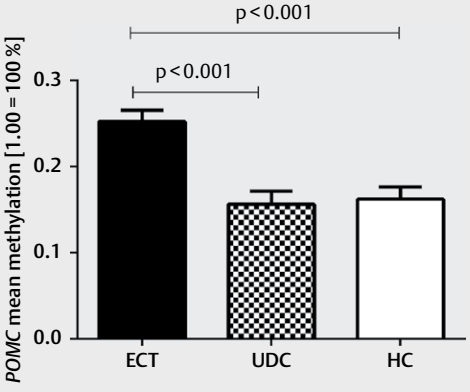
However, ECT-NR showed a significantly higher baseline *POMC*m when compared to the control groups (EMM(± SE): NR = 0.279(± 0.015); UDC = 0.162(± 0.013); HC = 0.168(± 0.012); MLM $p < 0.001$), whereas ECT-R just barely missed statistical significance (R: 0.215(± 0.018), $F(2,811) = 2.909$, $p = 0.055$). ECT-R and ECT-NR did not differ significantly in DNAm in the entire *POMC* gene (EMM(± SE): R: 0.219(± 0.019), NR: 0.266(± 0.0019); MLM $p = 0.110$).



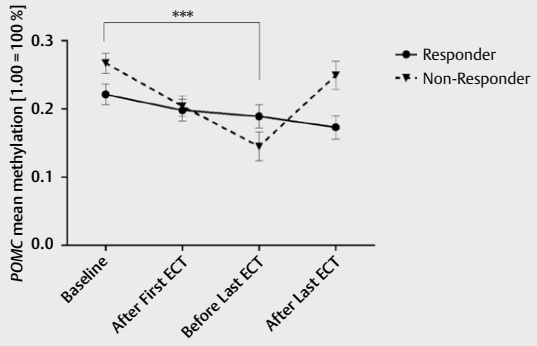
▶ **Fig. 1** Baseline differences in *NR3C1* mean methylation between the ECT group, UDC and HC. At baseline, the UDC group had lower *NR3C1*m when compared to ECT or HC. Error bars show the standard error of the mean. P-values are derived from mixed linear modeling.



▶ **Fig. 2** Time course of *NR3C1*m depending on outcome after a course of ECT. Responders and non-responders differed at baseline (***$p < 0.001$). Error bars show the standard error of the mean. P-value is derived from mixed linear modeling.



▶ **Fig. 3** Baseline differences in mean *POMC*m between the ECT group, UDC and HC. At baseline, the ECT group had a significantly higher mean *POMC*m promoter when compared to UDC or HC. Error bars show the standard error of the mean. P-values are derived from mixed linear modeling.



▶ **Fig. 4** Time course of *POMC*m depending on outcome after a course of ECT. Responders and non-responders are shown. The methylation rate decreased after the first ECT and in the case of response upon the last ECT. Error bars show the standard error of the mean. P-value derived from mixed linear modeling (***p < 0.001).

Distinct regions in *POMC*

CpG sites showed a significant interaction with *POMC*m ($F(16,983) = 16.197$, $p < 0.001$). We also found a significant interaction between the CpG site and group ($F(32,983) = 2.371$, $p < 0.001$). The differences in methylation at single CpG sites are shown in **Tables S5, S7**.

DNAm changes over time in ECT-R and ECT-NR (*POMC*)

According to ▶ **Fig. 4**, MLM revealed a significant decrease in DNAm over the course of ECT ($F(1, 1265) = 7.598$, $p = 0.006$; EMM (± SE): first ECT = 0.223(± 0.008), last ECT = 0.189(± 0.009); before/after x ECT number: $F(1,1265) = 13.128$, $p < 0.001$). Additionally, the CpG site showed an effect on DNAm ($F(16,1265) = 27.491$, $p < 0.001$). ECT-R and ECT-NR did not differ at baseline ($F(1,387) = 3.367$, $p = 0.067$) but showed a decrease in DNAm from baseline, with ECT-R having a pronounced dip after the last ECT (response x timepoint: $F(3,1265) = 4.078$, $p = 0.007$; EMM(± SE): ECT-R at baseline: 0.221(± 0.015), ECT-R after last ECT: 0.173(± 0.017); ECT-NR at baseline: 0.267(± 0.015), ECT-NR after last ECT : 0.249(± 0.021)).

