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Highlights

- Differences in DNA methylation are observed at the *NR3C1* exon 1F in depression.
- The degree of DNA methylation at the *NR3C1* exon 1F is correlated with increases in morning cortisol concentrations and childhood emotional abuse severity.
- No differences in DNA methylation are observed at the *FKBP5* sites measured in depression.

DNA methylation differences at the glucocorticoid receptor gene in depression are related to functional alterations in hypothalamic-pituitary-adrenal axis activity and to early life emotional abuse

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ABSTRACT

Depression is associated with alterations in hypothalamic-pituitary-adrenal (HPA) axis activity. A proposed mechanism to explain these alterations are changes in DNA methylation levels, secondary to early life adversity (ELA), at stress-related genes. Two gene regions that have been implicated in the literature, the glucocorticoid receptor gene (*NR3C1*) exon 1F and the *FKBP5* gene intron 7 were examined in 67 individuals (33 depressed patients and 34 controls). We investigated whether cortisol concentrations, evaluated in 25 depressed patients and 20 controls, and measures of ELA were associated with the degree of methylation at these candidate gene regions. Mean *NR3C1* exon 1F DNA methylation levels were significantly increased in the depressed cohort and the degree of methylation was found to be positively associated with morning cortisol concentrations. DNA methylation levels at specific CG sites within the *NR3C1* exon 1F were related to childhood emotional abuse severity. DNA methylation at CG38 was related to both HPA axis and childhood emotional abuse measures in the depressed group. No *FKBP5* differences were revealed. Our findings suggest that hypermethylation at the *NR3C1* exon 1F may occur in depression. This locus-specific epigenetic change is associated with higher basal HPA axis activity, possibly reflecting acquired glucocorticoid receptor resistance.

INTRODUCTION

Major depressive disorder (MDD) is a debilitating mood disorder with a lifetime prevalence rate of approximately 17% (Kessler et al., 2005). Despite extensive research, there are no established biological markers for the diagnosis of depression (Mossner et al., 2007). Longitudinal studies indicate that many adulthood-onset diseases are associated with adverse developmental or biological events experienced early in life. The latency between exposure to adversity and development of a disorder can span years, and even decades in some instances (Shonkoff et al., 2009). Exposure to early life adversity (ELA) has been identified as a major risk factor in the development of almost all psychiatric disorders (Kessler et al., 2010), including MDD (Kessler and Magee, 1993). It has been reported that adult patients with MDD have a two-fold higher rate of ELA compared to healthy controls and that ELA has been shown to predict earlier-onset depression, a higher risk of recurrent depression, more severe course of illness, greater chronicity and suicide (Williams et al., 2016). MDD patients with a history of ELA show different treatment responses reinforcing the idea of diverse pathways to the loose clinical syndrome of depression, one of which is ELA (Nemeroff et al., 2003).

The hypothalamic-pituitary-adrenal (HPA) axis is the main stress response system (Bellavance and Rivest, 2014). Through glucocorticoid receptors (GR) cortisol mediates a myriad of tissue-specific effects (Young et al., 2003). Importantly, cortisol signaling via GR is responsible for negative feedback inhibition of the HPA axis. A frequently replicated finding in depression research is the association of depression with hyperactivity of the HPA axis (Pariante and Lightman, 2008). It has been hypothesized that the increased HPA axis activity results from dysfunctional feedback because of reduced efficacy of central GR function (Young et al., 2003). The cortisol awakening response (CAR), the brisk increase of cortisol levels within 20-30 minutes of awakening in the morning (Fries et al., 2009), provides a naturalistic test for a cortisol stress response. Several studies have demonstrated an increased CAR in currently depressed individuals (Suzuki et al., 2013; Vreeburg et al., 2009), as well as in recovered patients with depression (Bhagwagar et al., 2003).

The GR co-chaperone protein FK506 binding protein 5 (FKBP5) is an immunophillin protein that accompanies the inactive GR complex in the cytoplasm. FKBP5 reduces the affinity of the GR complex for cortisol and thus decreases overall GR signaling (Rao et al., 2016). It is hypothesized that genetic variants

within the *FKBP5* gene may influence individuals' susceptibility to depression, with certain variants associated with increased FKBP5 protein levels (Szczepankiewicz et al., 2014), and consequently with relative decrease in GR complex activity. A recent meta-analysis supports the association between specific FKBP5 genetic variants and an increased risk of depression (Rao et al., 2016).

Epigenetic mechanisms are essential for the regulation of gene expression as they can modulate events at the level of transcription and translation (Portela and Esteller, 2010). Epigenetic mechanisms may provide an etiological link between ELA and the syndrome of depression (Dalton et al., 2014). The addition of a methyl group to the cytosine within a CG site in a critical regulatory region can inhibit transcriptional activity (Farrell and O'Keane, 2016).

