

**An investigation into the stress response axis,
serotonin system and adaptive immunity in
depression: a focus on early life adversity and
epigenetic mechanisms**

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I. Declaration

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I declare that this thesis has not been submitted as an exercise for a degree at this or any other university.

I declare that this thesis was composed by myself and the research presented is my own.

All the lab work and statistical analyses presented in this thesis was conducted by myself, with the exceptions of:

- (i) the diagnostic interviews which were performed by Prof. Veronica O’Keane and Dr. Chaitra Jairaj (Trinity College Dublin);
- (ii) the *FKBP5* genotyping data presented in Chapter 3 which was performed in the lab of Dr. Derek Morris (National University of Ireland Galway);
- (iii) the cortisol concentration data presented in Chapter 3,4 and 6 which was carried out in the lab of Prof. Brian Keevil (University Hospital of South Manchester);
- (iv) the cortisol awakening response parameters presented in Chapter 3,4 and 6 which were calculated by Dr. Kelly Doolin (Trinity College Dublin);
- (v) the baby saliva samples (Chapter 6) which were collected by Ms. Niamh O’Leary (Trinity College Dublin);
- (vi) the kynurenine pathway metabolite data presented in Chapter 4 and 5 which was carried out in the lab of Dr. Andre Liesener (Boehringer Ingelheim).

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II. Abstract

Major depressive disorder (MDD) is a debilitating psychiatric illness that affects at least 350 million individuals worldwide. Despite its high prevalence rate and its large global disease burden, the biological mechanisms leading to its presentation remain largely unknown. Evidence suggests that alterations reported in physiological systems implicated in the underlying pathogenesis of depression may be mediated, to an extent, via epigenetic mechanisms. It is believed that a large contributor to epigenetic alterations is exposure to early life adversity (ELA), which is of great interest given the high rates of childhood trauma reported by individuals suffering with depression.

Research has reported that depressed patients are frequently characterised by a hypercortisolemic state which is believed to be the result of a decrease in the inhibition of hypothalamic-pituitary-adrenal (HPA) axis activity, mediated by increases in DNA methylation at the glucocorticoid receptor/nuclear receptor subfamily 3, group C, member 1 (GR/*NR3C1*) gene. In accordance with these hypotheses, in this thesis, the assessment of HPA axis activity in a cohort consisting of depressed patients and healthy controls, measured by cortisol concentrations and dynamics, revealed increased morning cortisol concentrations and decreased morning cortisol reactivity in the depressed group. These increased morning cortisol concentrations were associated with both DNA methylation increases at the *NR3C1* promoter and decreased GR gene expression. Methylation at sites within the *NR3C1* gene were strongly associated with the severity of emotional abuse experienced in childhood, which is in accordance with the literature. Additionally, a similar DNA methylation profile was observed in an independent cohort of babies at six months postpartum. It was revealed that babies born to women who were clinically depressed during pregnancy had increases in DNA methylation at the *NR3C1* promoter, compared to babies born to women with no history of psychiatric illness. The degree of DNA methylation at the *NR3C1* promoter in babies at six months was strongly associated with a decrease in stress reactivity. Taken together, the findings from this group of studies provide strong evidence for adversity-driven DNA methylation changes at the glucocorticoid receptor gene in depression that are associated with altered HPA axis function.

Alterations in serotonin (5-HT) neurotransmission, as a consequence of tryptophan depletion and/or serotonin transporter (5-HTT) dysregulation in the brain, are

hypothesised to be another mechanism that contributes to an episode of depression. Epigenetic changes at the gene for the serotonin transporter, solute carrier family 6 member 4 (*SLC6A4*), have been hypothesised to mediate changes in 5-HTT function. Site-specific *SLC6A4* promoter DNA methylation was significantly decreased in depressed patients. Tryptophan concentrations were also significantly lower in the depressed, compared to the control group. Furthermore, decreases in DNA methylation at the *SLC6A4* promoter were associated with increased morning cortisol measures when examining the entire cohort and depressed cohort separately. This inverse relationship between increases in HPA axis activity and reduced 5-HT function, as found in my study, supports the idea of a functional relationship between increased HPA axis output and reduced 5-HT neurotransmission in depression.

The relative contribution of the adaptive immune system was also investigated through the assessment of DNA methylation levels at a CG site, cg06164961, in the *CD3E* gene and its association with the expression of T cell surface glycoproteins. Significant increases in DNA methylation at this site, which were highly associated with reduced gene expression, were observed in depression, with the most profound increases present in depressed patients with a history of adversity. Furthermore, it was revealed that the degree of DNA methylation at the *CD3E* gene was highly associated with decreases in circulating tryptophan concentrations; providing a novel mechanism to explain the complex interplay between 5-HT neurotransmission and adaptive immune system alterations in depression.

Overall, this thesis supports a role for dysregulated stress response, 5-HT and adaptive immune systems, mediated via epigenetic mechanisms, in the aetiology of depression. The findings suggest that the stress and adaptive immune systems particularly may change over time in depressed individuals with a history of childhood adversity, and that these changes may be mediated by epigenetic modifications. On the other hand, epigenetic findings relating to alterations in the 5-HT system would appear to be related more so to the depressed state. The findings of this thesis highlight the complex interactions, spanning from the epigenetic through to the systems level, that occur between these three physiological systems implicated in the pathophysiology of depression and emphasize the need for future studies to investigate the interplay of these systems, taking exposure to early life adversity into account.

De réir a chéile a thógtar na caisleáin.

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VII. List of abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HTT	Serotonin transporter
AHR	Aryl hydrocarbon receptor
BCL	B-cell lymphoma
BLIMP	B lymphocyte-induced maturation protein
BMI	Body mass index
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
CD3E	Cluster of differentiation 3 epsilon
CES-D	Center for Epidemiologic Studies Depression scale
CG/CpG	5'-cytosine-phosphate-guanine-3'
CRP	C-reactive protein
CTQ	Childhood Trauma Questionnaire
CXCR3	CXC chemokine receptor 3
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
ELA	Early life adversity
EOMES	Eomesodermin
FAM	Fluorescein amidite
FBS	Fetal bovine serum
FOX	Forkhead box
GATA3	GATA-binding protein 3

GITR	Glucocorticoid-induced TNF-receptor related protein
GR	Glucocorticoid receptor
HAM-D	Hamilton Depression Rating scale
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic-pituitary-adrenal
IDO	Indoleamine 2,3-dioxygenase
IFNγR	Interferon gamma receptor
IL	Interleukin
ISCED	International Standard Classification of Education
LPS	Lipopolysaccharide
LTα	Lymphotoxin- α
MAF	Musculoaponeurotic fibrosarcoma oncogene
MBD2	Methyl-CpG-binding domain protein 2
MDD	Major depressive disorder
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NR3C1	Nuclear receptor subfamily 3 group C member 1
NR3C2	Nuclear receptor subfamily 3 group C member 2
PBS	Phosphate buffered saline
PSQI	Pittsburgh Sleep Quality Inventory
PSS	Perceived Stress Scale
PTSD	Post-traumatic stress disorder
RNA	Ribonucleic acid
ROR	Retinoic acid receptor-related orphan receptor
RPMI	Roswell Park Memorial Institute medium

RUNX3	Runt related transcription factor 3
SERT	Serotonin transporter
SLC6A4	Solute carrier family 6 member 4
SMAD	Mothers against decapentaplegic homologue
SNP	Single-nucleotide polymorphism
SNRI	Serotonin-noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
STAT	Signal transducer and activator of transcription
TCA	Tricyclic antidepressant
TCR	T-cell receptor
T_{FH}	T follicular helper
TGFβ	Transforming growth factor-β
T_H	T helper
THPOK	TH-inducing POZ/Krupp

Chapter 1

Introduction

Elements of this chapter have been published. (Psychiatry Research. 2016 June; 242:349-356).

1.1 Major Depressive Disorder

Major depressive disorder (MDD) is a psychiatric disorder characterised by intense feelings of sadness and/or a diminished interest or pleasure in almost all activities (American Psychiatric Association, 2013). Women are more likely than men to experience depression with a 1.7-fold greater incidence in women reported in the literature (Albert, 2015). The 12-month and lifetime prevalence rates for MDD are approximately 7% (Kessler et al., 2005b) and 17% (Kessler et al., 2005a) respectively. Additionally, at least 50% of individuals who recover from a first episode of depression will go on to develop one or more additional episodes in their lifetime, and approximately 80% of individuals with a history of two episodes will experience another recurrence (Burcusa and Iacono, 2007). In Ireland, it is estimated that 450,000 are currently suffering from depression (Aware) and 451 people died by suicide in 2015 (CSO, 2015).

MDD is typically diagnosed based on criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (American Psychiatric Association, 2013) or the International Statistical Classification of Diseases and Health Related Problems, Tenth Edition (ICD-10) (WHO). Both diagnostic systems assess possible mood changes, hedonic capacity, weight or appetite changes, sleep problems, psychomotor activity, fatigability, feelings of worthlessness or guilt, concentration difficulties and the presence or not of suicidal ideation (Saito et al., 2010). Currently, a diagnosis of depression is determined by a trained professional, usually a general practitioner or psychiatrist, using the aforementioned diagnostic systems and clinical judgement. However, no biological markers for depression are currently available to aid in the diagnosis (Mossner et al., 2007).

Depression severity is categorised as mild, moderate or severe depending on the intensity of the presenting symptoms. Additionally, there are several different subtypes of the disorder. Currently, the DSM-5 has nine specifiers: anxious distress, mixed features, melancholic features, atypical features, mood-congruent psychotic features, mood-incongruent psychotic features, catatonia, peripartum onset and seasonal pattern (American Psychiatric Association, 2013). These various subtypes describe the many different presentations of depression. For example, depression with melancholic features is characterised by an unreactive mood, insomnia, reduced appetite and weight loss (Rush and Weissenburger, 1994), whereas, depression with atypical features is characterised by mood reactivity, hypersomnia and increased appetite and weight gain (Thase, 2007).

1.2 Pharmacological treatments for depression

For moderate to severe depression, treatment with selective serotonin reuptake inhibitors (SSRIs) is the most common treatment. SSRIs work by reducing the neuronal uptake of serotonin by the presynaptic serotonin transporter (SERT). This reduction of serotonin uptake theoretically results in prolonged stimulation of postsynaptic serotonin receptors. Other prescribed medications for the treatment of depression include serotonin-noradrenaline reuptake inhibitors (SNRIs), noradrenaline-dopamine reuptake inhibitors (NDRIs), tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs). Other medications are sometimes added to enhance antidepressant effects. In some cases, two antidepressants may be combined or medications such as mood stabilisers or antipsychotics may be used as an adjunct therapy (Mayo Clinic, 2016).

Although many pharmacological treatments are available for individuals suffering with depression, the response rates are relatively poor. From 2001-2006, the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) Study was conducted to determine the effectiveness of different treatments for people with major depression. The study found that approximately 50% of participants became symptom-free after two adequate courses of antidepressant treatment and that almost 70% of participants became symptom-free after all four treatment levels (Rush et al., 2006a). These findings indicate that approximately 50% of individuals requiring treatment for depression will need at least two trials of antidepressants to alleviate symptoms and 30% of individuals will be deemed treatment-resistant. Given the multiple aetiologies and subtypes of depression more targeted treatments should lead to more effective treatment approach for individuals suffering with depression.

1.3 Early life adversity as a risk factor for the development of depression

Research indicates that many adult 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 ELA has been shown to predict earlier-onset depression, a higher risk of recurrent depression, more severe course of illness and greater chronicity (Williams et al., 2016). MDD patients with a history of ELA display different responses to psychotherapy versus pharmacotherapy which suggests that there may be important differences in the pathophysiology of depression in individuals with and without ELA (Nemeroff et al., 2003).

Adversity experienced in the prenatal period is also associated with negative outcomes (Field et al, 2011; O'Connor et al., 2005). This is independent of psychological or behavioural mechanisms post-birth that could explain the association between an adverse environment and psychiatric disorder. Maternal depression during pregnancy is associated with impaired cognitive and emotional development of the offspring during childhood, and increased rates of psychiatric disorder during adolescence and adulthood (Davis et al., 2007; Huot et al., 2004; Pawlby et al., 2009). Research indicates that maternal depression during pregnancy skews the developing fetal stress systems in utero, possibly in response to high levels of the stress hormone cortisol transmitted via the placenta or mediated by placental corticotrophin-releasing hormone (CRH) (Kapoor et al., 2006a).

1.4 The hypothalamic-pituitary-adrenal axis and depression

The hypothalamic-pituitary-adrenal (HPA) axis is the body's stress response centre. It is composed of the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland and the adrenal cortex of the adrenal glands, located on the kidneys. The HPA axis is controlled by peptides released from the hypothalamus: corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). Various brain inputs, in particular from the hippocampus, amygdala and the raphe nuclei, converge on the PVN and the supraoptic nuclei to modify the release or inhibition of CRH or AVP. CRH, carried in a small blood vessel system to the anterior pituitary, stimulates the synthesis and release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH is released into the bloodstream and goes on to stimulate the production of glucocorticoids, such as cortisol in humans, from the zona fasciculata of the adrenal cortex (Bellavance and Rivest, 2014)[Figure 1]. Upon release, cortisol binds to both the high-affinity low-capacity mineralocorticoid receptors and the low-affinity high-capacity glucocorticoid receptors and mediates tissue-specific effects (Young et al., 2003). Cortisol feeds back to the hippocampus, the hypothalamus and the pituitary gland resulting in receptor-mediated negative feedback inhibition on the HPA axis. This negative feedback loop ensures the maintenance of relatively low cortisol levels during normal physiological conditions (Anacker et al., 2011).

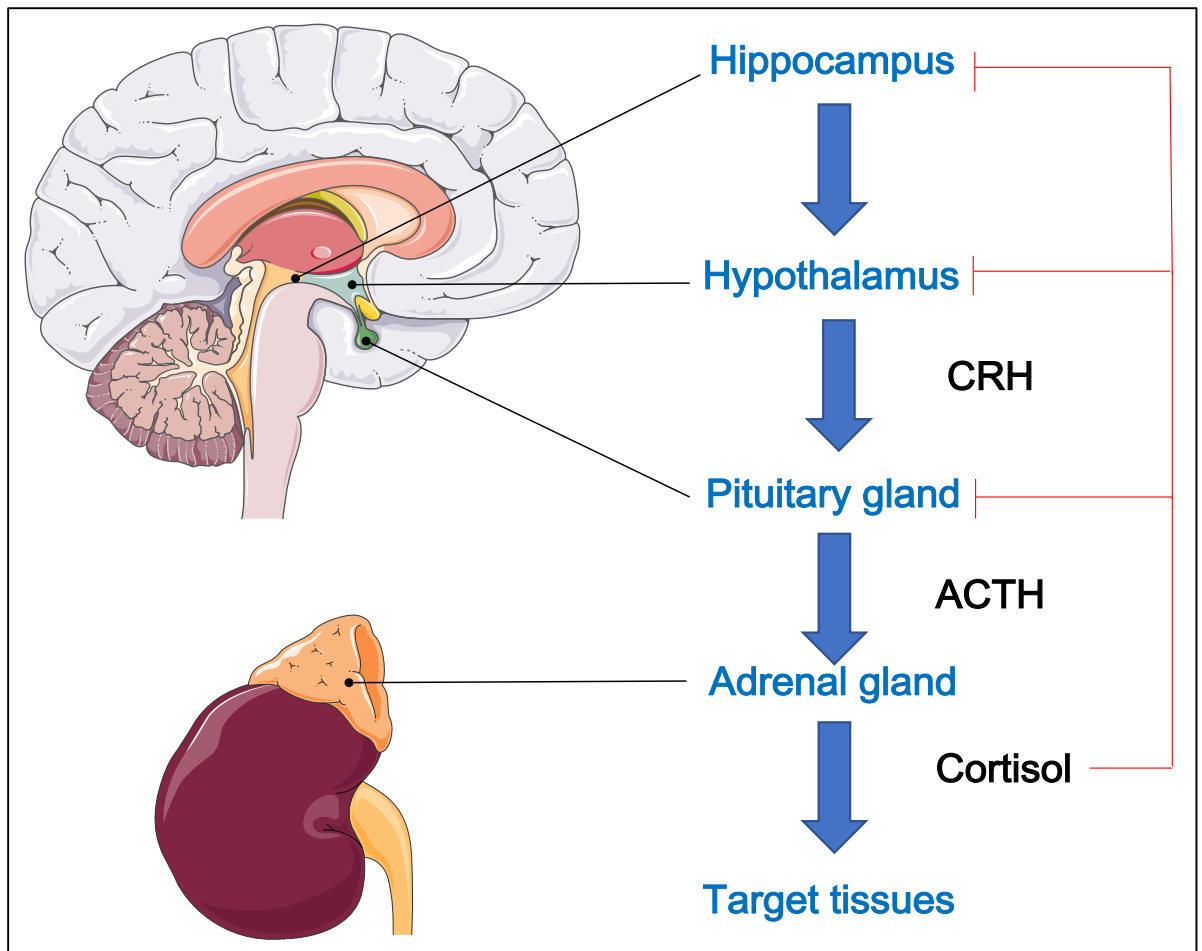


Figure 1.1 Schematic representation of the HPA axis. The hippocampus relays signals to the hypothalamus to stimulate the production and release of CRH. The release of CRH results in the release of ACTH from the pituitary gland which in turn stimulates the production and release of cortisol from the adrenal glands. Cortisol goes on to act on target tissues and will negatively feedback to inhibit both the hypothalamus and the pituitary gland. This reduces the secretion of CRH and ACTH thereby controlling activity of the HPA axis.