Discussion

The main finding of the present study is the altered DNAm status in *NR3C1* and *POMC* in the different groups analyzed. Regarding *NR3C1*, UDCs showed the lowest mean methylation rate compared with HCs and ECT patients, with ECT-NR having higher *NR3C1*m than ECT-R. This finding is particularly interesting because *NR3C1*m seems to play a role in MDD (and stress), although the direction of its effects (in terms of positive or negative correlation) is partially contradictory [9, 37]. However, depression was mostly associated with hypermethylation, whereas alcohol consumption, overweight, and high cortisol levels were associated with low or absent methylation [23]. As for our results, we can only speculate why UDC patients had the lowest *NR3C1*m. Altered (mostly increased) *NR3C1*m has previously been associated with a negative social environment in early childhood [11]. Since childhood maltreatment has been associated with MDD severity and treatment resistance [38], one might speculate that there were fewer patients with such experiences in the UDC group than in the ECT sample – or, more precisely – in ECT-NR [39]. However, the DNAm of HC and ECT patients did not differ significantly: in this case, we lacked data to test our hypothesis because participants were not asked about early life experiences. In the ECT group, the DNAm could be a sign of long-term illness, especially since baseline DNAm was positively correlated with age at diagnosis. Regarding treatment outcome, *NR3C1*m is suggested to relate to ECT response as ECT-NR had a higher mean DNAm rate than ECT-R independent of the region analyzed (all CpG sites vs. promoter only). In line with our results, a methylome-wide association study suggested that *NR3C1* plays a role in ECT response, but the finding did not survive correction for multiple testing. Additionally, the respective CpG site does not correspond to any of the CpG site analyzed in our study [40].

Epigenetic marks are suggested to be relatively stable, but to meet environmental demands, plasticity is transiently heightened even in an adult brain [10, 41]. In our study, we provide additional evidence that *NR3C1*m can be altered in the short and long-term by environmental cues, in this case, ECT (▶ **Fig. 2**). After one ECT session, *NR3C1*m levels decreased significantly (mainly driven by ECT-NR), but in the long-term (comparing DNAm directly before the first and last ECT), our results did not reach statistical significance.

After the last ECT, *NR3C1*m did not differ between ECT-R and -NR, leading to the conclusion that methylation rates of NR decreased during the ECT sessions, aligning with those of ECT-R. One could speculate that these patients might benefit from further ECT sessions, as they might need the first few ECTs to adjust before reaching a response. On the other hand, a response might not be achieved in ECT-NR because other crucial factors need to be fulfilled for ECT to be effective. Nevertheless, *NR3C1*m could be viewed as an epigenetic signature of ECT-NR.

As for *POMC*, ECT patients had the highest mean baseline DNAm rate compared with the other two groups. Our baseline results are in contrast to the rodent study by Wu et al. [26], who found hypomethylated regions within the promoter region of exon 1 after early life stress. These changes persisted for up to one year and were thought to contribute to susceptibility to depression or anxiety disorders [26]. However, it should be noted that Wu et al. [26] used male mouse pituitary glands, in contrast to our study in which we analyzed PBMCs and both sexes. Methylation patterns are known to be tissue-specific, and epigenetic changes in the blood do not necessarily reflect changes in the brain. Nevertheless, there is evidence that at least some intraindividual DNAm signatures correlate between PBMCs and different brain regions [42]. Higher methylated *POMC* was observed in a group of children who were regularly abused [43], as well as in adolescent patients with depressive disorders and non-suicidal self-injury behaviors [27].

Regarding the time course of ECT, we found a significant decrease in *POMC*m acutely after a single ECT session (directly before vs. 15 min after ECT) that was not related to the response. Interestingly, acute methylation changes after the last ECT differed between patients: ECT-R showed a decrease, and ECT-NR an increase in *POMC* promoter methylation. Rapid changes in *POMC*m have also

been observed in other studies, e. g., an increase in *POMC* promoter methylation 10 min after injection of diacetylmorphine in opioid-dependent patients [24]. The decreasing *POMC*m after the last ECT in ECT-R might reflect a normalized HPA axis.