In relation to MDD and alterations in HPA axis activity, an interesting epigenetic modification is methylation of a nerve growth factor-inducible protein A (NGFI-A) binding site located in exon 1F of the GR gene, the nuclear receptor subfamily 3 group C member 1 (*NR3C1*) (Swirnoff and Milbrandt, 1995), that is associated with ELA. Alterations at or near this NGFI-A binding site may explain one of the biological links between ELA and the development of depressive illnesses (Smart et al., 2015). *NR3C1* exon 1F hypermethylation in relation to ELA has been demonstrated in peripheral tissues, including cord blood, white blood cells and whole blood (Oberlander et al., 2008; Perroud et al., 2011; Radtke et al., 2011; Radtke et al., 2015) and would potentially result in dysregulation of HPA axis negative feedback were it occurring in the brain. It is possible that this hypermethylation is indeed occurring centrally as increased DNA methylation has been reported in the hippocampus of the orthologue 1_7 site in stressed rats (Weaver et al., 2004) and in the hippocampus of abuse victims who died by suicide (McGowan et al., 2009).

FKBP5 has recently attracted attention as a protein possibly implicated in the area of epigenetic-mediated gene-childhood trauma interaction leading to adult mental illness. Klengel et al. performed the first human study examining gene-ELA interaction and methylation at *FKBP5*. They showed that individuals suffering from post-traumatic stress disorder (PTSD) who were exposed to ELA and who carried a functional *FKBP5* risk T allele at rs1360780 had decreased levels of methylation at intron 7 of the *FKBP5* gene. This intronic demethylation was associated with an increase in FKBP5 induction by GR

activation, leading to symptoms of GR resistance (Klengel et al., 2013). One study examining *FKBP5* methylation in individuals with the risk T allele and remitted depression revealed a (non-significant) trend towards higher methylation levels compared to healthy controls (Höhne et al., 2015). There are a few more studies that examined FKBP5 methylation association to ELA but the direction of effect was inconsistent (Needham et al., 2015; Tyrka et al., 2015).

No studies, to our knowledge, have examined *NR3C1* and *FKBP5* methylation in the same individuals, and the possible associations between their DNA methylation levels and HPA axis function. The aims of this study were to determine if DNA methylation at the *NR3C1* exon 1F and/or *FKBP5* intron 7 were altered in individuals with depression compared to controls. We also explore possible relationships between *NR3C1* and *FKBP5* DNA methylation and HPA axis function as assessed through the CAR.

METHODS AND MATERIALS

Patient Recruitment and Assessment

Depressed patients were recruited from the Tallaght Mental Health Services. Any patient presenting with depression was considered for the study. They were screened for eligibility by a consultant psychiatrist. Screening involved assessing whether patients fulfilled criteria for a Major Depressive Episode (MDE) from the Mini International Neuropsychiatric Interview (M.I.N.I) (Sheehan et al., 1997); scored >17 on the Hamilton Depression Rating 21-Item Scale (HAM-D-21) (Hamilton, 1960), indicative of moderate depression; were not suffering from a psychotic or substance abuse disorder or from any chronic medical illnesses and were not taking any steroid medications, excluding the contraceptive pill. Healthy, control individuals were screened and recruited from the general population. Screening involved establishing that individuals had never experienced a mental health disorder, scored ≤7 on the HAM-D-21, were not suffering from any chronic medical illnesses, and were not taking any steroid medications, except the oral contraceptive pill. For both groups, 45 years, considered the beginning of middle age (Stevenson, 2010), was selected as the maximum age for inclusion to reduce the possibility of age-related effects on biological data. The groups were balanced for age and sex. If individuals met inclusion and exclusion criteria, they were invited to participate. If, following a thorough explanation of the study, and they remained willing to participate, they gave written informed consent. As this was not an intervention study, all treatment was continued as usual. Detailed demographic information was collected. The International Standard Classification of Education (ISCED) is a standardized scale used to gather information on educational achievement (UNESCO, 2011) and was used to grade participants' level of education. Participants were then administered a battery of clinical instruments: Centre for Epidemiological Studies Depression Rating Scale (CES-D) (Orme et al., 1986), suicidality module (Module C) of the M.I.N.I (Sheehan et al., 1997) and the Childhood Trauma Questionnaire (CTQ) (Bernstein, et al., 1997). The CTQ is a retrospective, self-report measure of ELA. The CTQ contains five subscales; three assessing abuse (emotional, physical and sexual) and two assessing neglect (emotional and physical). Other traumatic events (deaths, bullying etc.) that may have occurred during childhood were not

assessed. The study was approved by the Tallaght Hospital/St. James's Hospital Joint Research Ethics Committee (2013/23/02) following the principles in the 1964 Declaration of Helsinki(World Medical Association, 2013).