A common finding throughout the literature to date is the association of depression with hyperactivity of the HPA axis (Calfa et al., 2003). This HPA axis hyperactivity has been measured in several ways. Cortisol levels in plasma have been used to assess HPA axis activity. Increased cortisol levels have been observed in plasma (Gibbons and McHugh, 1962; Young et al., 2000) of depressed patients. Relative HPA axis hypersecretion is more likely in more severe depression, with the highest rates being found in those suffering with psychotic depression (Belanoff et al., 2001).

The dexamethasone suppression test (DST) has long been used to assess cortisol feedback function in depressed individuals (Carroll and Davies, 1968). In the DST, dexamethasone is given in various doses and cortisol levels, usually, 12 hours later, are measured.

Dexamethasone is a synthetic glucocorticoid that should provide negative feedback and suppress the secretion of ACTH leading to reduced ACTH levels and decreased cortisol levels. However, reduced suppression of ACTH and cortisol has been reported in various studies examining the DST in depressed and suicidal cohorts (Fountoulakis et al., 2004; Nuller and Ostroumova, 1980; Yerevanian et al., 2004).

The cortisol awakening response (CAR) refers to the brisk increase of cortisol levels within 20-30 minutes of awakening in the morning (Fries et al., 2009). Several studies have demonstrated an increased CAR in currently depressed individuals (Bhagwagar et al., 2005; Vreeburg et al., 2009) as well as recovered patients with depression (Bhagwagar et al., 2003).

1.4.1 The glucocorticoid receptor

The glucocorticoid receptor (GR), or nuclear receptor subfamily 3 group C member 1 (NR3C1), mediates the actions of the glucocorticoids. It belongs to the steroid nuclear superfamily of transcription factor proteins and is a ligand-dependent transcription factor, regulating the expression or suppression of glucocorticoid-responsive genes (Nicolaidis et al., 2010). The GR is a modular protein composed of distinct regions: an N-terminal transactivation domain (NTD), a DNA-binding domain (DBD), a hinge region and a C-terminal ligand-binding domain (LBD). The NTD accommodates a potent transactivation domain, known as activation function-1 (AF-1), which is ligand-independent. AF-1 is involved in the glucocorticoid receptor interacting with molecules required for the initiation of transcription, such as co-activators and basal transcription factors e.g. RNA-polymerase II, TATA-binding protein (Nicolaidis et al., 2010). The DBD is the most conserved domain among the nuclear receptors. It contains two zinc finger motifs that form its core structure (Helsen and Claessens, 2014). The zinc finger modules of the DBD within the GR recognise and bind to specific DNA sequences known as glucocorticoid-response elements (GREs) in the promoter region(s) of target genes (Nicolaidis et al., 2010; Oakley and Cidlowski, 2013). The DBD also plays important roles in receptor homodimerisation and interaction with cofactor proteins (Zhou and Cidlowski, 2005). The C-terminal LBD recognises and binds steroid hormone ligands, chaperones and other proteins. Further, it contains a ligand-dependent activation function (AF)-2. AF-2 folds to complete binding surfaces for other proteins e.g. co-activators and co-repressors (Kumar and Thompson, 2005).

The human glucocorticoid receptor cDNA was isolated in 1985 (Hollenberg et al., 1985). The gene is located on chromosome 5q31-32 and is composed of 9 exons. The 184 nucleotides of exon 1 represent exclusively the 5'-untranslated region (5'UTR). The GR NTD is encoded

mainly by exon 2, the DBD is encoded by exons 3 and 4, and the hinge region and LBD is encoded by exons 5-9 (Nicolaides et al., 2010; Oakley and Cidlowski, 2013). Alternative splicing in exon 9 generates two highly homologous receptor isoforms, labelled α and β (Zhou and Cidlowski, 2005). It is believed that, in normal physiological conditions, the default splicing pathway is the one leading to GR α and that the alternative splicing event leading to GR β is minimally activated (Pujols et al., 2002).

In the absence of glucocorticoids, the GR resides in the cytoplasm of the cell in an inactive state. It remains in this inactive state due to its binding to a multi-protein complex, comprised of heat-shock proteins and immunophilins, e.g. FK506 binding protein (FKBP5) (C. Anacker et al., 2011). When glucocorticoids are synthesised and released from the adrenal cortex, they readily cross the cell membrane, due to their lipophilicity. Once they enter the cell and bind to the GR, the receptor dissociates from the multi-protein complex, exposing its nuclear localisation signals, which are recognised by a group of nuclear translocation proteins e.g. ATP-stimulated translocator promoter (ASTP), pyrodoxal 5'-phosphate and molybdate thus allowing the glucocorticoid-glucocorticoid receptor (GC-GR) complex to freely translocate into the nucleus (Smoak and Cidlowski, 2004). Once inside the nucleus, the GC-GR dimerises and interacts with GREs in the promoter regions of glucocorticoid-regulated genes. Once bound to GREs, co-activators can arrive and further secure the GC-GR-DNA interaction by triggering the assembly of the general transcription machinery, resulting in an increase in gene transcription, or transactivation i.e. the activation of target genes.

In contrast, the GC-GR complex can bind to negative GREs (nGREs) on the promoter region of target genes to suppress gene transcription. The exact mechanism of nGRE-mediated repression has not been fully elucidated, but it has been hypothesised that it results from blocking the assembly of the basal transcriptional machinery (Nicolaides et al., 2010). The GC-GR is also capable of translocating to the cell nucleus as a monomer and interacting with other transcription factors like nuclear factor kappa B (NF κ B) and cyclic AMP response element binding protein (CREB). Binding to these transcription factors typically results in transrepression i.e. the repression of target genes. A well-known example of GC-GR-mediated transrepression is the repression of inflammatory cytokine expression. Other target genes include those for; IL-1 β , IL-2, TNF- α and inducible NO-synthase (iNOS), underlying the well documented immunosuppressive effects of the glucocorticoids (C. Anacker et al., 2011).

The link between HPA axis hyperactivity and depression (Section 1.3) has been hypothesised to result from defective regulatory feedback inhibition of glucocorticoids at the level of the hippocampus. It has been hypothesised that the dysfunctional feedback results from a

reduced efficacy of central glucocorticoid and/or glucocorticoid receptor function (O'Keane et al., 2012a). Results from an early study examining GRs in lymphocytes indicated lower GR levels in depressed patients relative to controls (Gormley et al., 1985). In the same study, changes in receptor concentration were positively correlated with post dexamethasone serum cortisol levels. A subsequent investigation examining GR level and function in depression noted alterations of GR mRNA level in the frontal cortex (Webster et al., 2002). However, other reports have found no differences in GR levels and/or function between depressed patients and healthy controls (Lopez et al., 1998). This could be partly due to methodological differences between studies or due to the fact that HPA axis dysregulation may only represent a subtype of depression.

1.4.2 The mineralocorticoid receptor in depression

The mineralocorticoid receptor (MR), or nuclear receptor subfamily 3 group C member 2 (NR3C2), is encoded by the *NR3C2* gene, which is located on chromosome 4q31.23 and covers 366,523bp. When considering the role of the HPA axis in depression, the majority of research to date has focused on the GR. The GR has been considered to be more important in the regulation of the response to stress when endogenous levels of glucocorticoids are high, such as depression. However, as negative feedback regulation of the HPA axis is dependent on both GRs and MRs, some studies have examined the potential role that the MR may have in the pathophysiology of depression. It has been suggested that there may be an imbalance in the MR/GR ratio in MDD patients that could play a role in the serotonin alterations observed in MDD (Young et al., 2003). Brain MR expression has been shown to be reduced in the hippocampus, inferior frontal gyrus and cingulate gyrus of individuals who had suffered with depression (Klok et al., 2011), further supporting the hypothesis that MR differences may contribute to the depressive phenotype. Individuals with psychotic major depression have diminished feedback inhibition of the HPA axis in response to the MR agonist fludrocortisone compared to healthy controls (Lembke et al., 2013) which highlights the possible role that MR dysregulation may have in the aetiology of depression.

1.4.3 FKBP5 in depression

The 51 kDa FK506 binding protein 5 (FKBP5) protein is encoded by the *FKBP5* gene (Binder, 2009). This gene is located on chromosome 6p21.31, covers 16400 kb and is comprised of 13 exons (Zannas et al., 2016). FKBP5 is one of the immunophilin proteins that make up the

inactive glucocorticoid receptor complex in the cytoplasm. FKBP5 negatively regulates glucocorticoid function by two main mechanisms:

- (1) when FKBP5 is bound to the receptor complex, cortisol binds with lower affinity and the nuclear translocation of the receptor is less efficient (Menke et al., 2013a)
- (2) FKBP5 expression is induced by steroids, including glucocorticoids, as part of an intracellular ultra-short negative feedback loop for glucocorticoid activity (Binder, 2009).

Thus, when cortisol levels are relatively high, FKBP5 levels are increased, leading to reduced activation of cortisol-induced transcriptional activity. Possession of an FKBP5 risk allele may define a neuroendocrinologically distinct subtype of depression. In vivo experiments performed on peripheral blood cells of individuals with the minor T-allele rs1360780 resulted in impaired GC-GR sensitivity, whereas the same cells from individuals who did not possess the risk T-allele did not display this impaired sensitivity following stimulation with dexamethasone. It is interesting to note that genotype-dependent differences were not observed at baseline (Menke et al., 2013a). The authors pointed out that this mirrors previous animal studies performed by Hartmann et al., 2012 showing that mice lacking the FKBP5 gene only displayed alterations in endocrine measures following acute or chronic stress.

In another study, the same T-allele variation has been associated with enhanced expression of FKBP5 protein following GR activation in depressed individuals, which leads to GR resistance, diminished negative feedback and prolonged stress hormone activation following a stressor (Binder et al., 2004a); mirroring previous work that has examined neuroendocrine differences in individuals with depression.

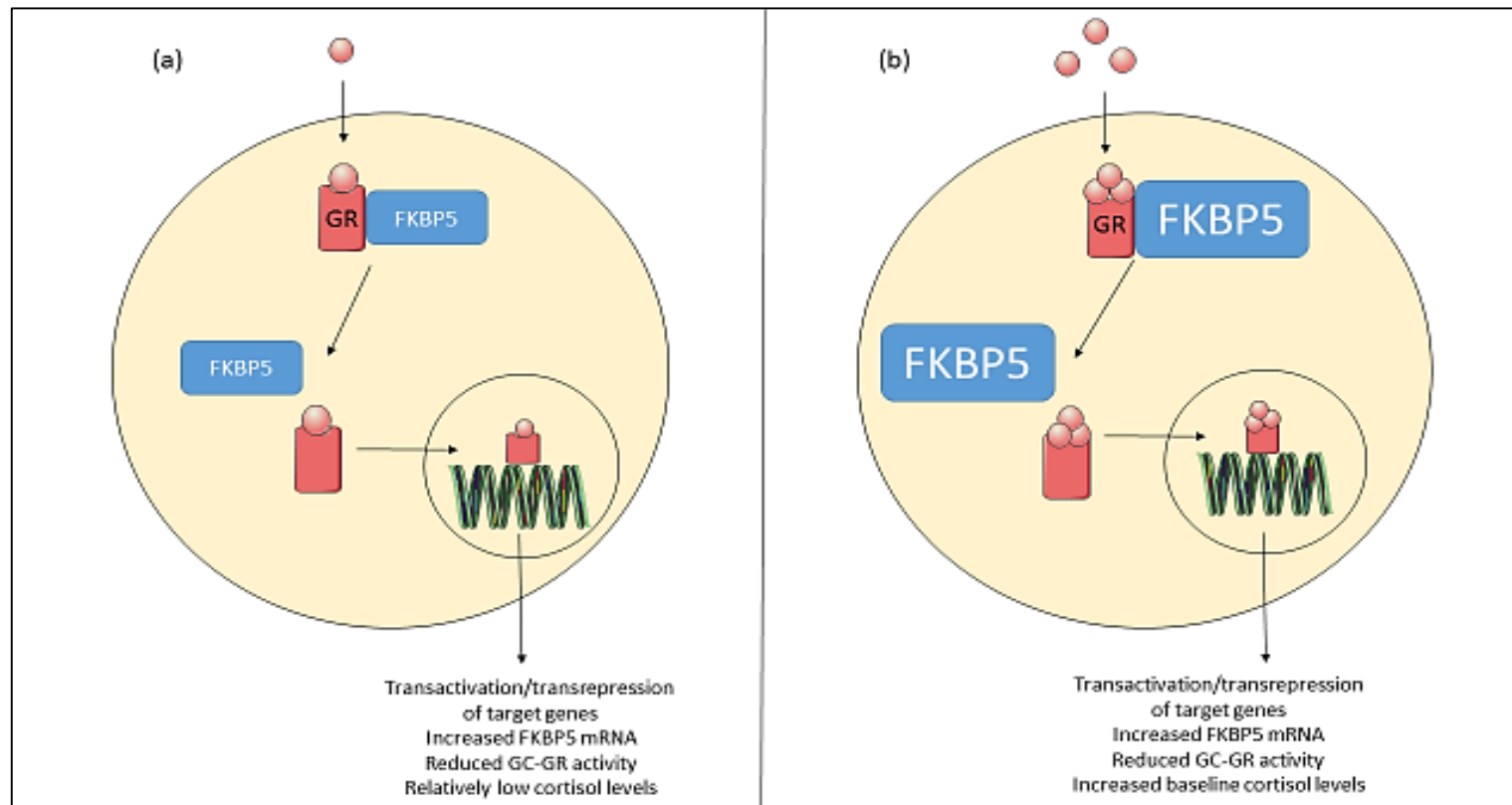


Figure 1.2 Glucocorticoid receptor and FKBP5 mechanism of action. Under (a) normal physiological conditions, the GR resides in the cytosol bound to a complex which includes FKBP5. A stress response occurring under normal HPA axis requires relatively low levels of cortisol to stimulate the activation and release of the GR from the GR-FKBP5 complex. The GC-GR complex translocates to the nucleus and effects target genes. This is accompanied by an induction of FKBP5 mRNA an appropriate HPA axis feedback. It is thought that possession of a risk allele and/or suffering from chronic stress/depression can lead to dysregulation of this mechanism. This is depicted in (b) where increased levels of FKBP5 in the cytosol results in higher levels of cortisol required to dissociate the GC-GR complex from FKBP5 leading to reduction in GC-GR activity, increased baseline cortisol levels and dysregulated GC-GR function, including GC-mediated HPA axis feedback. Diagram taken from Farrell and O'Keane (2016).