A strength of the current study is the presence of a control group without current antidepressant treatment (UDC) to specify differences in severe MDD between ECT patients and those without medication. In many other studies, control groups (not undergoing ECT) have lower MADRS and BDI scores than ECT patients, thus limiting the comparability between groups. Regarding the limitations of the current project, a larger replication sample is needed to confirm our findings. Interpretation of our study is further hindered by (a) the partial heterogeneity of the groups studied (ECT patients were older than control subjects), (b) conflicting results regarding *NR3C1*m in the literature, (c) the lack of data complementary to our DNAm analysis (neither mRNA nor protein expression was evaluated), (d) the very small differences in methylation levels, (e) the fact that DNAm between brain and periphery may greatly differ, and (f) the weak association between DNAm and gene expression in general. Regarding the latter (f), however, recent studies found that the DNAm of *NR3C1-1F* and *POMC* were inversely correlated with *NR3C1* and *POMC* (and thus ACTH), respectively [44–46].

It should be additionally considered that our analysis was performed using PBMCs as source material. Several studies, including our own [47], have shown that the relative proportions of immune cell subtypes change with ECT or drug intake. Hence, the changes in DNAm detected in the current study might also reflect changes in circulating immune cell subsets rather than being true differences/changes in DNAm. Lastly, another limitation of candidate gene studies is the probability of producing false positive results.

A follow-up study (using other sequencing methods specialized in detecting small DNAm changes, e. g., the next generation sequencing) should complement these analyses.

Our findings suggest that *NR3C1*m and *POMC*m might play a role in the pathophysiology of depression. In future studies, patients should be deeply phenotyped, e. g., with regard to childhood maltreatment and dysregulation of the HPA axis.

Supplementary Materials: **Table S1**: Primers used for polymerase chain reaction and sequencing. **Table S2**: Polymerase chain reaction programs for *NR3C1* amplification. **Table S3**: *NR3C1* CpG sites. **Table S4**: PCR programs for *POMC* amplification. **Table S5**: CpG sites on *POMC*. **Table S6**: *NR3C1* DNA methylation in distinct regions at baseline. **Table S7**: *POMC*m in distinct regions at baseline. **Fig. S1**: Sequencing product of *NR3C1*. **Fig. S2**: Sequencing product of *POMC*.

Author Contributions

HBM: Supervision of patient recruitment, processed data, including their analysis and drafting the manuscript. NM: Supervised and conducted the experiments and revised the manuscript critically for important intellectual content. FE: Recruiting of patients, data management, and conducting the experiments under supervision. KJ: Provided enormous support regarding the experiments, revised the manuscript for important intellectual content. TF: Recruitment and treatment of the patients, revised the manuscript for important intellectual content. RS: Revision of the statistical analysis and revision of the manuscript for important intellectual content. SB: Substantial contribution to the conception of the work, study revision for important intellectual content. HF and AN: Supervised the study; Essential contribution to the conception and design of the work, analysis, and interpretation of the data, and revising the manuscript for important intellectual content. All authors have read and agreed to the published version of the manuscript.

Funding

This project was supported by PRACTIS-Clinician Scientist Program of Hannover Medical School, funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, ME3696/3–1) – Project-ID 413617135 and in-house funding from Hannover Medical School. The funders had no role in study design, collection, analyses, or interpretation of data, in manuscript writing, or in the decision to publish the results.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Hannover Medical School (protocol code 2842–2015, date of approval: 18.08.2015).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Study data can be made available upon reasonable request to the corresponding author.

Funding Information

This project was supported by PRACTIS-Clinician Scientist Program of Hannover Medical School, funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, ME3696/3-1) – Project-ID 413617135 and in-house funding from Hannover Medical School.

Acknowledgements

Parts of this study were conducted within the doctoral thesis of Franziska Eberle. An abstract for a poster presentation at the AGNP/DGBP Congress 2022 in Germany has been published in Pharmacopsychiatry. The authors thank Kyra Liepach and Mareike Sack for their support throughout the study. Furthermore, we are deeply thankful to all participants in our study.

Conflict of Interest

Conflicts of Interest: HBM and RS took part in an educational event sponsored by Livanova. HF received speaker's honararia and served as advisor for Recordati Pharma GmbH and Janssen-Cilag Gmbh. AN received lecture fees from Novartis and Merck. NM is currently working for SCENTS Health GmbH, but had no conflict of interest to declare while conducting the experiments and preparing the manuscript., FE, KJ, TF, SB declare no conflict of interest.

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