DNA methylation analysis of HPA axis genes

DNA methylation of the *NR3C1* exon 1F and *FKBP5* intron 7 was measured in whole blood by pyrosequencing, performed on bisulfite-treated DNA. A schematic representation of both regions investigated can be found at Figure S1. Blood was collected into PAXgene® Blood DNA tubes and DNA was extracted using the FlexiGene® DNA Kit (Qiagen, Valencia, California) using the manufacturer's recommended protocol. 1 µg DNA was bisulfite-treated using the EZ DNA Methylation Gold™ Kit (Zymo Research, Irvine, California) using the manufacturer's recommended protocol. A 319bp fragment corresponding to our region of interest within the *NR3C1* exon 1F was amplified by touchdown PCR using the following primers: NR3C1F-FW-biotin: 5Biosg/GTTTTTAATTTTTTAGGAAAAAGGGT and NR3C1F-REV: ACCCCTTTCCAAATAACACACTTC. A 341bp fragment corresponding to our region of interest within the *FKBP5* intron 7 was amplified by standard PCR using the following primers: FKBP5-FW: TGGGATAATAATTTGGAGTTATAGTGTAGG and FKBP5-REV-biotin: 5Biosg/AAATTTATCTCTTACCTCCAACACTAC. For both PCR reactions, a master mix solution was made up using EpiMark® Hot Start *Taq* DNA Polymerase (NEB® Inc., Ontario, Canada). A no template control was included to monitor contamination of extraneous nucleic acids. The pyrosequencing reaction was performed using the PyroMark Q96 platform (Qiagen, Valencia, California). Sequencing primers used were as follows: NR3C1F_LR_S3: AACTCCCCAATAAATCTAAAAC (*NR3C1* exon 1F CG36-CG39), NR3C1F_LR_S4: AAAAAACTCCATAACCCTCT (*NR3C1* exon 1F CG41) and FKBP5_S4: GTTGATATATAGGAATAAAATAAGA (*FKBP5* intron 7 CG6-CG7). Percentage methylation levels and quality control were analyzed using PyroMark CpG Software 1.0.1.1 (Qiagen, Valencia, California).

FKBP5 rs1360780 genotyping

The rs1360780 allele was genotyped from whole blood DNA using a Taqman SNP Genotyping Assay performed on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, California) as previously described (Tozzi et al., 2016).

Salivary cortisol analysis

Saliva samples were collected within one week of recruitment into the study. Participants were instructed not to eat, drink or brush their teeth 30 minutes prior to saliva collection. Saliva samples were collected in Salivette® tubes (Sarstedt, Nümbrecht, Germany) at five time points throughout the day. These time points were 0, 30 and 60 minutes after waking (to assess CAR), and 12 and 12.5 hours after waking (to assess diurnal variation). Cortisol concentrations were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Jones et al., 2012).

Statistical Analysis

Data are presented as mean with standard deviation (SD) or standard error of the mean (SEM) where appropriate. All data were tested for normality using the Shapiro-Wilk test. DNA methylation and cortisol values were log transformed to normalize distribution. DNA methylation and cortisol data were analyzed using analyses of variance with covariates where appropriate. In our preliminary analysis it was observed that smoking and education were driving differences in DNA methylation between the two groups, and were therefore included as covariates in the final analysis. Similarly, in the preliminary cortisol analysis, it was observed that BMI and education were driving differences between the two groups, and therefore were included as covariates in the final analysis. Non-parametric data were analyzed using the Mann-Whitney U test. Associations between categorical variables were tested using the Chi-Square ($\chi 2$) test. Correlational analysis was carried out using Spearman's rho statistics. Given the exploratory nature of the correlational analyses, Bonferroni corrections were not applied due to the heightened risk of Type II errors associated with such corrections. Statistical analyses were considered significant when $p \leq 0.05$.

Graphs were generated using GraphPad Prism Software Version 6.00 (La Jolla, California). All data were analyzed using SPSS Version 22 (IBM, Armonk, New York).

RESULTS

Demographic and clinical data of patients with depression and healthy controls

There were no significant differences in age, gender or BMI between the groups. The depressed group had a higher prevalence of smokers (p=0.02) and lower duration of education (p<0.001), compared to that of the control group. Consistent with a diagnosis of depression, patients with MDD had significantly higher HAM-D-21 and CES-D scores compared to healthy controls. A score of 19-22 in the HAM-D-21 is considered to represent severe depression. The mean HAM-D score in the patient group was 21.94 (SD=5.49) indicating that overall this group was severely depressed. Furthermore, a score of 9-16 in the suicidality module of the M.I.N.I indicated that there was a moderate suicide risk. The mean score in the patient group was 10.06 (SD=12.16), thus indicating a moderate risk for a suicide. Any participant who met criteria for "moderate" severity on any of the CTQ subscales was considered to have experienced ELA. The depressed group had a higher prevalence of individuals who had ELA, and had significantly higher CTQ global, emotional abuse, physical abuse, emotional neglect and physical neglect compared to the control group. Within the depressed sample, 20 individuals were receiving pharmacological treatment for depression at the time of recruitment. Of these, 14 were taking selective serotonin reuptake inhibitors (SSRIs), two were taking serotonin-noradrenaline reuptake inhibitors (SNRIs) and four were receiving other antidepressant treatments [Table 1].