1.5 The immune system and depression

1.5.1 The innate immune system and depression

The innate immune system provides a first and rapid line of defence against many common microorganisms and is essential for the control of common bacterial infections (Janeway et al., 2001). Since the “macrophage theory of depression” was proposed over 25 years ago (Smith, 1991), there has been a considerable body of research that links increased activation of the innate immune system and inflammation to the development of depression (Miller, 2010). Numerous meta-analyses have revealed increased levels of pro-inflammatory cytokines including IL-1 β and IL-6 as well as the acute-phase protein, C-reactive protein (CRP) in depression (Dowlati et al., 2010; Howren et al., 2009; Valkanova et al., 2013). It has also been demonstrated that when pro-inflammatory cytokines (IFN- α and IL-2 immunotherapy) are administered to non-depressed individuals, depressive symptoms appear (Capuron et al., 2001). The anti-inflammatory effects of antidepressants have also been examined. Tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and serotonin-noradrenaline reuptake inhibitors (SNRIs) have been shown to display anti-inflammatory properties (Kubera et al., 2001).

Associations between increased pro-inflammatory and/or decreased anti-inflammatory cytokines levels with stress have been observed (Raison et al., 2006). One theory to explain this phenomenon is decreased glucocorticoid sensitivity. Miller et al., found that glucocorticoid sensitivity was decreased in depressed women relative to non-depressed controls (Miller et al., 2005). Due to the fact that glucocorticoids usually act as potent anti-inflammatory agents, this finding suggests that individuals suffering from depression may not have the capacity to fully control inflammation during stress (Raison and Miller, 2003).

There are four pathways through which cytokines effect changes in the central nervous system (CNS):

1. Activation of primary afferent neurons (e.g. vagus nerve)
2. Diffusion of peripheral cytokines through the brain's circumventricular organs (CVOs)
3. Cytokine transporters at the blood-brain barrier (BBB)
4. IL-1 activates receptors on perivascular macrophages and endothelial cells, resulting in the local release of prostaglandin E₂ (Almond, 2013).

1.5.2 The adaptive immune system and depression

Despite the expanding literature available on innate immune system alterations and depression, far less attention has been focused on the potential role of adaptive immune responses in the development of MDD. Adaptive immune responses are carried out by white blood cells called lymphocytes. There are two broad classes of adaptive immune responses: cell-mediated immune responses and antibody responses, and they are carried out by different classes of lymphocytes, called T-cells and B-cells, respectively (Alberts et al., 2002). Mounting data indicate that T-cell function is adversely affected in depression and that T-cells, through their neuroprotective and anti-inflammatory processes, may have a role in both the development and treatment of depression (Miller, 2010).

1.5.2.1 T-cells and their subtypes

T-cells are characterised by the presence of a T-cell receptor (TCR) on their cell surface (J Kuby et al., 2007). The TCR is a multi-subunit complex composed of TCR α , TCR β , cluster of differentiation (CD3) γ , CD3 δ , CD3 ϵ , CD3 ζ and CD3 η units (TCR-CD3 complex) (Wegener et al., 1992).

During their development in the thymus, T-cells are differentiated into two main separate subtypes: CD4⁺ helper T-cells or CD8⁺ cytotoxic T-cells. CD4⁺ helper T-cells are activated upon presentation of major histocompatibility complex (MHC) II molecules (Vyas et al., 2008). Further differentiation of various CD4⁺ helper T-cells results in a variety of effector responses which work alongside the innate immune system to remove pathogens (Hughes, 2014a). CD8⁺ cytotoxic T-cells are activated upon presentation of MHC I molecules (Vyas et al., 2008). Once activated, cytotoxic T-cells kill infected and transformed cells. The direct killing of cells is mediated by secretion of perforin and granzymes which cause apoptosis in target cells (Dong and Martinez, 2010).

Table 1.1 T cell subsets; their surface phenotype, transcription factors and effector molecules.
Adapted from Dong and Martinez, 2010.

T cell subtype	Surface phenotype	Transcription factors	Effector molecules secreted
Naïve CD4 ⁺ αβ T cell	Aβ TCR, CD3, CD4, CCR7, CD62L ^{hi} , IL-7R	THPOK	-
CD8 ⁺ αβ T cell	Aβ TCR, CD3, CD8, CCR7, CD62L ^{hi} , IL-7R	RUNX3	-
Helper T _H 1 cell	Aβ TCR, CD3, CD4, IL-12R, IFNγR, CXCR3	T-bet, STAT4, STAT1	IFNγ, IL-2, LTα
T _H 2 cell	Aβ TCR, CD3, CD4, IL-4R, IL-33R, CCR2, IL-17RB, CRTH2	GATA3, STAT6, DEC2, MAF	IL-4, IL-5, IL-13, IL-10
T _H 9 cell	Aβ TCR, CD3, CD4	PU.1	IL-9, IL-10
T _H 17 cell	Aβ TCR, CD3, CD4, IL-23R, CCR6, IL-1R, CD161	RORγt, STAT3, RORα	IL-17A, IL-17F, IL-21, IL-22, CCL20
T _H 22 cell	Aβ TCR, CD3, CD4, CCR10	AHR	IL-22
T _{FH} cell	Aβ TCR, CD3, CD4, CXCR5, SLAM, OX40L, CD40L, ICOS, IL-21R, PD1	BCL-6, STAT3	IL-21
Cytotoxic T cell	Aβ TCR, CD3, CD8	EOMES, T-bet, BLIMP1	Perforin, granzyme, IFNγ
Regulatory T _R 1 cell	Aβ TCR, CD3, CD4	Unknown	IL-10
Natural T _{Reg} cell	Aβ TCR, CD3, CD4, CD25, CTLA4, GITR	FOXP3, STAT5, FOXO1, FOXO3	IL-10, TGFβ, IL-35
Inducible T _{Reg} cell	Aβ TCR, CD3, CD4, CD25, CTLA4, GITR	FOXP3, FOXO1, FOXO3, STAT5, SMAD2, SMAD3, SMAD4	IL-10, TGFβ
Memory Central memory T cell	CCR7 ^{hi} , CD44, CD62L ^{hi} , TCR, CD3, IL-7R, IL-15R	BCL-6, BCL-6B, MBD2, BMI1	IL-2, CD40L, IL-4, IFNγ, IL-17A
Effector memory T cell	CD62L ^{low} , CD44, TCR, CD3, IL-7R, IL-15R, CCR7 ^{low}	BLIMP1	Rapid and high production of inflammatory cytokine

1.5.2.2 Evidence for T-cell alterations in depression

Early investigations examining the effects of stress and depression on T cell responses in humans revealed decreased proliferation of peripheral blood mononuclear cells (PBMCs) in response to specific T cell mitogens (Kronfol et al., 1983; Schleifer et al., 1984). Although subsequent studies attempting to replicate these findings have not always been successful, meta-analyses by Miller's group concluded that there are decreased T-cell responses in individuals who are stressed and/or are suffering with depression (Irwin and Miller, 2007; Miller, 2010). A number of possible mechanisms by which T cell function is altered in stress and depression have been proposed.

1.5.2.3 Decreased tryptophan and T-cells in depression

Increased T-cell apoptosis has been observed in situations of chronic stress in humans (Sakami et al., 2002). One possible explanation for this observed increase in apoptosis is tryptophan depletion. Tryptophan is essential for the successful proliferation of T-cells and T-cells will undergo apoptosis in tryptophan-depleted environments (Fallarino et al., 2002a). Mellor and Munn proposed that indoleamine 2,3-dioxygenase (IDO)-mediated local tryptophan depletion was responsible for the maintenance of allogenic pregnancies by limiting tryptophan supply to proliferating T-cells, preventing a T-cell response (i.e. rejection) to the developing fetus (Munn et al., 1998). As increased levels of pro-inflammatory cytokines, which have been reported in depressed individuals (Howren et al., 2009), are known to induce the enzymatic activity of IDO (Dantzer et al., 2011), this suggested mechanism could explain the reduced T-cell number and/or function seen in depression.

1.5.2.4 Glucocorticoid resistance and T-cells in depression

Another possible mechanism by which T cell function is attenuated in depression is via inhibition by glucocorticoids. Glucocorticoids are known to have numerous effects on the immune system and, under normal physiological conditions, can induce apoptosis in T cells, especially developing T cells in the thymus (McEwen et al., 1997). As mentioned in Section 1.4, increased secretion of the glucocorticoid cortisol has been reported consistently in the literature (Knorr et al., 2010a) and this is believed, in some cases, to result in glucocorticoid resistance (C. Anacker et al., 2011). Studies have demonstrated that glucocorticoid-induced inhibition on T-cell proliferation (Bauer et al., 2003a) was reduced, and decreased T-cell redistribution in depressed individuals (Bauer et al., 2002a). Miller has suggested that this failure of T-cells to respond to neuroendocrine trafficking signals could impact the ability of T-

cells to mobilise to the brain and transmit neuroprotective functions during stress (Miller, 2010).

1.5.2.5 Regulatory T-cells and depression

Regulatory T-cells (T_{reg}) are crucial for the maintenance of immune system homeostasis. Their modes of action can be grouped into the following: suppression by inhibitory cytokines (e.g. IL-10, TGF β), suppression by cytotoxicity (i.e. disruption of cells), suppression by metabolic disruption and suppression by modulation of dendritic-cell maturation or function (Vignali et al., 2008). Decreased IL-10 levels have been reported in depression (Dhabhar et al., 2009a) and as T_{reg} cells possess the ability to produce anti-inflammatory cytokines, a decrease in these cells could contribute to the pathophysiology of depression. A study in patients with PTSD reported an altered T_{reg} cell ratio compared to that in controls (Jergović et al., 2014).

1.6 Monoamines and depression

1.6.1 The monoamine hypothesis of depression

The monoamine hypothesis of depression states that the underlying pathophysiology in depression is a reduction in serotonin (5-HT), norepinephrine (NE), and/or dopamine (DA) neurotransmission in the brain (P. Delgado, 2000). As far back as 1959, the depressive effects of reserpine - an antihypertensive drug that blocks the vesicular monoamine transporter (VMAT) thus reducing the availability of synaptic monoamines - were observed (Quetsch et al., 1959). Many subsequent studies have gone on to investigate whether individuals suffering with depression have impaired monoamine transmission and/or decreased monoamine levels.

Table 1.2 Examples of studies which have noted altered monoamine systems in depression

Neurotransmitter	Alteration
Dopamine (DA)	Reduced dopamine transporter (DAT) density and elevated D ₂ /D ₃ receptor binding in post-mortem amygdala of depressed patients (Klimek et al., 2002)
Epinephrine (EPI)	Reduced EPI measured in cerebrospinal fluid of (CSF) depressed patients (Christensen et al., 1980)
Norepinephrine (NE)	Reduced norepinephrine transporters (NETs) in the locus coeruleus in depressed patients (Kilmek et al., 1997)
Serotonin (5-HT)	Reduced 5-HT measured in CSF of severely depressed patients (Hou et al., 2006)

Another compelling fact giving support to the monoamine hypothesis is that most pharmacological agents prescribed for the treatment of depression modulate the monoamine system (see Table 1.2). As mentioned previously in Section 1.2, these drugs eventually relieve depressive symptom severity in almost 70% of individuals (Rush et al., 2006a). Currently available antidepressant medications, however, all have limitations, including, prominently, a delayed onset of antidepressant response (typically a few weeks), resulting in considerable morbidity, disruption to personal and professional life, as well as an increased suicide risk (Machado-Vieira et al., 2010). Also, approximately 30% of individuals presenting with MDD will go on to have treatment resistant depression (TRD), which means that these individuals do not improve or only show a partial response to standard pharmacological treatments (Al-Harbi, 2012).

1.6.2 Serotonin and depression

Serotonin (5-hydroxytryptamine/5-HT) is the monoamine most frequently implicated and investigated in depression research. 5-HT is a monoamine neurotransmitter primarily found in the gastrointestinal (GI) tract and central nervous system (CNS) (Ormsbee and Fondacaro, 1985). 5-HT is synthesised from the amino acid L-tryptophan (Ruddick et al., 2006). Tryptophan is an essential amino acid, meaning that it cannot be synthesised de novo in the body and must be supplied through the diet. Tryptophan is first converted to 5-hydroxytryptophan (5-HTP) by the rate limiting enzyme tryptophan hydroxylase. 5-HTP is then metabolised to 5-HT via the enzyme aromatic amino acid decarboxylase (AAAD). The majority of 5-HT (approximately 90%) is located in the gut, where it is synthesised from tryptophan in the enterochromaffin cells of the gastrointestinal tract. The tryptophan that does not get metabolised to 5-HT in the gut is absorbed and is made available in the circulation, where it exists in both a free and albumin-bound fraction. At this stage, it can cross the blood-brain-barrier (BBB) via a large amino acid transporter (O'Mahony et al., 2015). In the CNS, tryptophan is taken up by serotonergic neurons, found primarily in nine clusters located mostly in the raphe nuclei of the midbrain, pons and medulla. The tryptophan is converted to 5-HT via the metabolic pathway and stored in synaptic vesicles. It is "docked" at the pre-synaptic nerve terminals, until its release by an action potential. Release of 5-HT into the synaptic cleft activates serotonergic neurons at the post-synaptic neuron (Gilmore et al., 2007). Once released from the pre-synaptic neuron, 5-HT may also bind to 5-HT autoreceptors located at the pre-synaptic terminal (Hjorth et al., 2000).

1.6.2.1 The 5-HT transporter (5-HTT)

The 5-HT transporter (5-HTT) is primarily responsible for terminating the action of 5-HT in the synaptic space, in the brain as well as other tissues, via reuptake of released 5-HT. It terminates the action of 5-HT by transporting it back into the pre-synaptic neuron where it is transported into vesicles using a sodium-dependent mechanism. The 5-HTT protein is encoded by the solute carrier family 6 member 4 (*SLC6A4*) gene (GeneCards, 2017a). In 1996, a functional polymorphism in the promoter region of *SLC6A4* was discovered (Heils et al., 1996), referred to as *5-HTTLPR*: a 44bp deletion/insertion generated two alleles of *5-HTTLPR*, with the 14-repeat short (s) variant having less transcriptional activity and lower serotonin uptake than the 16-repeat long (l) variant. Researchers speculated that the different transcriptional activity consequent on the polymorphism would modify serotonergic neurotransmission and thus could be implicated in affective disorders (Goldman et al., 2010).

The literature regarding the *5-HTTLPR* has been mixed. Early reports of associations between short form of the allele carriers and depressive disorders were promising (Collier et al., 1996) whereas more recent meta analyses have been unable to replicate these findings (Clarke et al., 2010). Other research has focused on the interaction that the *5-HTTLPR* polymorphism may have in relation to stress. Caspi et al., reported that individuals with one or two copies of the short allele displayed more depressive symptoms, diagnosable depression and suicidality in relation to stressful life events than individuals who were homozygous for the long allele (Caspi et al., 2003). A 2009 meta-analysis indicated that the *5-HTTLPR* polymorphism moderated the effect of environmental adversity in the development of depression. However this finding was dependent on the use of objective or contextual methods to assess adversity, rather than self-report instruments (Uher and McGuffin, 2009).

5-HT is degraded primarily by monoamine oxidase (MAO)-A, although the closely related enzyme MAO-B is present in some serotonin-containing cells in the brain, as well as in blood platelets (Murphy et al., 1998). Alterations in MAO levels may also play a part in the pathophysiology of depression. MAO-A expression has been shown to be elevated in the brains of those who had suffered from depression (Meyer et al., 2006) suggesting that increased degradation of 5-HT could contribute to depressive symptomology.

Due to the interest in 5-HT depletion in pathophysiology of depression, a significant body of research has examined the potential role of the precursor tryptophan in the development of MDD. Because the synthesis of 5-HT is dependent on the bio-availability of tryptophan (Fernstrom, 1983), it has been hypothesised that individuals with depression have decreased tryptophan concentrations resulting in reduced availability for 5-HT synthesis. Early studies identified that depressed patients exhibited significantly lower plasma tryptophan levels than healthy controls (Cowen et al., 1989a), and a recent meta-analysis indicates that this is a largely reproduced finding in the literature (Ogawa et al., 2014). Tryptophan is in competition with all large neutral amino acids for the BBB transport system (Young, 2007). This make it possible to induce temporary tryptophan depletion by administering tryptophan-free drinks that have a high content of other neutral amino acids. Taking such a drink results in an approximate decrease in plasma tryptophan of 75%. In some studies, tryptophan depletion has been shown to produce significant mood lowering in healthy controls (Smith et al., 1987). Other studies have shown an effect of tryptophan on mood in depressed individuals, and not in controls (Smith et al., 1997). Yet other studies have reported that depletion of tryptophan

has no significant effect on mood (Knott et al., 1999). Some of the discrepancies between these findings could be attributable to baseline mood states in the studies subjects (Bell et al., 2001).

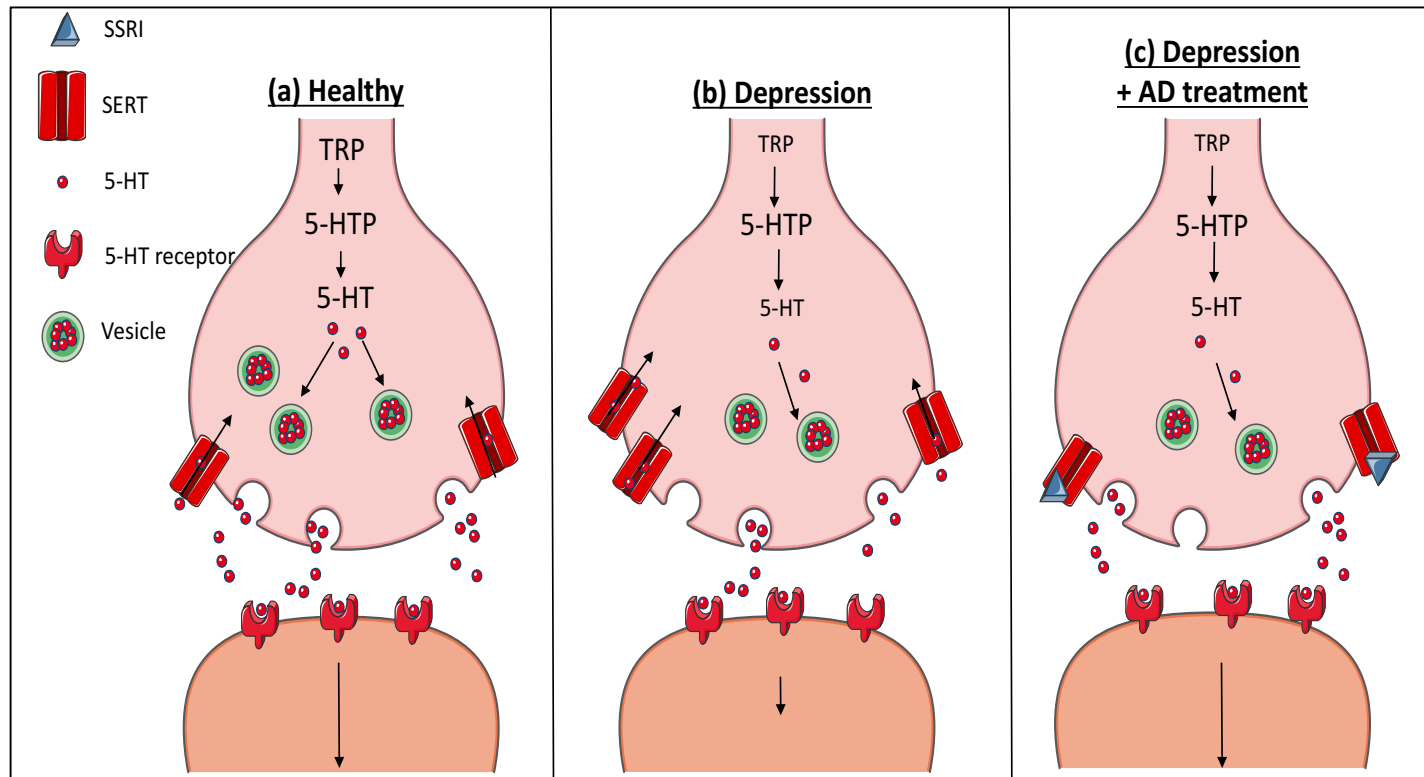


Figure 1.3 Serotonin hypothesis of depression (a) under normal physiological conditions the amino acid tryptophan is converted to serotonin by the enzymes tryptophan hydroxylase and aromatic-l-amino-acid decarboxylase. This serotonin is packaged into vesicles and is released via exocytosis when necessary. Once released, the serotonin binds to post-synaptic 5-HT receptors before being taken up back into the pre-synaptic neuron for repackaging into vesicles via SERT or is broken down by MAO. (b) The serotonin hypothesis of depression states that decreased levels of tryptophan result in a subsequent reduction of serotonin synthesis. Thus less 5-HT is packaged into vesicles and less 5-HT is available to be released into the synaptic cleft for post-synaptic receptor stimulation. It is also hypothesised that SERT may be overactive in depression, leading to a further decrease in available synaptic 5-HT. (c) The exact mechanism of many antidepressants is unknown, however, it is believed that SSRIs work primarily by blocking SERT thus reducing the re-uptake of 5-HT into the pre-synaptic neuron, allowing for increased post-synaptic 5-HT receptor binding. Diagram created using Servier Medical Art.

1.6.3 The kynurenine pathway

Although tryptophan is essential for the production of 5-HT, approximately 90% of tryptophan is used for the synthesis of kynurenine (Stone and Darlington, 2002). The conversion of tryptophan to kynurenine requires the induction of either of the enzymes, indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO). IDO can be induced in response to pro-inflammatory cytokines (IL-6, IFN- γ , IL-1 β) and TDO can be induced in response to increased cortisol levels. Whether converted via IDO or TDO, kynurenine can then be metabolised to either kynurenic acid (KYNA) by kynurenine aminotransferase (KAT) or to 3-hydroxykynurenine (3-HK) by kynurenine monooxygenase (KMO). 3-HK is further metabolised to 3-hydroxyanthranilic acid (3-HAA) by kynureninase (KYNU) or to xanthurenic acid (XA) by KAT. 3-HAA is further converted to quinolinic acid, the precursor for nicotinamide adenine dinucleotide (NAD⁺), by 3-hydroxyanthranilate 3,4-dioxygenase (HAAO)[See Figure 5]. In the CNS, KYNA is reported to have neuroprotective effects. Other kynurenine pathway products give rise to metabolites with reactive oxidative properties including 3-HK, 3-HAA and quinolinic acid that act as agonists at the NMDA receptor and may contribute to excitotoxicity and neurotoxicity (O'Farrell and Harkin, 2017).

It has been hypothesised that the decreased tryptophan concentrations observed in depressed patients could result from an increase in the metabolism of tryptophan in the kynurenine pathway, induced by altered inflammatory processes (Muller and Schwarz, 2007). Evidence suggests that certain processes of the kynurenine pathway may be implicated in depression. A role for IDO-related activation of the pathway in depression has been of great interest in the past 15 years. In the presence of an increased inflammatory state, it has been proposed that a subsequent increase in IDO may play a role in the pathogenesis of depression by shunting tryptophan down the kynurenine pathway rather than toward 5-HT synthesis (Miller and Raison, 2016). Processes downstream of kynurenine metabolism have also been shown to be dysregulated in individuals suffering with depression. Studies examining kynurenic acid, which is believed to have anti-excitotoxic effects, have shown decreases in its plasma concentration in depressed patients (Myint et al., 2007). The NMDA receptor agonist quinolinic acid, which may contribute to excitotoxicity when in excess, has been shown to be significantly higher in the CSF of individuals who attempted suicide (Erhardt et al., 2013). In one study, although there was no difference in the concentrations of individual kynurenine metabolites among currently MDD, remitted MDD patients and healthy controls, a reduction of kynurenic acid to quinolinic acid ratio in both the depressed and remitted MDD patients was observed when compared to healthy controls (Savitz et al., 2015). However, not all studies

support the hypothesis of kynurenine pathway induced tryptophan depletion in depression (Hughes et al., 2012a).

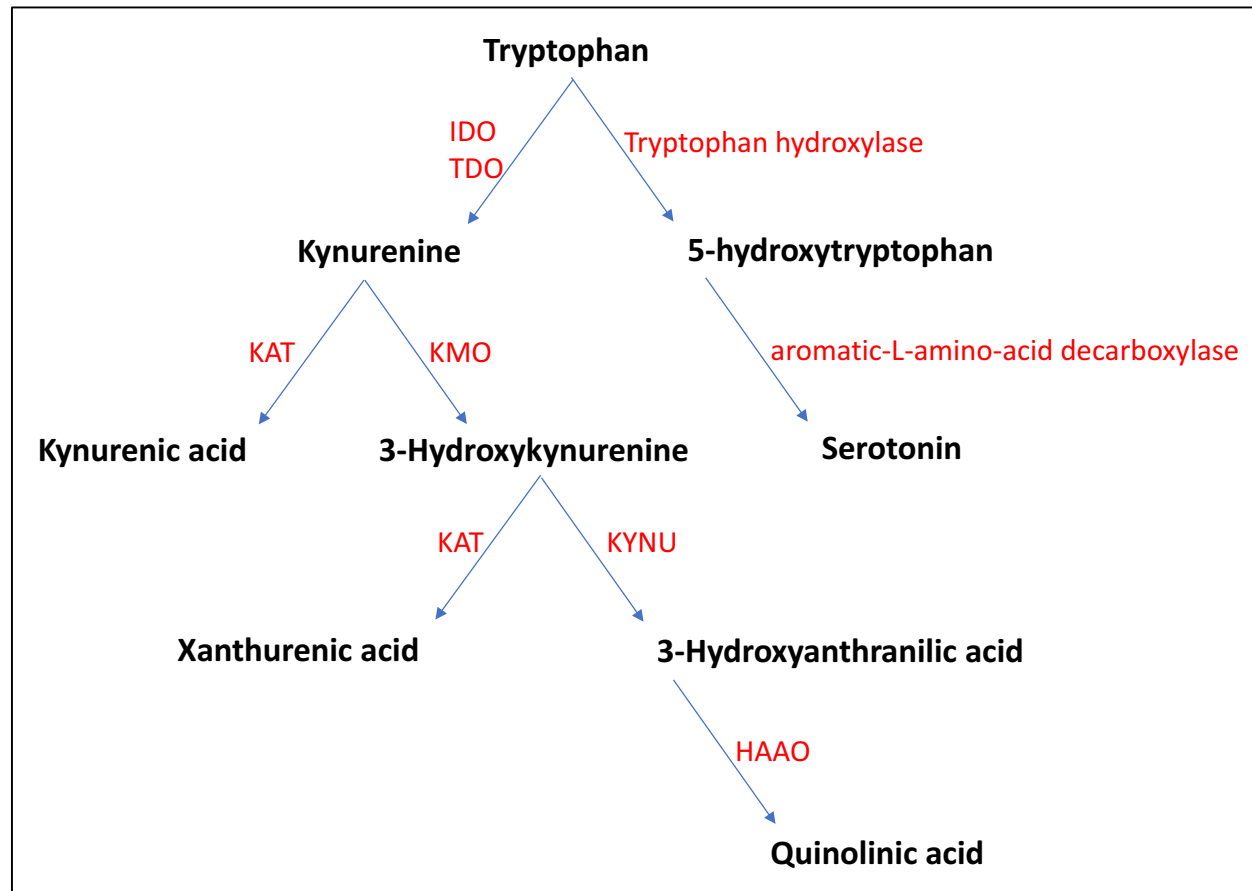


Figure 1.4 Tryptophan metabolic pathway. Tryptophan is metabolised down two pathways: (1) Approximately 10% of tryptophan is converted to serotonin via the enzymes tryptophan hydroxylase and aromatic-L-amino-acid decarboxylase (2) the majority (~90%) is metabolised down the kynurenine pathway via activation of IDO and/or TDO. Kynurenine is then further metabolised into many various products, including kynureninc acid and quinolinic acid. IDO=indoleamine 2,3-dioxygenase, TDO=tryptophan 2,3-dioxygenase, KAT=kynurenine aminotransferase, KMO=kynurenine 3-monooxygenase, KYNU=kynureninase, HAAO=3-hydroxyanthranilate.

1.7 Epigenetics

In the first half of the twentieth century, C. H. Waddington composed the term epigenetics to link the two fields of developmental biology and genetics. Today, epigenetic events are defined as: “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states”. This implies some alteration in the function of DNA rather than in the base pair pattern. The “Central Dogma of Molecular Biology” (Crick, 1970), although somewhat outdated today as a result of some major discoveries (including, but not limited to non-coding RNAs and the importance of introns), remains an important concept in appreciating the importance of epigenetic mechanisms. The Central Dogma explains the flow of information from DNA to RNA: transcription, and from RNA to protein: translation. Epigenetic mechanisms are essential for regulation of DNA structure and gene expression (Urduingio et al., 2009) as they can modulate events at the level of transcription and translation. Epigenetic modifications can be placed into three categories: DNA methylation, histone modifications and nucleosome positioning (Portela and Esteller, 2010).

1.7.1 DNA methylation

DNA methylation refers to the addition of a methyl group to a CpG (region of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases along its length) site. DNA methyltransferases (e.g. DNMT1, DNMT3a, DNMT3b) are the enzymes which establish and maintain methylation patterns (Kulis and Maher, 2010). DNMT1 is mostly responsible for the maintenance of DNA methylation. DNMT3A and DNMT3B generally perform de novo methylation of unmethylated or hemimethylated DNA to assist in maintenance (Liao et al., 2015). Addition of a methyl group to a CpG site typically results in gene silencing as the chromatin becomes tightly coiled and transcriptional machinery cannot access DNA. The majority of CpG dinucleotides in the human genome are methylated. However, unmethylated CpGs are grouped into clusters, known as CpG islands, in the promoter region of many genes (Lim and Maher, 2010). DNA methylation often occurs at these CpG islands. However, DNA methylation does not occur exclusively at these regions. DNA methylation can also occur in regions known as CpG shores (regions of CpGs that lie in close proximity to CpG islands). DNA methylation of these CpG shores is associated with transcriptional inactivation. It is important to note that, although DNA methylation is typically associated with transcriptional repression, in some instances it can be coupled with transcriptional activation (Portela and Esteller, 2010). This has been observed at gene bodies, where the methylation positively correlated with gene expression (Hellman and Chess, 2007).

1.7.2 Histone modifications

Histones are subject to several different post-translational modifications including, but not limited to acetylation, methylation, phosphorylation, ubiquitination and sumoylation (Portela and Esteller, 2010). Histone acetylation (or deacetylation) typically refers to the addition (or removal) of acetyl groups to the highly conserved N-termini of the histones H3 and H4. Histone acetylation is facilitated by histone acetyltransferases (HATs) which catalyse the transfer of acetyl groups from acetyl-CoA to lysine residues, resulting in ϵ -N-acetyllysine. Acetylation of histones H3 and H4 loosens nucleosome fibres which leads to more accessible chromatin. This allows for transcriptional machinery to access promoters and initiate transcription more freely (Eberharter and Becker, 2002). Deacetylation of histones is driven by histone deacetylases (HDACs). HDACs increase ionic interactions between positively charged histones and negatively charged DNA, which yields a more compact chromatin structure and represses gene transcription by limiting the accessibility of transcriptional machinery (Ropero and Esteller, 2007).

Histone methylation can occur on all basic residues: lysines, arginines and histidines (Martin and Zhang, 2005a; Di Lorenzo and Bedford, 2011; Pattaroni and Jacob, 2013). This methylation is catalysed by enzymes belonging to three distinct families of proteins: protein arginine N-methyltransferase 1 (PRMT1) family, SET-domain-containing protein family and the non-SET domain proteins DOT 1 and DOT 1L (disruptor of telomeric silencing) (Martin and Zhang, 2005b). Histone methylation has been associated with both transcriptional activation and repression (Hublitz et al., 2009). Histone phosphorylation can occur at all four nucleosomal histone tails. The tails contain acceptor sites that can be phosphorylated by numerous protein kinases and dephosphorylated by phosphatases. Histone phosphorylation can take place on serine, threonine and tyrosine residues. Similar to methylation, the phosphorylation of histone tails has been associated with both transcriptional activation and repression (Rossetto et al., 2012).

Histone ubiquitination is most abundant at H2A and H2B. H2A ubiquitination is frequently correlated with gene silencing, while H2B is mostly associated with transcription activation (Cao and Yan, 2012). Histone sumoylation has been shown to mediate transcriptional repression and gene silencing (Shiio and Eisenman, 2003).