DNA methylation levels of NR3C1 exon 1F in depressed patients and healthy controls

Mean DNA methylation at *NR3C1* exon 1F was significantly higher in the depressed patients relative to healthy controls (F=3.89, d=0.49, p=0.05). There were no significant differences in DNA methylation at any of the individual CG sites measured [Table 2].

DNA methylation levels of FKBP5 intron 7 promoter in depressed patients and healthy controls

There were no statistically significant differences in overall, or individual CG site, DNA methylation at *FKBP5* intron 7 in depressed patients relative to healthy controls [Table 2]. At the analysis of rs1360780 the homozygous TT samples were rare in our group, therefore they were grouped with CT samples. In the

depressed group, 16 possessed the CC genotype and 17 possessed the CT/TT genotype. In the control group, 21 possessed the CC genotype and 13 possessed the CT/TT genotype. There was no significant difference in the prevalence of the risk allele between the groups. There were no significant differences in methylation when individuals were divided based on their FKBP5 rs1360780 allele status [Table 3].

Salivary cortisol levels in depressed patients and healthy controls

Out of the 67 participants recruited into the study with DNA methylation data available, 45 (25 depressed patients and 20 healthy controls) had corresponding cortisol data [Table S1]. Twenty-four participants provided insufficient sample quantity or quality, and were excluded from the analysis. Analysis revealed a significant increase in wakening (T0) cortisol concentrations and overall morning cortisol output in depressed patients compared to healthy controls. There were no differences in evening cortisol values between the groups [Table 4].

Associations between cortisol concentrations and NR3C1 DNA methylation

There was a significant relationship between methylation at the *NR3C1* exon 1F CG38 site and cortisol levels at T0, and with average morning cortisol output [Table 5].

Associations between ELA and NR3C1 DNA methylation

Spearman's *rho* correlation analysis revealed a significant positive association between the severity of emotional abuse experienced during childhood and the degree of DNA methylation at *NR3C1* exon 1F CG37 (r=0.53, p=0.01) and CG38 (r=0.43, p=0.04) [Figure 2]. This finding was only present in depressed individuals who reported ELA and not in the overall sample. No associations were revealed between *NR3C1* DNA methylation and physical/sexual abuse or emotional/physical neglect. No associations were revealed between *FKBP5* DNA methylation and any adversity parameter examined.

DISCUSSION

This study sought to determine whether there were differences in DNA methylation levels at the *NR3C1* exon 1F and/or the *FKBP5* intron 7 were present in a sample of depressed individuals, and if so, whether these differences were associated with changes in HPA axis measures, as measured by the CAR/diurnal cortisol-HPA axis test. The overall percentage of methylation at the *NR3C1* exon 1F region was significantly increased in the depressed patients relative to healthy controls. The degree of this methylation was related to increased cortisol concentrations at T0, as well as overall morning cortisol secretion in the entire study cohort. Within the *NR3C1* exon 1F, DNA methylation levels at CG38 were related to the same two cortisol measures. CG38 DNA methylation was also related to childhood emotional abuse severity in the depressed group. No significant differences between depressed and control groups, in the total group and when stratifying for a risk polymorphism, were seen in *FKBP5* DNA methylation measures.

NR3C1 DNA methylation and the HPA axis

Although *NR3C1* DNA methylation levels were low in general, the overall percentage methylation at the *NR3C1* exon 1F region was significantly increased in the depressed patients relative to healthy controls. This finding is in keeping with the literature generally (Palma-Gudiel et al., 2015), with the same exon 1F region being previously implicated in various stress-related psychopathologies including depression, bipolar disorder and PTSD (Argentieri et al., 2017). The mechanism(s) underlying how ELA produces targeted methylation of specific gene regions is currently unknown. We know that the epigenome is responsive to external influences and it has been hypothesized that this plasticity enables optimal adaptation to changing environmental conditions (Ryan et al., 2016). It is possible that this plasticity gives trauma the opportunity to impact the epigenome in a manner that explains the long-term effects of trauma on the risk of developing psychiatric disorders. Future studies should attempt to elucidate the molecular mechanisms underlying trauma-induced DNA methylation changes at specific gene regions. The depressed group had increased cortisol concentrations upon wakening, as well as an overall increase in morning cortisol output relative to the healthy controls. Although not all previous studies have been

consistent (Gunnar and Vazquez, 2001), elevated wakening cortisol levels have often been found in a significant number of previous studies examining the CAR tests in depression (Bhagwagar et al., 2005; Suzuki et al., 2013; Vreeburg et al., 2009). While there were no significant methylation differences between the depressed and control groups at individual *NR3C1* exon 1F CG sites, significant relationships were revealed between individual CG sites and CAR parameters, particularly at the CG38 site. Interestingly, the relationship between methylation at the CG38 site and the CAR measures existed for the two CAR values that distinguished the depressed from the control group (i.e. T0 and morning average). It is worth highlighting that CG37 and CCG38 are at the location of the NGFI-A binding site (Palma-Gudiel et al., 2015) that has been previously associated with depression pathophysiology(Smart et al., 2015). This indicates a possible functional relationship between DNA methylation alterations at CG38 of the *NR3C1* exon 1F and HPA axis activity.

NR3C1 methylation and ELA

As previously mentioned, ELA has been identified as a major risk factor in the development of almost all psychiatric disorders (Kessler et al., 2010), including MDD (Kessler and Magee, 1993). To date, a number of studies have shown that increases in DNA methylation at the glucocorticoid receptor gene have been associated with exposure to childhood abuse (McGowan et al., 2009; Perroud et al., 2011; Radtke et al., 2015). Overall, these studies have revealed positive associations with the degree of methylation at the exon 1F region and the severity of abuse exposure during childhood. In our study, we observed a significant positive association between the severity of emotional abuse experienced during childhood and the degree of DNA methylation at NR3C1 exon 1F CG37 and CG38 sites. This finding is particularly noteworthy as a diagnosis of depression is more likely to be related to reports of childhood emotional abuse than to physical or sexual abuse (Gibb et al., 2007). This finding was only present in depressed patients who reported ELA and was not present in the overall cohort, suggesting an association between ELA severity, rather than depression *per se*, and methylation status of the CG37 and CG38 sites NR3C1 exon 1F. As previously mentioned, the CG37 and CG38 sites are at the NGFI-A binding site, which has been implicated in childhood trauma-associated DNA methylation changes(McGowan et al., 2009). The

findings of our present study solidifies these previous finding. Overall, although causality cannot be determined, the *NR3C1* methylation findings in this group of depressed patients suggests that the CG38 site of exon 1F is methylated in association with emotional abuse in childhood and that this may lead to increased morning HPA axis activity.

FKBP5 intron 7 DNA methylation in depression

As FKBP5 intron 7 methylation alterations have previously been observed in individuals with a history of ELA who suffer with post-traumatic stress disorder (PTSD) (Klengel et al., 2013), we sought to assess differences in methylation at the same region in our depressed cohort. Analysis revealed no significant differences in DNA methylation at FKBP5 intron 7 in depressed patients relative to healthy controls. As the previous methylation findings have been allele-specific in nature, the functional FKBP5 SNP rs1360780 was genotyped and groups were divided according to whether or not they possessed the risk T-allele. This allele is associated with increased FKBP5 induction by glucocorticoids (Fujii et al., 2014). In our cohort, no significant allele-specific differences in DNA methylation were observed in depressed patients relative to healthy controls; nor did the risk-allele status separate out depressed from control groups. No associations between *FKBP5* methylation and cortisol concentrations or CAR parameters were observed. Overall, this study did not reveal significant differences in *FKBP5* intron 7 methylation in depressed individuals and healthy controls, even when considering the presence of a risk polymorphism. These findings suggest that although *FKBP5* DNA methylation is implicated in certain stress-related disorders, it may not be a candidate marker for state depression.

Study limitations and strengths

A limitation of this study is its cross-sectional design. Findings from cross-sectional studies need to be interpreted with caution as it cannot be assumed that model parameters are stable over time (Bowen and Wiersema, 1999). There is extensive evidence that the depressive phenotype changes over the course of an illness (O'Keane et al., 2012), therefore, future longitudinal studies are necessary to solidify these findings. Although this study is at least comparable to similar studies in terms of sample size, the

statistical power is limited because of the relatively small sample size. Three saliva samples were collected in the morning to assess individual CAR measurements. We found that subjects were less likely to comply if more than three samples were required. Three samples are deemed to be adequate for the assessment of the CAR (Stalder et al., 2016), but additional morning samples can provide a better overview of HPA axis activity. Furthermore, due to lapses in participant compliance, not all participants had cortisol concentration values. This needs to be considered when interpreting the results of correlation analyses. The study's relatively small sample size needs to be highlighted as a limitation in the context of statistical power. Underpowered statistical analyses run the risk of Type II errors (i.e. false negative results) and this should be considered when interpreting the results presented in this manuscript. Additionally, given the novelty of DNA methylation analysis in psychiatry research, much of the work presented is exploratory in nature. As a result of this exploratory work, no corrections were made to address multiple comparisons, which run the risk of Type I errors (i.e. false positive results). This should be considered when interpreting the results. Future work should attempt to (i) increase sample size to increase statistical power, and (ii) use conservative post-hoc corrections (i.e. Bonferroni) to ensure the reporting of robust, statistically significant, positive findings. We did not include antidepressant use as a covariate in our analyses as they did not impact upon the DNA methylation or cortisol results across the groups. This could be due to of the relatively small sample size, and needs to be considered when interpreting the findings. Finally, this study would have benefited from the inclusion of a higher number of individuals exposed to early life adversity. Future studies should attempt to increase the number of non-depressed controls who have been exposed to early life adversity, in an attempt to disentangle the effects of early life adversity in the pathogenesis of depression.