1.7.3 Nucleosome positioning

The nucleosome is the basic repeating structural and functional unit of chromatin. It contains eight histone proteins and approximately 146 base pairs of DNA. Two of each of the histones

H2A, H2B, H3 and H4 come together to form a histone octamer, which binds and wraps DNA (Annunziato, 2008). Nucleosomes act as a physical barrier to transcription, as they block the access of transcriptional machinery to DNA and also inhibit the elongation of the transcripts by polymerases. The packaging of DNA into nucleosomes is thought to affect all stages of transcription. The precise position of nucleosomes around transcription start sites (TSS) has an important influence on transcription initiation (Portela and Esteller, 2010).

1.7.4 Depression...why epigenetics?

Meta-analyses have estimated that the heritability of depression is approximately 37% (Sullivan et al., 2000) but genome wide association studies (GWAS) have not reproduced gene loci that significantly contribute to the disorder. Depression has also been shown to have a discordance rate in monozyotic twins of approximately 50%, further highlighting the limited contribution that genetics have in the aetiology of depression. Evidence has repeatedly linked environmental factors, particularly exposure to ELA, with an increased risk in developing depression, as outlined earlier (Sun et al., 2013). Epigenetic mechanisms could provide an aetiological link between ELA and the syndrome of depression (Dalton et al., 2014).

1.7.4.1 Epigenetic alterations at *NR3C1* and depression

In relation to major depressive disorder and alterations in HPA axis activity, the most extensively investigated epigenetic modification to date has been hypermethylation of a nerve growth factor-inducible protein A (NGFI-A) binding site located in the hippocampal *NR3C1* gene exon 1F (1₇) promoter. NGFI-A is a member of a family of immediate-early gene-encoded transcription factors. It binds to and activates transcription at the sequence GCGGGGGCGG (Swirnoff and Milbrandt, 1995a). The majority of the available literature that reports alterations at the NGFI-A binding site in the *NR3C1* 1F promoter focuses on the link between early life adversity and the development of depressive illness (Klengel et al., 2014).

The findings to date indicate a probable role of exon 1F promoter hypermethylation in the pathogenesis of depression (Smart et al., 2015). This hypermethylation essentially blocks the binding of NGFI-A to its binding site at the promoter and subsequently affects the level of GR protein in the cytoplasm of the cell. If this were happening in brain limbic tissues, this would result in dysregulation of HPA axis negative feedback, uncontrolled cortisol release and ultimately depression. It is important to mention that this mechanism demonstrated by

various studies in peripheral tissues (Oberlander et al., 2008a; Perroud et al., 2011a; Radtke et al., 2015), may not also be occurring at the level of the hippocampus/brain, so a level of caution should be applied when interpreting methylation data from peripheral samples. This is because methylation occurs in a tissue-specific manner (Lokk et al., 2014).

Hypermethylation at the *NR3C1* gene, as a result of early life adversity, could potentially dysregulate glucocorticoid effects in other physiological systems, as the GC-GR complex is involved in a myriad of functions, including cellular metabolism, immunosuppression and glucose homeostasis (Necela and Cidlowski, 2004). This is interesting as dysregulation of many of these functions have been reported, if not implicated, in depression (Nagasawa et al., 2012; Nikolaus et al., 2000; Raison and Miller, 2001).

A breakthrough paper by Weaver et al., (2004a) reported that pups born to low-maternal care mothers were more fearful, had more exaggerated HPA axis responses to stress, and had corresponding reduced hippocampal GR protein expression and diminished glucocorticoid feedback sensitivity compared to the offspring born to the high care mothers. These results were mostly reversed by cross-fostering, i.e., high care offspring to low care mothers and vice versa, suggesting a non-genomic transmission of these differences and the possibility of an epigenetic mechanism. They concluded that these effects were the result of an increase in the methylation of the NGFI-A binding site in the exon 1₇ promoter of the *NR3C1* gene. On the grounds of these intriguing results, many researchers began translating this work into human studies. The human *NR3C1* gene has a highly similar 5' structure to that of the rat, and the human exon 1F, discovered by Turner and Muller (2005), is the orthologue to the rat 1₇ region (Moser et al., 2007).

McGowan et al., (2009) carried out one of the first translational studies on the *NR3C1* gene exon 1F promoter. They reported that those who had a history of childhood abuse and who died by suicide had increased cytosine methylation at specific sites on the NGFI-A binding site at the 1F promoter, decreased levels of the GR mRNA, and decreased mRNA transcripts that possessed the GR exon 1F variant. There were no differences in the epigenetic and related measures between those who were not abused and died by suicide and the controls, suggesting that these changes are related to childhood adversity, rather than suicide completion.

Oberlander et al., 2008a examined the link between prenatal exposure to maternal depression and levels of methylation at the NGFI-A binding site of the *NR3C1* gene in newborns and their HPA stress reactivity at 3 months of age. They found increased cytosine

methylation at the NGFI-A binding site and increased salivary cortisol stress responses in the babies born to depressed women. These findings were associated with maternal third trimester depressed or anxious mood, again supporting the hypothesis of an epigenetic process linking antenatal mood to increased methylation at sites on the *NR3C1* gene. Perroud et al., (2011a) examined the correlation between the extent of methylation of the *NR3C1* exon 1F promoter and the severity of childhood maltreatment in adults with depression, borderline personality disorder (BPD) and depression plus co-morbid post-traumatic stress disorder (PTSD). They found that childhood sexual abuse, its severity and the number of maltreatments correlated with *NR3C1* methylation. Radtke et al., (2011) demonstrated that maternal exposure to intimate partner violence during pregnancy influenced the methylation status of exon 1F promoter in the *NR3C1* gene in adolescent offspring. This was the first study to show that prenatal exposure to stress could have a long-term outcome on the methylation status in human offspring. Radtke et al., (2015) have also recently identified a positive association between methylation of a CpG site located in the *NR3C1* 1F exon and childhood maltreatment. The methylation of this site was correlated to depressive symptoms as well as borderline personality disorder features. The authors remarked that the participants in their study were exposed to moderate levels of maltreatment, highlighting the risks that moderate stress, and not just severe maltreatment, can have on mental health status.

Although many studies published to date indicate a role of hypermethylation of the *NR3C1* gene in depression, particularly when patients have experienced early life adversity, the findings are not consistent. A study by Alt et al., (2010) investigating glucocorticoid receptor expression in post-mortem brain tissues in individuals who had suffered from depression, found that there was no significant difference in methylation patterns between both groups (controls and MDD) and between different brain regions for any of the CpG sites that they examined (including the exon 1F promoter). However, NGFI-A expression was significantly decreased in the hippocampus, the cingulate gyrus and the nucleus accumbens of MDD patients. In their discussion, the authors suggest that some of their MDD patients may have been victims of childhood abuse events and build a case that increased *NR3C1* methylation, sometimes observed in depression, may represent a subtype following exposure to early life adversity.

A series of studies from the Yehuda group have examined *NR3C1* methylation in PTSD. Lower methylation levels at the *NR3C1* exon 1F promoter have been observed in individuals suffering from PTSD (Yehuda et al., 2014b) as well as offspring with both maternal and paternal PTSD (Yehuda et al., 2014a). The differences noted in the literature between increased and

decreased *NR3C1* methylation observed in depression and PTSD respectively, demonstrate that methylation at the 1F promoter can be increased or decreased in response to adversity. (Bremner, 1993; Kessler and Magee, 1993). Neuroendocrine findings are frequently abnormal in opposing directions in depression and PTSD, notably for HPA axis findings, with melancholic depression most commonly associated with hyperactivity of the HPA axis and PTSD, as well as atypical depression, associated with hypoactivity of the HPA axis (O'Keane et al., 2012a).

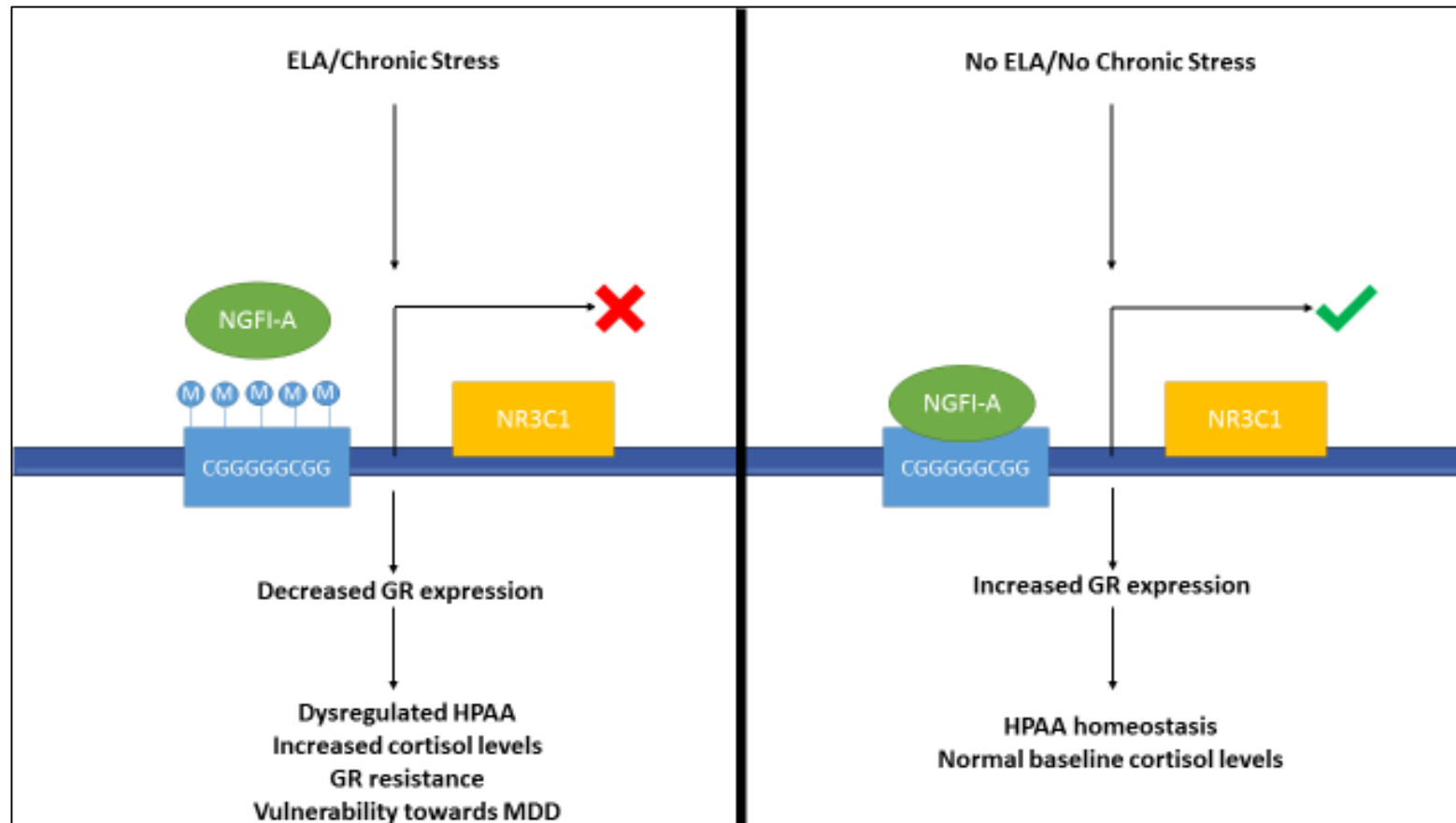


Figure 1.5 Regulation of glucocorticoid receptor expression by NGFI-A binding site hypermethylation. Recent evidence supports the theory that early life adversity results in hypermethylation of a NGFI-A binding site within the *NR3C1* exon 1F promoter. This hypermethylation leads to a subsequent decrease in GR expression, resulting in reduced GR-mediated negative feedback of the HPA axis. Dysregulation of the HPA axis is a robust finding in the search for depression aetiologies. ELA: early life adversity, NGFI-A: nerve growth factor-inducible protein A, NR3C1: nuclear receptor subfamily 3 group C member 1, GR: glucocorticoid receptor, HPA: hypothalamic-pituitary-adrenal

1.7.4.2 Epigenetic alterations at *FKBP5* and depression

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., (2013a) carried out the first human study looking specifically at methylation of the *FKBP5* gene-early life adversity interaction. They identified that individuals suffering from PTSD who were exposed to early life adversity and who carried a functional *FKBP5* risk T-allele (rs1360780) had decreased methylation at intron 7 of the *FKBP5* gene, compared to individuals who did not have a history of adversity and/or who did not carry the risk allele. This intronic demethylation was associated with an increase in *FKBP5* induction by GR activation, leading to glucocorticoid receptor resistance, a reduced binding of GCs to the GR and ultimately dysregulation of the stress hormone system. The majority of work examining epigenetic alterations at the *FKBP5* gene has focused on PTSD and or trauma (Yehuda et al., 2014a), so the field of alterations in methylation at this site specifically in depression is relatively novel. A recent study examining *FKBP5* DNA methylation in individuals with the risk T allele (rs1360780) and remitted depression had a (non-significant) trend towards higher DNA methylation levels compared to healthy controls (Höhne et al., 2015). However, much more work needs to be done examining *FKBP5* methylation status in depression to assess whether it is a useful target in assessing depression risk, severity and/or treatment response.

1.7.4.3 Epigenetic alterations at the *SLC6A4* promoter and depression

As described in Section 1.6.4, a considerable body of work has investigated the role that the serotonin transporter (5-HTT) may have in the pathophysiology of depression. Most of this work has studied gene-by-environment interactions (GxE) between the 5HTTLPR polymorphism and stressful life events. However, with improvements in epigenetic technologies, more focus has been channelled on differences in DNA methylation at the 5-HTT gene; solute carrier family 6 member 4 (*SLC6A4*). The *SLC6A4* gene is located on chromosome 17q.11 and has 15 known exons (GeneCards, 2017a).

A longitudinal study examining depression, specifically post-stroke depression (PSD), revealed significant increases in DNA methylation at the *SLC6A4* gene promoter in individuals with mild and major PSD when compared with non-PSD individuals. This increase in methylation was associated with PSD both 2 weeks and 1 year after stroke and was significantly associated with the worsening of depressive symptoms. Interestingly, these methylation differences were

only significant in the presence of the s/s 5-HTTLPR genotype, indicating that methylation changes in this region may be allele-specific (Kim et al., 2013).

Another recent investigation examined *SLC6A4* promoter methylation and stress-related change in hippocampal volume in depression relative to healthy controls. This study found that *SLC6A4* DNA methylation was negatively correlated with hippocampal volume and was positively correlated with childhood adversity (Booij et al., 2015). As decreases in hippocampal volume have been observed in depression (Sheline et al., 2003) and a high proportion of individuals with depression have experienced ELA (Williams et al., 2016), this study highlights the importance of trying to examine as many interacting factors in elucidating the complexity associated with depression aetiology.

Finally, in a study examining *SLC6A4* promoter methylation variation and depressive symptoms in a sample of monozygotic twin pairs, DNA methylation variation was significantly correlated with differences in Beck Depression Inventory (BDI) scores. Approximately, a 10% increase in mean DNA methylation levels were associated with a 4.4 increase in the difference in BDI scores (Zhao et al., 2013).

1.8 Hypothesis, Aims and Objectives

Investigations into DNA methylation alterations in psychiatric disorders are still in their infancy and although exploratory in nature, the overall hypothesis of the thesis was:

- (i) that stress and/or adversity experienced early in life (in utero or childhood) have the potential to impact upon mental health later in life;
- (ii) that this stress may mediate its effects via epigenetic mechanisms;
- (iii) that these epigenetic mechanisms may impact upon stress and immune related genes, resulting in altered HPA axis activity, changes in immune system markers and dysregulations in kynurenine pathway (KP) metabolism.

The overall aim of this thesis was to examine relationships among systems previously implicated in the pathophysiology of depression including the HPA axis, the tryptophan/kynurenine pathway and the adaptive immune system, with a particular focus on ELA and epigenetic mechanisms.