This study had a number of strengths. To our knowledge, this is the first study to assess both *NR3C1* and *FKBP5* DNA methylation in the same sample and to examine this methylation in relation to HPA axis activity and ELA in clinically depressed and healthy samples. Another strength of the study is the use of LC-MS/MS for quantification of cortisol concentrations. More commonly used immunoassays are less specific and measure the inactive metabolite cortisone, resulting in false-high cortisol levels being reported in the literature. LC-MS/MS is considered the best available technique for the specific analysis of

salivary cortisol (Antonelli et al., 2015). A final strength of the study is the use of whole blood samples. Whole blood is easily accessible in the clinical environment, and may be potentially useful in the future for diagnosis and prognosis of depression. The use of whole blood should also be considered as a limitation as it is known that DNA methylation patterns differ across tissues (Kriebel et al., 2016), and this should be considered when interpreting the findings presented in this manuscript.

Conclusion

While there were no differences in DNA methylation levels at the *FKBP5* sites of interest, methylation at the *NR3C1* exon 1F region was significantly increased in the depressed group. Our findings of increases in morning cortisol concentrations, in a group with MDD compared to controls, are compatible with overall findings of HPA axis hyperactivity in individuals suffering with MDD. This study provides evidence that this hyperactivity is associated with increased methylation at the *NR3C1* exon 1F, within which the CG38 was identified as being particularly associated with CAR over-activity. The degree of methylation at CG38 was also specifically associated with childhood emotional abuse severity in depressed patients. The CG38 site resides at a NGFI-A site that has been previously investigated in relation to HPA axis alterations in depression. These results suggest that epigenetic alterations at the *NR3C1* gene, but not the *FKBP5* gene, may contribute to the finding of both increased morning cortisol levels in depression and ELA. The study provides novel insights into the molecular basis of depression and how this may evolve from ELA.

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Author Contributions

Veronica O'Keane, Andrew Harkin, Linda Booij, and Thomas Frodl conceived and designed the experiments; Veronica O'Keane screened and recruited all study participants; Chloë Farrell, Kelly Doolin, Niamh O'Leary, Chaitra Jairaj, Darren Roddy, and Leonardo Tozzi performed sample collection; Zsófia Nemoda designed primers for DNA methylation analysis; Moshe Szyf provided expert advice for all epigenetic methodology and analysis; Derek Morris performed *FKBP5* genotyping; Chloë Farrell performed DNA methylation analysis; Chloë Farrell analysed the data; Chloë Farrell wrote the manuscript.

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Tables and Figures

Table 1. Demographic and clinical data for all recruited depressed patients and healthy controls

	Patients (n=33)	Controls (n=34)	Statistics (p-value)
Age (years)	28.27 (8.77)	28.21 (7.47)	Z=-0.08, p=0.94
Gender (M/F)	9/24	13/21	χ^2=0.91, p=0.44
BMI	26.31 (6.89)	23.15 (3.87)	Z=-1.65, p=0.10
Smoking (Y/N)	12/21	4/30	χ^2=5.58, p=0.02*
ISCED Education	3.12 (1.58)	6.00 (1.97)	Z=-4.85, p<0.001***
HAM-D-21	21.94 (5.49)	2.16 (2.93)	Z=-6.88, p<0.001***
CES-D	38.39 (9.54)	6.82 (8.77)	Z=-6.53, p<0.001***
CTQ Global Score	42.30 (15.31)	29.35 (7.81)	Z=-4.01, p<0.001***
CTQ EA	10.25 (4.28)	6.15 (2.22)	Z=-4.39, p<0.001***
CTQ PA	6.88 (2.98)	5.50 (1.33)	Z=-2.41, p=0.02*
CTQ SA	7.47 (6.26)	5.53 (3.09)	Z=-1.27, p=0.20
CTQ EN	11.06 (5.14)	6.74 (2.21)	Z=-3.35, p=0.001***
CTQ PN	7.03 (2.72)	5.50 (1.58)	Z=-2.81, p=0.004**
ELA (Y/N)	15/18	3/31	χ^2=11.44, p=0.001***
Suicidality	10.06 (12.16)	0.00 (0.00)	Z=-5.86, p<0.001***

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education, HAM-D, CES-D, PSQI, CTQ Global Score, Suicidality) and Chi squared (χ^2) test (Gender, Smoking, ELA, Antidepressant use). BMI=Body Mass Index; ISCED=International Standard Classification of Education; HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for

Epidemiologic Studies Depression Scale; CTQ=Childhood Trauma Questionnaire; EA=Emotional Abuse; PA=Physical Abuse; SA=Sexual Abuse; EN=Emotional Neglect; PN=Physical Neglect. *p≤0.05, **p≤0.01, ***p≤0.001 vs. control.

Table 2. *NR3C1* exon 1F and *FKBP5* intron 7 methylation data for depressed patients and healthy controls

	Patients (n=33)	**Controls (n=34)**	**Statistics (p-value)**
Mean *NR3C1*F*	4.07 (0.57)	3.81 (0.48)	F=3.89, *d*=0.49, p=0.05
***NR3C1*F CG36**	2.72 (1.24)	2.44 (1.00)	F=1.80, *d*=0.25, p=0.19
***NR3C1*F CG37**	3.12 (1.02)	3.27 (1.23)	F=0.02, *d*=0.13, p=0.90
***NR3C1*F CG38**	5.58 (0.94)	5.49 (0.93)	F=0.11, *d*=0.10, p=0.75
***NR3C1*F CG39**	5.38 (0.76)	5.27 (0.77)	F=0.84, *d*=0.14, p=0.36
***NR3C1*F CG41**	3.57 (1.74)	2.92 (0.95)	F=3.07, *d*=0.46, p=0.09
Mean *FKBP5*	60.11 (4.50)	60.03 (6.09)	F=0.28, *d*=0.02, p=0.60
***FKBP5* CG6**	60.35 (11.57)	62.32 (5.24)	F=0.10, *d*=0.22, p=0.92
***FKBP5* CG7**	57.99 (5.43)	57.74 (8.64)	F=0.34, *d*=0.04, p=0.57

Data are expressed as mean whole blood DNA methylation percentage with SD in parentheses. Statistical analysis was performed using analysis of variance, with smoking and education as covariates, on log transformed methylation data. *d*=Cohen's *d*.

Table 3. ***FKBP5*** **intron 7 methylation data for depressed patients and healthy controls, categorized on their rs1360780 allele status**

	Patients CC (n=16)	Patients CT/TT (n=17)	Controls CC (n=21)	Controls CT/TT (n=13)	Statistics (p-value)
Mean *FKBP5*	60.88 (3.89)	59.40 (5.25)	60.82 (6.99)	58.90 (4.52)	F=0.79, p=0.51
***FKBP5* CG6**	58.61 (16.72)	61.57 (4.30)	62.82 (5.62)	61.61 (4.76)	F=0.31, p=0.82
***FKBP5* CG7**	58.97 (4.13)	57.24 (6.67)	58.83 (10.51)	56.18 (4.86)	F=0.85, p=0.47

Data are expressed as mean methylation percentage with SD in parentheses. Statistical analysis was performed using analysis of variance, with smoking and education as covariates on log transformed methylation data.

Table 4. Morning and evening cortisol concentrations in depressed patients and healthy controls

	Patients (n=25)	Controls (n=20)	Statistics (p-value)
CAR			
Cortisol T0*	10.88 (6.42)	7.01 (3.98)	F=4.60, *d*=0.72, p=0.04
Cortisol T30	13.86 (7.27)	10.07 (4.87)	F=2.75, *d*=0.61, p=0.11
Cortisol T60	8.59 (5.70)	7.65 (4.38)	F=0.53, *d*=0.18, p=0.47
Cortisol AUCi	100.94 (266.61)	101.67 (245.08)	F=0.72, *d*=0.01, p=0.40
Cortisol AM average*	11.41 (5.97)	8.54 (3.47)	F=3.96, *d*=0.59, p=0.05
Evening cortisol			
Cortisol T720	2.15 (1.99)	3.00 (4.89)	F=0.05, *d*=0.23, p=0.83
Cortisol T750	2.48 (2.49)	3.48 (5.36)	F=1.13, *d*=0.24, p=0.30
Cortisol PM average	2.05 (1.63)	3.13 (4.83)	F=0.18, *d*=0.30, p=0.68

Data are expressed as mean cortisol concentrations (nMol/L) with SD in parentheses. Statistical analysis was performed using analysis of variance, with BMI and education as covariates on log transformed data. *d*=Cohen's *d*.

Table 5. Correlational analysis of *NR3C1* exon 1F DNA methylation and cortisol concentrations in all participants.

	***NR3C1*F CG36**	***NR3C1*F CG37**	***NR3C1*F CG38**	***NR3C1*F CG39**	***NR3C1*F CG41**	**mean*NR3C1*F**
Cortisol T0	r=0.29 (p=0.07)	r=0.18 (p=0.26)	r=0.32 (p=0.04)*	r=-0.13 (p=0.47)	r=-0.06 (p=0.73)	r=0.25 (p=0.12)
Cortisol AM	r=0.34 (p=0.03)*	r=0.16 (p=0.32)	r=0.37 (p=0.02)*	r=-0.02 (p=0.90)	r=0.03 (p=0.88)	r=0.31 (p=0.04)*

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant.