The aim of the first study was to determine if DNA methylation at *NR3C1*, *FKBP5* and *SLC6A4* were altered in individuals with MDD, and if so, whether these alterations were associated with changes in target gene expression, cortisol output, stress and inflammatory response system markers including tryptophan catabolites of kynurenine pathway. In Chapters 3-5, the studies attempt to isolate effects of ELA from depression. Analysis was performed on the depressed cohort while dividing into groups based on whether or not they were exposed to ELA.

Subsequent studies were carried out in peripheral blood mononuclear cells (PBMCs) obtained from depressed patients and healthy controls to assess epigenetic changes to the T Cell *CD3E* gene promoter and associated transcriptional and metabolic markers under baseline and stimulated conditions. DNA methylation levels were determined at the *CD3E* gene promoter and expression of T-cell associated genes were examined. Responses of PBMCs to the T-cell mitogen, anti-CD3, were determined.

Stress experienced in utero has been proposed to be the earliest form of adversity. Chapter 6 sought to investigate the effects of maternal depression during pregnancy on the new born. *NR3C1* and *FKBP5* DNA methylation status as well as cortisol concentrations were measured in both mother and baby to assess the potential role that clinical depression during pregnancy has on the epigenetic signature and subsequent stress response.

Chapter 2

Materials and Methods

2.1 Materials

Clinical Sample Collection

9 ml ethylene diamine triacetic acid (EDTA) VACUETTE®	Cruinn Diagnostics, IRL
9 ml sodium heparin VACUETTE®	Cruinn Diagnostics, IRL
Blood Collection Set + Blood Holder 19cm 23G x 3/4"	Cruinn Diagnostics, IRL
Cortisol-Salivette® saliva collection device	Sarstedt, IRL
PAXgene® Blood DNA tube (IVD)	Qiagen, UK
PAXgene® Blood RNA tube (IVD)	Qiagen, UK

Cell Culture

Anti-CD3	VWR International, IRL
Cryovials (2 ml)	Nunc®, USA
Dexamethasone	Sigma Aldrich, IRL
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, IRL
Dulbecco's phosphate buffered saline (DPBS) (10X)	Sigma Aldrich, IRL
Fetal bovine serum (FBS)	Bio-Sciences, IRL
Haemocytometer	VWR International, IRL
Histopaque®-1077	Sigma Aldrich, IRL
Lipopolysaccharide (LPS)	Sigma Aldrich, IRL
Mr. Frosty™ Freezing Container	VWR International, IRL
Penicillin-Streptomycin	Bio-Sciences, IRL
Plastic syringe (50 ml)	Becton Dickenson, UK
Roswell Park Memorial Institute (RPMI) 1640 Medium	Bio-Sciences, IRL
Serological pipette	Sarstedt, IRL

Sterile 48 well Nunc® plates	Sarstedt, IRL
Sterile Falcon™ tubes (15 ml)	Sarstedt, IRL
Sterile Falcon™ tubes (50 ml)	Sarstedt, IRL
Sterile microtubes (1.5 ml)	Sarstedt, IRL
Sterile Pasteur pipettes (3 ml)	Sarstedt, IRL
Syringe filter (0.2 µm)	Millipore, USA
Trypan blue	Sigma Aldrich, IRL

General Laboratory Chemicals

β-Mercaptoethanol	Sigma Aldrich, IRL
2-Propanol	Sigma Aldrich, IRL
3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma Aldrich, IRL
Potassium chloride (KCl)	Sigma Aldrich, IRL
Potassium phosphate monobasic (KH ₂ PO ₄)	Sigma Aldrich, IRL
Sodium chloride (NaCl)	Sigma Aldrich, IRL
Sodium phosphate dibasic (NaH ₂ PO ₄)	Sigma Aldrich, IRL
Sulfuric acid 98% (H ₂ SO ₄)	VWR International, IRL
Tween-20	Sigma Aldrich, IRL

General Laboratory Plastics and Hardware

Eppendorf™ tubes (1.5 ml)	Sarstedt, IRL
F96 MaxiSorp™ immunoplates for ELISA	Nunc®, USA
Micropipettes (20 µl, 200 µl, 1000 µl)	Fisher Scientific, UK
Microtubes (1.5 ml)	Sarstedt, IRL
Multichannel pipette (30 – 300 µl)	Fisher Scientific, UK
Parafilm®	Sarstedt, IRL

Pasteur pipettes	Sarstedt, IRL
Pipette tips (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Sarstedt, IRL
Polymerase chain reaction (PCR)	
Fast 96 well optical reaction plates	Applied Biosystems, UK
Filter tips (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Sarstedt, IRL
High capacity cDNA kit	Applied Biosystems, UK
Molecular grade absolute ethanol	Sigma Aldrich, IRL
Molecular grade water	Sigma Aldrich, IRL
NucleoSpin® RNA extraction kit	Macherey-Nagel, GER
Optical adhesive covers	Applied Biosystems, UK
PAXgene® blood RNA kit	Qiagen, UK
RNaseZap® decontamination solution	Ambion, UK
RNase-free water	Sigma Aldrich, IRL
Taqman® Fast advanced master mix	Applied Biosystems, UK
Taqman® gene expression assays	Applied Biosystems, UK

Enzyme-linked immunosorbent assay (ELISA)

Human CRP Quantikine ELISA kit	R&D Systems, UK
Human IL-10 ELISA MAX™ ELISA kit	Biolegend, UK
Human IL-6 ELISA MAX™ Deluxe kit	Biolegend, UK

Pyrosequencing

Agarose	Sigma Aldrich, IRL
Deoxynucleotide (dNTP) solution mix	NEB®, USA

EpiMark® Hot Start Taq Polymerase	NEB®, USA
EpiMark® Hot Start Taq Reaction Buffer	NEB®, USA
EZ DNA Methylation-Gold™ kit	Zymo Research, USA
FlexiGene® DNA kit	Qiagen, UK
GeneRuler 100 bp DNA ladder	Fisher Scientific, UK
Primers	IDT, USA
PyroMark® Annealing buffer	Qiagen, USA
PyroMark® Binding buffer	Qiagen, USA
PyroMark® Denaturation solution	Qiagen, USA
PyroMark® Gold Q96 aATPαS	Qiagen, USA
PyroMark® Gold Q96 dCTP	Qiagen, USA
PyroMark® Gold Q96 dGTP	Qiagen, USA
PyroMark® Gold Q96 dTTP	Qiagen, USA
PyroMark® Gold Q96 Enzyme mixture	Qiagen, USA
PyroMark® Gold Q96 Substrate mixture	Qiagen, USA
PyroMark® Wash buffer	Qiagen, USA
Streptavidin Sepharose high performance beads	GE Healthcare, USA
Tris-Borate-EDTA (TBE) buffer	Fisher Scientific, UK

2.2 Methods

2.2.1 Patient recruitment

2.2.1.1 Tallaght Mental Health Services

Depressed patients were recruited from the Tallaght Mental Health Services. Any patient presenting to the clinic with depression was considered for the study. They were screened for eligibility by Professor Veronica O’Keane, Consultant Psychiatrist, Tallaght Hospital, Dublin. The screening involved assessing whether the patient fulfilled criteria for a Major Depressive Episode from the Mini International Neuropsychiatric Interview (M.I.N.I), scored ≥ 17 on the Hamilton Depression Rating Scale (HAM-D) [see Section 2.2.1.3 for detailed scale information], was not suffering from a psychotic or substance abuse disorder, was not suffering from any chronic medical illnesses, and was not taking any steroid medications, except the oral contraceptive pill. Healthy, control individuals were recruited from the general population of Dublin. These controls were screened for eligibility by myself and Dr. Kelly Doolin. This screening involved establishing that individuals had never experienced a mental health complaint, 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 into the study to reduce the possibility of age-related effects on biological data. The groups were subsequently balanced for age and gender.

Once the participant met the inclusion and exclusion criteria, they were invited to participate in the study. If, following a thorough explanation of the study using the Patient Information Sheet (Appendix 3a), and willingness to participate, they gave written informed consent (Appendix 3b). As this was not an intervention study, all treatment continued for the patients as usual. Demographic information including BMI, smoking status and level of education were collected (Appendix 4a). The International Standard Classification of Education (ISCED) is a standardised scale used to organise information on educational achievement (UNESCO, 2011) and was used to grade participants level of education (Table 2.1). Patients were then administered a battery of clinical instruments (Section 2.2.1.5-2.2.1.7). The study was undertaken following the principles outlined in the 1964 Declaration of Helsinki (World Medical Association, 2013) and the ethical components of this study were approved by the Tallaght Hospital/St. James’s Hospital Joint Research Ethics Committee (2013/23/02).

Table 2.1 ISCED levels

ISCED level	Education completed
0	Early childhood education
1	Primary Education
2	Lower secondary education
3	Upper secondary education
4	Post-secondary non-tertiary education
5	Short-cycle tertiary education
6	Bachelor's or equivalent level
7	Master's or equivalent level
8	Doctoral or equivalent level

Table 2.2 Demographic data for all recruited depressed patients and healthy controls

	Patients (n=67)	Controls (n=59)	Statistic (p-value)
Age (years)	28.01 (8.61)	27.76 (6.15)	Z=-0.308, p=0.758
Gender (M/F)	20/46	23/36	$\chi^2=1.164$, p=0.347
BMI	25.97 (8.44)	23.13 (3.46)	Z=-1.349, p=0.177
Smoking (Y/N)	27/39	12/47	$\chi^2=6.141$, p=0.020*
ISCED Education	3.03 (1.79)	6.15 (1.61)	Z=-7.44, p<0.001***

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education. *p ≤0.05 vs. control, ***p≤0.001 vs. control.

Table 2.3 Clinical data for all recruited depressed patients and healthy controls

	Patients (n=67)	Controls (n=59)	Statistic (p-value)
HAM-D	22.76 (5.00)	2.96 (3.11)	Z=-9.30, p<0.001***
CES-D	38.85 (9.46)	7.14 (8.12)	Z=-9.17, p<0.001***
PSQI	12.62 (4.23)	4.04 (2.46)	Z=-8.37, p<0.001***
CTQ Global Score	45.43 (16.82)	30.19 (7.54)	Z=-5.51, p<0.001***
ELA (Y/N)	32/31	7/50	$\chi^2=20.2$, p=0.001***
Antidepressant (Y/N)	38/29	0/59	

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (ELA, Antidepressant use). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. control.

2.2.1.2 The Mini International Neuropsychiatric Interview

The Mini International Neuropsychiatric Interview (M.I.N.I) is a short, structured diagnostic interview developed by both psychiatrists and clinicians for DSM-IV and ICD-10 psychiatric disorders (Sheehan et al., 1998). It contains questions for the diagnosis of the following; A: Major Depressive Episode (with optional section for Melancholic Features), B: Dysthymia, C: Suicidality, D: (Hypo) Manic Episode, E: Panic Disorder, F: Agoraphobia, G: Social Phobia, H: Obsessive-Compulsive Disorder, I: Posttraumatic Stress Disorder, J: Alcohol Abuse and Dependence K: Non-Alcohol Psychoactive Substance Use Disorders, L: Psychotic Disorders and Mood Disorder with Psychotic Features, M: Anorexia Nervosa, N. Bulimia Nervosa and P. Antisocial Personality Disorder.

For the purposes of the study, Sections A, B and C were assessed and recorded for each patient (Appendix 4c).

2.2.1.3 Hamilton Depression Rating Scale

The Hamilton Depression Rating Scale (HAM-D), first developed in 1960 (Hamilton, 1960) is a clinician-rated scale used to assess depression severity. Questions rate severity of depression by probing mood, feeling of guilt, suicidal ideation, insomnia, psychomotor agitation or retardation, anxiety, weight disturbances and somatic symptoms. To meet criteria for the study, individuals had to score at least 17 in the HAM-D. Both myself and Prof. O'Keane completed HAM-D scales for each participant (Appendix 4b). To ensure tester consistency/inter-rater reliability concordance correlation coefficients (ρ_c) were calculated and only those with $\rho_c \geq 0.7$ were included in analysis.

Table 2.4 HAM-D severity rankings

Depression Severity	Score
Normal	0-7
Mild Depression	8-13
Moderate Depression	14-18
Severe Depression	19-22
Very Severe Depression	23+

2.2.1.4 Atypical depression specifiers from DSM-IV

The DSM-IV characterises depression with atypical features using the following criteria:

- A. Mood reactivity (i.e. mood brightens in response to actual or potential positive events)
- B. Significant weight gain or increase in appetite;
Hypersomnia;
Leadened paralysis (i.e. heavy, leaden feelings in the limbs);
A long standing pattern of interpersonal rejection sensitivity that results in significant social or occupational impairment.

If Section A and two components of Section B were present, the depressive episode was considered to be atypical (Appendix 4b).

2.2.1.5 Centre for Epidemiological Studies Depression Rating Scale

The Centre for Epidemiological Studies Depression Rating Scale (CES-D) is a self-rated depression scale (Orme et al., 1986). The CES-D is used to measure current levels of depressive symptomology, with an emphasis on the affective component. Other components of the scale assess feelings of guilt and worthlessness, feelings of helplessness, psychomotor retardation, loss of appetite and sleep disturbances (Appendix 4d). Possible range of scores is 0 to 60, with the higher scores indicating the presence of more symptomatology (Radloff, 1977).

2.2.1.6 Pittsburgh Sleep Quality Index

The Pittsburgh Sleep Quality Index (PSQI) is a self-rated instrument used to measure the quality and patterns of sleep in adults (Buysse et al., 1988). The PSQI measures seven components of sleep quality: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medications and daytime dysfunction (Appendix 4e). A score of 5 or greater is indicative of poor sleep quality.

2.2.1.7 The Childhood Trauma Questionnaire

The Childhood Trauma Questionnaire (CTQ) is a retrospective, self-report measure of early life adversity (Appendix 4f). The CTQ contains five subscales, three assessing abuse (emotional, physical and sexual) and two assessing neglect (emotional and physical). Each question pertains to one of the aforementioned subscales and has five possible answers: Never True, Rarely True, Sometimes True, Often True and Very Often True which are scored in a Likert-type fashion. CTQ scores range from 25-125. There is also a minimisation subscale to check

for response bias (Bernstein et al., 1997). Other traumatic events that may occur during childhood, including deaths, major illnesses or bullying, were not assessed.

Table 2.5 CTQ subscale scoring

	None	Low	Moderate	Severe
Emotional Abuse	5-8	9-12	13-15	16
Physical Abuse	5-7	8-9	10-12	13
Sexual Abuse	5	6-7	8-12	13
Emotional Neglect	5-9	10-14	15-17	18
Physical Neglect	5-7	8-9	10-12	13

2.2.1.8 The Perceived Stress Scale (Perinatal study only – Chapter 6)

The Perceived Stress Scale (PSS) is a self-rated instrument for measuring the perception of stress (Appendix 4g). It is a measure of the degree to which situations in one's life are perceived as stressful. Items in the questionnaire are designed to assess how unpredictable, uncontrollable, and overloaded respondents find their lives (Cohen et al., 1983). Each item is rated on a five-point scale ranging from never (0) to almost always (4). Higher scores are indicative of more perceived stress with scores around 13 considered normal and scores higher than 20 indicative of high stress.

2.2.3 DNA methylation analysis

2.2.3.1 PAXgene whole blood collection

2.5 ml whole blood was collected into PAXgene® Blood DNA tubes, which contain a proprietary K₂ EDTA preservative. Filled tubes were stored at room temperature for 24 hours, then moved to -20°C for 24 hours and were finally stored at -80°C until batch DNA extraction was performed.

2.2.3.2 DNA extraction from whole blood

DNA was extracted from whole blood collected into PAXgene® Blood DNA tubes using the FlexiGene® DNA Kit (Qiagen) as per the manufacturer's recommended protocol. Prior to DNA extraction, frozen blood samples were thawed for two hours at room temperature (RT). The thawed blood samples were then inverted 10 times to ensure homogeneity.

To access intracellular DNA 5 ml Buffer FG1 (lysis buffer) was added to a 15 ml Falcon™ tube before 2 ml whole blood was added and inverted 5 times. The Buffer FG1-whole blood mixture was centrifuged at 2000 x g for 5 minutes at RT. The liquid supernatant was discarded and the Falcon tube was inverted on a clean sheet of absorbent paper for 2 minutes.

To degrade proteins and enzymes 1 ml Buffer FG2/QIAGEN Protease (denaturation buffer) was added to the tube and was immediately vortexed until the pellet was completely homogenised. The tube was inverted 3 times, before being placed in a water bath for 10 minutes at 65°C.

1 ml isopropanol (100%) was added and was mixed by gentle inversion until the DNA precipitate became visible as white threads. The tube was then centrifuged at 2000 x g for 3 minutes at RT. The supernatant was carefully discarded, as to not disturb the DNA pellet, and the tube was inverted onto a clean piece of absorbent paper to remove any excess supernatant.

To wash the DNA 1 ml 70% ethanol (EtOH) was added and the tube was vortexed for 5 seconds before centrifugation at 2000 x g for 3 minutes at RT. The supernatant was discarded and the tube was inverted onto a clean piece of absorbent paper for at least 5 minutes. The tube was constantly monitored to ensure that the DNA pellet remained in the tube.

The pellet was then air-dried by standing the tube, lid off, in the laminar flow hood for 5 minutes. 200 µl Buffer FG3 (hydration buffer) was added to the DNA pellet before 5 seconds of vortexing at a low speed. The DNA was dissolved by incubating the tube in a water bath for 1 hour at 65°C.

2.2.3.3 DNA quantification and equalisation

The DNA quantity and quality of each sample was assessed based on optical density (OD) using the NanoDrop™ 1000 spectrophotometer (Thermo Scientific). The machine was blanked with 1 µl Buffer FG3 before 1 µl each sample was placed on the spectrophotometer and DNA concentration was measured at absorbance wavelengths of 260 nm. The quality of the DNA was assessed using the A260/280 ratio, with a range of 1.8-2.0 as an indicator of good quality DNA. To prevent cross-contamination between samples, the spectrophotometer was wiped down with clean tissue paper after each sample was measured. DNA samples were equalised to contain 25ng/µl in RNase-free water for subsequent bisulfite conversion.

2.2.3.4 Bisulfite conversion of DNA

DNA treatment with sodium bisulfite converts unmethylated cytosines to uracils which become thymines in subsequent PCR amplification, whereas methylated cytosines remain unchanged (Jones et al., 2012). The main steps of bisulfite conversion are DNA denaturation, incubation with sodium bisulfite at an elevated temperature, desulfonation of sulfonyl uracil adducts at an alkaline pH, and removal of the desulfonation solution (Darst et al., 2010).

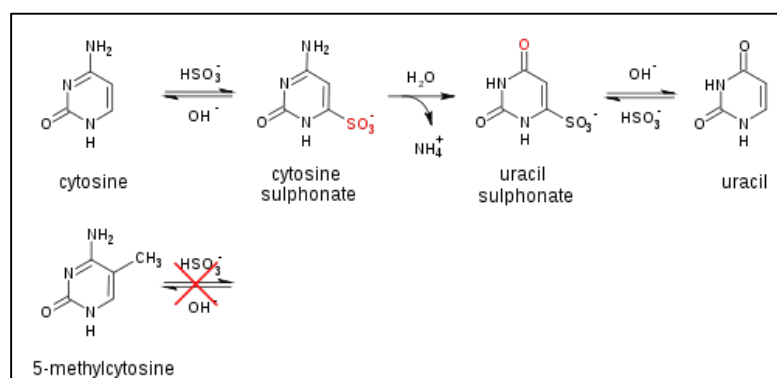


Figure 2.1 Bisulfite conversion of DNA. Bisulfite conversion, changing unmethylated cytosine to uracil, while 5-methylcytosine remains unaffected. HSO_3^- =bisulfite; NH_4^+ =ammonium. Image by Jujubix (2011).

Genomic DNA (gDNA) was bisulfite-treated using the EZ DNA Methylation-Gold™ Kit (Zymo Research) as per the manufacturer's recommended protocol. To convert unmethylated cytosines to uracils, 110 µl CT Conversion Reagent was added to 40 µl equalised DNA sample in a PCR tube and was centrifuged. The sample tube was placed into a thermal cycler (Biometra) and the following steps were performed: 98°C for 10 minutes, 64°C for 2.5 hours, 4°C for up to 20 hours.

After removal from the thermal cycler, the sample was desulfonated. 600 µl M-Binding Buffer was added to a Zymo-Spin™ IC Column. The entire 150 µl bisulfite-converted DNA sample was added to the column, which was inverted 5 times to ensure that the M-Binding Buffer and the bisulfite-converted DNA mixture was homogenous. The sample was centrifuged at 13,000 rpm for 1 minute at RT. The flow-through was discarded and the column was placed back into the same collection tube.

100 µl M-Wash Buffer was added to the column before centrifugation at 13,000 rpm for 1 minute at RT. 200 µl M-Desulfonation Buffer was added to the column and was left to incubate for 20 minutes at RT. After the incubation, the column was centrifuged at 13,000 rpm for 1 minute at RT. The flow-through was discarded and the column was placed back into the same collection tube.

200 µl M-Wash Buffer was added to the column and centrifuged at 13,000 rpm for 1 minute at RT. This was followed by the addition of another 200 µl M-Wash Buffer and centrifugation at 13,000 rpm for 2 minutes. The flow-through was discarded.

The column was transferred into a labelled 1.5 ml microcentrifuge tube. The lid of the column was opened for 2-4 minutes to allow ethanol from the wash stages to evaporate. 15 µl M-Elution Buffer was added to the column and was left to incubate for 5 minutes before centrifugation at 13,000 rpm for 1 minute at RT. An additional 15 µl M-Elution Buffer was added to the column and was left to incubate for 5 minutes before a final centrifugation at 13,000 rpm for 2 minutes at RT.

2.2.3.5 Bisulfite converted DNA quantification and equalisation

Bisulfite converted DNA (bisDNA) contains uracil residues, which leaves the DNA largely single-stranded as new converted uracil residues do not bind to thymine residues. For this reason, bisDNA quantity and quality of each sample was assessed using the RNA option on the NanoDrop™ 1000 spectrophotometer (Thermo Scientific). The machine was blanked with 1 µl

M-Elution Buffer before 1 µl each sample was placed on the spectrophotometer and bisDNA concentration was measured at absorbance wavelengths of 260 nm. The quality of the bisDNA was assessed using the A260/280 ratio, with a range of 1.8-2.1 as an indicator of good quality bisDNA. To prevent cross-contamination between samples, the spectrophotometer was wiped down with clean tissue paper after each sample was measured. bisDNA samples were equalised to a concentration of 10ng/µl in RNase-free water.

2.2.3.6 Polymerase Chain Reaction (PCR) for *NR3C1*, *FKBP5*, *SLC6A4* and *CD3E*

NR3C1 exon 1F promoter PCR

A 319 bp fragment corresponding to our region of interest within the *NR3C1* exon 1F promoter was amplified by touchdown PCR using the bisulfite-converted DNA. A master mix solution was made up of 5 µl EpiMark® Hot Start Taq Reaction Buffer (New England Biolabs® Inc.), 16.4 µl RNase-free water, 0.5 µl dNTP mix (10 mM), 0.5 µl forward primer: NR3C1F_LR_F1-biotin (10 µM), 0.5 µl reverse primer: NR3C1F_LR_R1 (10 µM) and 0.1 µl EpiMark® Hot Start Taq DNA Polymerase (New England Biolabs® Inc.). 23 µl master mix solution and 2 µl bisDNA was added to a PCR tube and placed in the thermal cycler. A no-template control (NTC) was included to monitor contamination of extraneous nucleic acids. PCR primers are outlined in detail in Table 2.10.

Table 2.6 Touchdown PCR thermal cycler settings used for *NR3C1* exon 1F

Phase 1	Step	Temperature	Time
1	Denature	95°C	1 minute
2	Denature	95°C	30 seconds
3	Anneal	66°C-55°C	40 seconds
4	Elongate	68°C	40 seconds
Repeat steps 2 – 4 (12 times, reducing temperature -1°C each cycle)			
Phase 2	Step	Temperature	Time
5	Denature	95°C	30 seconds
6	Anneal	54°C	40 seconds
7	Elongate	68°C	40 seconds
Repeat steps 5 – 7 (35 times)			
Termination	Step	Temperature	Time
8	Elongate	68°C	5 minutes
9	Stop reaction	4°C	Until removed from thermal cycler

***FKBP5* intron 7 PCR**

A 341 bp fragment corresponding to our region of interest within the *FKBP5* intron 7 was amplified by PCR using the bisulfite-converted DNA. A master mix solution was made up of 5 µl EpiMark® Hot Start Taq Reaction Buffer (New England Biolabs® Inc.), 15.6 µl RNase-free water, 0.5 µl dNTP mix (10 mM), 0.4 µl forward primer: FKBP5_in7_F2 (10 µM), 0.4 µl reverse primer: FKBP5_in7_R2-biotin (10 µM) and 0.1 µl EpiMark® Hot Start Taq DNA Polymerase (New England Biolabs® Inc.). 22 µl master mix solution and 3 µl bisDNA was added to a PCR tube and placed in the thermal cycler. A no-template control (NTC) was included to monitor contamination of extraneous nucleic acids. PCR primers are outlined in detail in Table 2.10.

Table 2.7 PCR thermal cycler settings used for *FKBP5* intron 7

Phase 1	Step	Temperature	Time
1	Denature	95°C	30 seconds
2	Denature	95°C	30 seconds
3	Anneal	59°C	1 minute
4	Elongate	68°C	1 minute
Repeat steps 2 – 4 (45 times)			
Termination	Step	Temperature	Time
5	Elongate	68°C	5 minutes
6	Stop reaction	4°C	Until removed from thermal cycler

***SLC6A4* promoter PCR**

A 162 bp fragment corresponding to our region of interest within the *SLC6A4* promoter was amplified by PCR using the bisulfite-converted DNA. A master mix solution was made up of 6 µl EpiMark® Hot Start Taq Reaction Buffer (New England Biolabs® Inc.), 19.1 µl RNase-free water, 0.6 µl dNTP mix (10 mM), 0.6 µl forward primer: SLC6A4_N2_F2 (10 µM), 0.6 µl reverse primer: SLC6A4_N2_R2-biotin (10 µM) and 0.1 µl EpiMark® Hot Start Taq DNA Polymerase (New England Biolabs® Inc.). 27 µl master mix solution and 3 µl bisDNA was added to a PCR tube and placed in the thermal cycler. A no-template control (NTC) was included to monitor contamination of extraneous nucleic acids. PCR primers are outlined in detail in Table 2.10.

Table 2.8 PCR thermal cycler settings used for *SLC6A4* promoter

Phase 1	Step	Temperature	Time
1	Denature	95°C	1 minute
2	Denature	95°C	30 seconds
3	Anneal	59°C	1 minute
4	Elongate	68°C	1 minute
Repeat steps 2 – 4 (40 times)			
Termination	Step	Temperature	Time
5	Elongate	68°C	10 minutes
6	Stop reaction	4°C	Until removed from thermal cycler

CD3E PCR

A 302 bp fragment corresponding to our region of interest within the *CD3E* gene was amplified by PCR using the bisulfite-converted DNA. A master mix solution was made up of 4 µl EpiMark® Hot Start Taq Reaction Buffer (New England Biolabs® Inc.), 12.7 µl RNase-free water, 0.4 µl dNTP mix (10 mM), 0.4 µl forward primer: CD3E_F1 (10 µM), 0.4 µl reverse primer: CD3E_R2-biotin (10 µM) and 0.1 µl EpiMark® Hot Start Taq DNA Polymerase (New England Biolabs® Inc.). 18 µl master mix solution and 2 µl bisDNA was added to a PCR tube and placed in the thermal cycler. A no-template control (NTC) was included to monitor contamination of extraneous nucleic acids. PCR primers are outlined in detail in Table 2.10.

Table 2.9 PCR thermal cycler settings used for *CD3E*

Phase 1	Step	Temperature	Time
1	Denature	95°C	30 seconds
2	Denature	95°C	30 seconds
3	Anneal	60°C	1 minute
4	Elongate	68°C	1 minute
Repeat steps 2 – 4 (45 times)			
Termination	Step	Temperature	Time
5	Elongate	68°C	5 minutes
6	Stop reaction	4°C	Until removed from thermal cycler

Table 2.10 List of primers used for PCR of bisDNA

	Forward Primer	Reverse Primer
NR3C1 exon 1F	NR3C1F_LR_F1-biotin: 5Biosg/GTTTTTAATTTTGGGA AAAAGGGT	NR3C1F_LR_R1: ACCCCTTTCCAAATAACACACTTC
FKBP5 intron 7	FKBP5_in7_F2: TGGGATAATAATTTGGAGTTATA GTGTAGG	FKBP5_in7_R2-biotin: 5Biosg/AAATTTATCTCTTACCTCCAACACTAC
SLC6A4 exon 1	SLC6A4_N2_F: TTGTTAGGTTTTAGGAAGAAAGA GAGA	SLC6A4_N2_R2-biotin: 5Biosg/AAAAAAACTACACAAAAAAACAAAT ATAC
CD3E	CD3E_F1: ATGGTTTTAGGGTTTTATTTTGA GTAGAT	CD3E_R2-biotin: 5Biosg/ACATAACACMAACAATTCCTTACAT

Table 2.11 List of sequencing primers

	Sequencing primer(s)
NR3C1 exon 1F	NR3C1F_LR_S3: AACTCCCAATAAATCTAAAAC NR3C1F_LR_S4: AAAAACTCCATAACCCTCT
FKBP5 intron 7	FKBP5_in7_S4: GTTGATATATAGGAATAAAATAAGA
SLC6A4 exon 1	SLC6A4_N2_S1: ATCGATGGTATCGATGGAGTAGTCGAGCTAGAGATGTCGTGATCGAG SLC6A4_N2_S2: GTCGTTGTAGTCAGTCGGTCGCTAGGATGTCGTA
CD3E	CD3E_S3B: AGTTTTATGAAATAAAGATGTAGT

2.2.3.7 Agarose Gel Electrophoresis

To ensure the presence of our desired biotinylated PCR amplicon, 3 µl PCR product was mixed with an equal volume of loading dye. Samples and a DNA ladder were then loaded to a 2% agarose gel (2 g agarose in 100 ml TBE buffer) and run at 100V for 30 minutes. The gel was removed from the electrophoresis tank and was stained with ethidium bromide before visualisation using an ultraviolet (UV) transilluminator.

2.2.3.8 Pyrosequencing using the PyroMark® Q96

Pyrosequencing is a sequencing-by-synthesis method that quantitatively monitors the real-time incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a proportional light signal (Tost and Gut, 2007). Pyrosequencing is a method commonly used in DNA methylation analysis, mutation analysis, genotyping and drug resistance typing. The technique is based on the detection of released pyrophosphate (PPi) during DNA synthesis. It employs a series of four enzymes to accurately detect nucleic acid sequences during synthesis. A sequencing primer is hybridised to a single-stranded biotin-labelled DNA template and in the presence of the following enzymes: DNA polymerase, ATP sulfurylase, luciferase and apyrase, the following substrates: adenosine 5'-phosphosulfate (APS) and luciferin and four deoxynucleotide triphosphates (dNTPs). The cascade starts with the release of PPi following the incorporation of a nucleotide to the DNA template by DNA polymerase. PPi is then converted to ATP by ATP sulfurylase in the presence of APS. This ATP provides the energy required for the luciferase-mediated conversion of luciferin to the light-emitting oxyluciferin. The light emitted by oxyluciferin is detected by a charge coupled device (CCD) camera and is read as peaks on a pyrogram. Finally, apyrase degrades unused ATP and non-incorporated dNTPs in the reaction mixture (Fakruddin, 2012).