Chloë Farrell

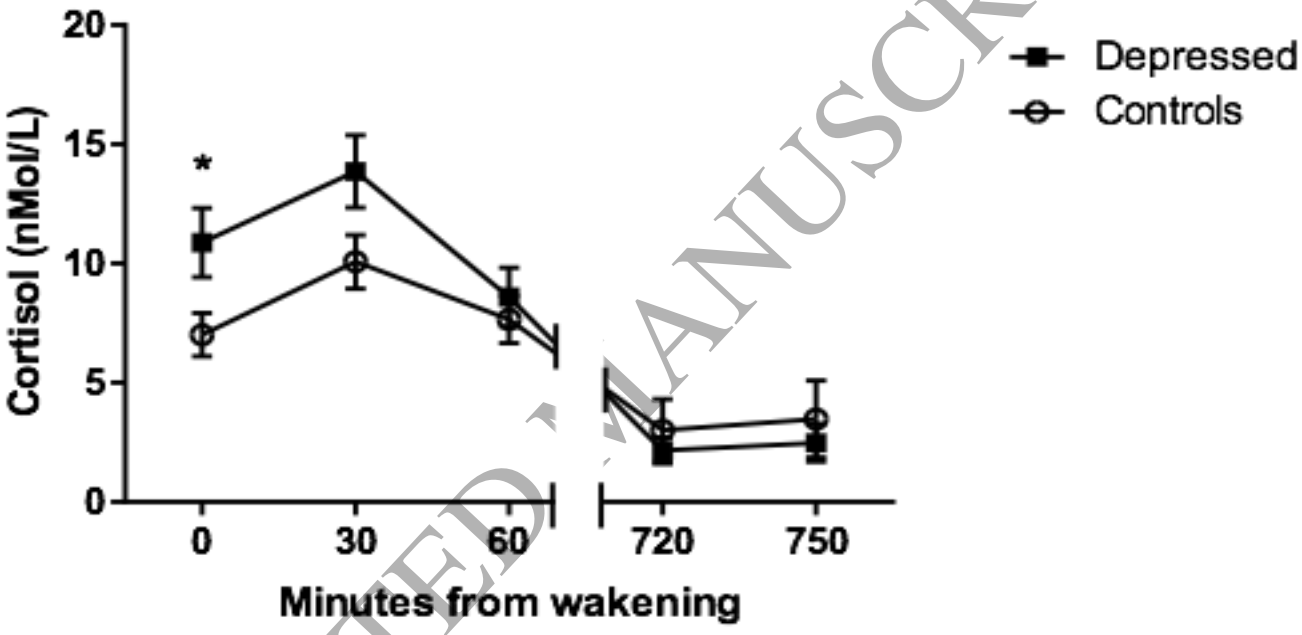

Figure 1. Morning and evening cortisol concentrations in depressed patients and healthy controls. Cortisol concentrations at three morning and two evening time points in depressed patients and healthy controls. Data are expressed as mean cortisol concentrations (nMol/L) with SEM, analysis of variance, with BMI and education as covariates on log transformed data, *p≤0.05 vs. controls.

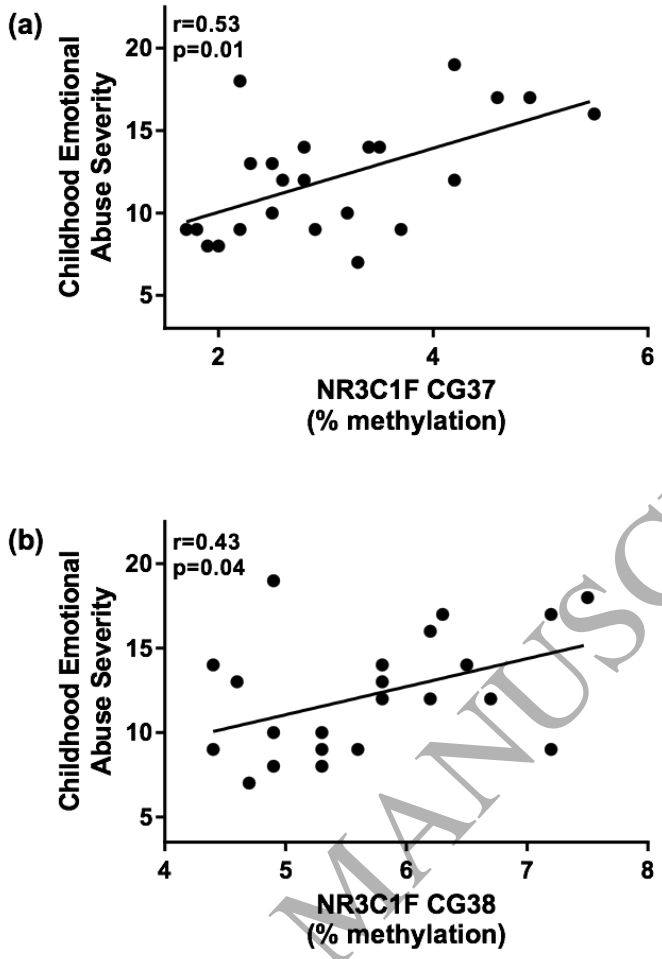

Figure 2. Relationship between NR3C1 exon 1F DNA methylation and early life severity. A significant relationship exists between (a) *NR3C1* exon 1F CG37 DNA methylation and emotional abuse and (b) *NR3C1* exon 1F CG38 DNA methylation and emotional abuse.