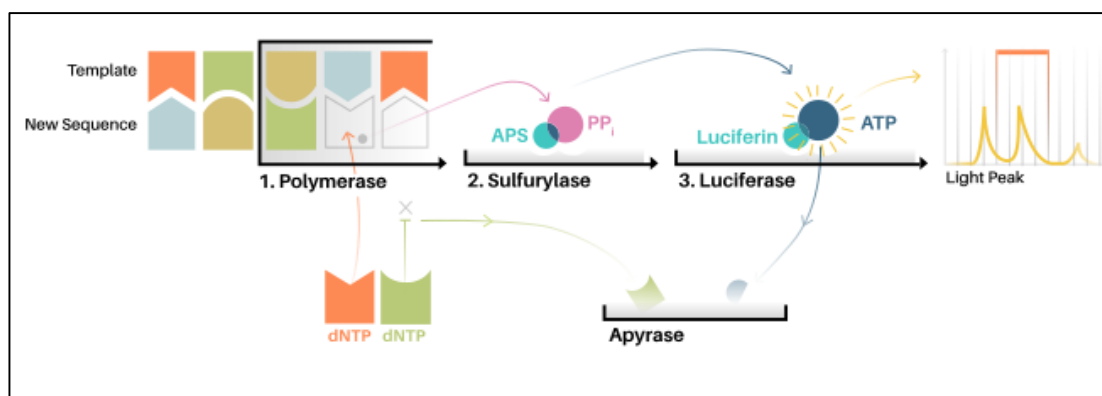


Figure 2.2. Pyrosequencing reaction cascade. Main stages of pyrosequencing: (1) Incorporation of dNTP to DNA template by DNA polymerase, (2) Conversion of PPi to ATP by ATP sulfurylase, (3) Luciferase-mediated conversion of luciferin to oxyluciferin, (4) degradation of excess dNTPs by apyrase. Image by Jacopo Pompili, DensityDesign Research Lab.

A mixture of 2 µl streptavidin sepharose beads, 40 µl binding buffer and 18 µl water per sample was prepared. 60 µl of this bead mixture was added to the wells of a 96-well PCR plate before addition of 20 µl biotinylated PCR product to each well. The PCR plate was sealed before constant agitation for at least 10 minutes at 1400 rpm to allow homogenous binding of PCR product to the beads. While the bead mix was shaking, 12 µl of 0.3 µM sequencing primer (diluted in annealing buffer) was added to each well of a PyroMark® Q96 Plate Low.

After shaking, the PCR plate and PyroMark® Q96 Plate Low were placed on to the work table in the same orientation as when the samples were loaded. Vacuum was turned on and the filter probes of the Q96 were carefully lowered into the PCR plate to capture the beads containing the PCR product. The probes were flushed for 5 seconds with 70% ethanol, then for 5 seconds with denaturation solution and finally for 10 seconds with wash buffer. The vacuum was closed and the beads were released into the PyroMark® Q96 Plate Low containing the 0.3 µM sequencing primer. The bead-primer mix was heated at 80°C for 2 minutes and was then placed into the PyroMark® Q96 machine where the pyrosequencing reaction was performed. Percentage methylation levels and quality control were analysed using PyroMark® CpG Software 1.0.11 (Qiagen).

2.2.4 FKBP5 DNA Genotyping

rs1360780 was genotyped from DNA extracted from whole blood (see Section 2.2.3.1-2.2.3.3). A TaqMan single-nucleotide polymorphism (SNP) Genotyping Assay was performed on a 7900HT Sequence Detection System (Applied Biosystems). The call rate, which is defined as the fraction of called SNPs per sample over the total number of SNPs in the dataset, for the TaqMan genotyping was >95% and all samples were in Hardy-Weinberg Equilibrium (HWE) ($p > 0.05$). The HWE is a principle stating that the genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors. This equilibrium can be disturbed by a number of forces (mutations, natural selection, genetic drift). These disruptive forces commonly occur in nature meaning that the HWE describes an idealised state, and genetic variations in nature can be measured as changes from this equilibrium state (Scitable, 2014). HWE is commonly used as a quality control measurement whereby inferences from genetic studies may be compromised if HWE is violated (Salanti et al., 2005). Along with the patient samples, a number of HapMap CEU DNA sample positive controls (www.hapmap.org) and non-template controls (NTC) were genotyped for quality control purposes.

The genotyping was kindly performed by Dr. Derek Morris and Ms. Laura Whitton, Discipline of Biochemistry, National University of Ireland Galway, Ireland.

2.2.5 In vitro studies

2.2.5.1 Aseptic technique

All cell culture work, including the preparation of cell culture reagents, was carried out in a laminar flow-hood (Hera safe, category 2) and aseptic technique was used throughout. Before and after use, the interior of the laminar flow-hood was sprayed with 70% ethanol (EtOH) to maintain a sterile environment. All materials brought into the flow-hood were sprayed with 70% EtOH beforehand to prevent the risk of contamination from the normal atmosphere. Other aseptic techniques utilised included the use of sterile and disposable plastic-ware and any glassware used for culture work was sterilised prior to use by autoclaving at 121°C for 2 hours. After use, the laminar flow-hood was exposed to ultraviolet (UV) light for 1 hour. Disposable powder-free latex gloves were worn at all times and were lightly sprayed with 70% EtOH before use in the laminar hood. Cells were kept in a Nuaire incubator at 37°C, 95% oxygen, 5% carbon dioxide and all items introduced to the incubator were sprayed with 70% EtOH. Both the laminar flow-hood and the incubator were cleaned on a regular basis with 70% EtOH to ensure a sterile working environment.

2.2.5.2 Preparation of culture media and test compounds

Anti-CD3: Anti-CD3 0.5 mg/ml stock solution was stored at 4°C. Prior to use, the stock was diluted to a working concentration of 0.1 µg/ml in 1X Dulbecco's phosphate-buffered saline (DPBS).

Culture Media: Using a 0.2 µM syringe filter, fetal bovine serum (FBS) and penicillin-streptomycin were filter-sterilised and added to sterile Rosewell Park Memorial Institute (RPMI) 1460 media which contained L-Glutamine and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to give complete RPMI (cRPMI). Final concentrations of FBS and penicillin-streptomycin in the media were 10% and 1% respectively.

Dexamethasone: 10 mg dexamethasone was dissolved in 10 ml 100% EtOH. 10 ml of the 1 mg/ml solution was added to 490 ml cRPMI to yield a stock solution of 20 µg/ml. Prior to use, the stock solution was diluted to a working concentration (100 nM and 10 nM) in pre-warmed cRPMI.

Dulbecco's Phosphate Buffered Saline (DPBS): 5 ml sterile DPBS (10X) was added to 45 ml double distilled water (d.d.H₂O) to yield a 1X working concentration of DPBS.

Lipopolysaccharide (LPS): 25 mg LPS [Escherichia coli serotype 0111:B4] was dissolved in 1 ml d.d.H₂O. The solution was vortexed until the LPS was fully dissolved. 500 µl of the 25 mg/ml solution was added to 62 ml cRPMI to yield a 200 µg/ml stock solution. The stock solution was filter-sterilised through a 0.2 µm syringe filter. Prior to use, the stock solution was diluted to a working concentration of 1 µg/ml in pre-warmed cRPMI.

2.2.5.3 Peripheral blood mononuclear cells (PBMCs)

2.2.5.3.1 PBMC collection, isolation and cryopreservation

Whole blood samples were taken from depressed patients and healthy controls in sodium heparin tubes. These blood samples were processed within 1 hour following blood draw to ensure maximum cell viability and recovery. 18 ml whole blood was gently layered over 18 ml Histopaque®-1077, a density gradient medium commonly used in mononuclear cell collection. This was centrifuged for 30 minutes at 500 x g and room temperature (RT) with the breaks off to allow gradient formation. The mononuclear cells, seen as a cloudy “buffy coat” at the interface of the plasma and Histopaque®-1077, were collected using a Pasteur pipette. The cells were transferred into a sterile 15 ml falcon tube and centrifuged for 10 minutes at 400 x g and RT with the breaks on to allow formation of a cell pellet. The supernatant was discarded and the cell pellet was re-suspended in 5 ml RPMI pre-warmed to 37°C before centrifugation for 10 minutes at 400 x g and RT. This step was repeated twice more for a total of three washes. At this stage, a small sample (~100 µl) was aliquoted so that the cells could be counted and cell membrane integrity could be assessed by trypan blue exclusion (as described in Section 2.2.5.3.3). The cell pellet was then re-suspended in 5 ml freezing medium (90% FBS + 10% DMSO) and aliquoted into cryovials. To ensure cell membrane integrity, the PBMCs were frozen down slowly using a Mr. Frosty™ Freezing Container at -80°C for 24 hours. The cells were then transferred into liquid nitrogen and stored until all samples had been collected, to allow for batch analysis.

2.2.5.3.2 PBMC thawing

Cells were thawed quickly to ensure viability. Following removal from liquid nitrogen storage, the frozen cells were placed immediately into a 37°C water bath for 1 minute. 1 ml cRPMI, pre-warmed to 37°C, was slowly added drop-by-drop into the cryovials. The vials were

inverted before the contents of each vial was added to 5 ml cRPMI at 37°C. Following centrifugation at 330 x g for 10 minutes at RT the supernatant was discarded and cells were re-suspended in 1 ml cRPMI at 37°C.

2.2.5.3.3 PBMC count and integrity

The cells were counted on the Sysmex KX-21N Coulter counter (Kobe, Japan) and cell membrane integrity was assessed using trypan blue dye exclusion. The trypan blue test is used to determine the number of viable cells in a cell suspension. It is based on the principle that live cells possess intact cell membranes that do not take up dyes, whereas dead cells do not have intact cell membranes and therefore take up dyes into the cytoplasm. A viable cell will have a clear cytoplasm and a nonviable cell will have a blue cytoplasm (Strober, 2001). 10 µl 0.4% trypan blue was mixed with 10 µl PBMC suspension in a reaction tube and allowed to incubate for 1 minute. 10 µl trypan blue-PBMC suspension was placed onto a disposable haemocytometer. A light microscope (OLYMPUS CKY41) was used to count the viable (unstained) and unviable (stained) cells. Based on these results, the PBMCs were equalised to a concentration of 1×10^6 cells/ml in cRPMI.

2.2.5.3.4 PBMC stimulation

For anti-CD3 stimulation, 200 µl of 0.1 µg/ml anti-CD3 solution was added to coat the appropriate wells on a 48-well plate and incubated overnight at 4°C. The following morning, excess anti-CD3 was aspirated from the wells and 450 µl cell suspension was added. The plates were incubated for 48 hours at 37°C and 5% CO₂ in a sterile incubator.

For LPS stimulation, 450 µl cell suspension was added to the appropriate wells. The cells were stimulated with 50 µl LPS, resulting in a final concentration of 1 µg/ml, and incubated for 48 hours at 37°C and 5% CO₂ in a sterile incubator.

For dexamethasone stimulation, 450 µl cell suspension was added to the appropriate wells. The cells were stimulated with 50 µl of either 10nM or 100nM (final concentration) dexamethasone and incubated for 24 hours at 37°C and 5% CO₂ in a sterile incubator.

As all samples were stimulated systematically over a number of weeks, stimulation plates were prepared in advance and frozen, thereby reducing variability between experiments if

treatments were prepared on a daily basis. The same batch of culture media, FBS and DPBS was used throughout, thereby reducing variability between plates.

2.2.5.3.5 Harvesting PBMCs for mRNA analysis

Following stimulation and incubation for the appropriate time, the 48-well plates were removed from the incubator. The culture media was pipetted up and down to gather cells from the wells before being transferred into 1.5 ml eppendorf tubes and centrifuged for 10 minutes at 12,000 rpm and 4°C. Keeping the RNA pellet on ice, the supernatant was removed and aliquoted. 353.5 µl RA1 lysis buffer containing 1% β-mercaptoethanol was added to the cell pellet and vortexed until the pellet had fully dissolved. The RNA samples were stored at -80°C until batch RNA extraction could be performed.

2.2.6 Real-Time Polymerase Chain Reaction (RT-PCR)

2.2.6.1 RNA isolation from PBMCs

Total RNA was isolated from PBMCs using NucleoSpin® RNA II kits (Macherey Nagel) as per the manufacturer's recommended protocol. Cells suspended in lysis buffer (350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol) were defrosted on ice. The cell lysate was filtered through a NucleoSpin® Filter Column, placed in 2 ml processing tubes, by centrifugation for 1 minute at 11,000 x g. To adjust RNA binding conditions, 350 µl 70% EtOH was added to the lysate and was mixed by pipetting up and down. The lysate-EtOH mix was transferred to a NucleoSpin® RNA Column placed in a 2 ml collection tube. RNA was bound to the column after centrifugation for 30 seconds at 11,000 x g. The column was placed into a new 2 ml collection tube and 350 µl Membrane Desalting Buffer was added and centrifuged for 1 minute at 11,000 x g to dry the membrane. A DNase reaction mixture (10 µl reconstituted rDNase and 90 µl Reaction buffer for rDNase per sample) was prepared to remove any remaining DNA from the column. 95 µl DNase reaction mixture was added directly to the center of the silica membrane of the column and was left to incubate at room temperature for 15 minutes. 200 µl Buffer RAW2 was added to the column to inactivate the rDNase. The column was centrifuged for 30 seconds at 11,000g. The column was placed into a new 2 ml collection tube and 600 µl Buffer RA3 was added before centrifugation for 30 seconds at 11,000 x g. Flow-through was discarded and the column was placed back into the collection tube. The column was washed once more with 250 µl Buffer RA3. The column was centrifuged for 2 minutes at 11,000 x g to dry the membrane completely. The column was placed into a 1.5 ml nuclease-free collection tube and RNA was eluted in 40 µl RNase-free water by centrifugation for 1 minute at 11,000 x g. The RNA samples were divided into 20 µl aliquots and were stored at -80°C until required for quantification, equalisation and further analysis.

2.2.6.2 PAXgene whole blood collection for mRNA analysis

2.5 ml whole blood was collected into PAXgene Blood RNA tubes. Once the sample was collected, the tube was inverted 10 times to mix whole blood with the proprietary stabilisation reagent inside the tube. This reagent allows for the stabilisation and preservation of RNA samples in whole blood. The samples were left at room temperature for 48 hours, were then stored at -20°C for 48 hours before long-term storage at -80°C until batch RNA extraction could be performed.

2.2.6.3 Isolation of total RNA from whole blood

Prior to starting, the bench was wiped down using RNase Zap decontamination solution to eliminate contamination and other RNases. The PAXgene Blood RNA kit was used to isolate RNA from PAXgene Blood RNA tubes as per the manufacturer's recommended protocol. The PAXgene Blood RNA tubes were removed from the -80°C freezer and were left to thaw for 2 hours at room temperature before inversion 10 times. The tubes were centrifuged at 3,000 x g for 10 minutes at RT. The supernatant was discarded and 4 ml RNase-free water was added to the pellet. The pellet was vortexed until it had completely dissolved and the tube was centrifuged at 3,000 x g for 10 minutes at RT. The supernatant was discarded and 350 µl resuspension buffer (Buffer BR1) was added to each pellet and vortexed until dissolved. To ensure complete protein digestion, the sample was transferred into a 1.5 ml microcentrifuge tube and 300 µl binding buffer (Buffer BR2) and 40 µl Proteinase K was added. Proteinase K is a broad-spectrum serine protease that inactivates RNases, DNases and enzymes. This was then quickly vortexed and incubated for 10 minutes at 55°C in the shaker-incubator at 300 rpm. The lysate was transferred directly into a PAXgene shredder spin column placed in a 2 ml processing tube and centrifuged for 3 minutes at 19,000 x g to homogenise the cell lysate and remove residual debris. To adjust RNA binding conditions, the supernatant was transferred to a new 1.5 ml microcentrifuge tube and 350 µl 96% EtOH was added. 700 µl of each sample was transferred into the PAXgene RNA spin column placed in a 2 ml processing tube and centrifuged for 1 minute at 19,000 x g, enabling RNA binding to the PAXgene silica membrane. To remove any remaining contaminants, 350 µl wash buffer (Buffer BR3) was added to the PAXgene spin column and was centrifuged for 1 minute at 19,000 x g. The flow-through was discarded and the spin column was placed into a new 2 ml processing tube. To ensure complete elimination from the column-bound RNA, a stock solution containing DNase and DNA digestion buffer (10 µl and 70 µl per sample, respectively) was added directly onto the membrane of the PAXgene column and left for 15 minutes. To remove the DNase, the column was washed with 350 µl wash buffer 1 (Buffer BR3), centrifuged for 1 minute at 19,000 x g and the flow-through was discarded. 500 µl wash buffer 2 (Buffer BR4) was then added to the column, centrifuged for 1 minute at 19,000 x g. The flow-through was discarded before the addition of another 500 µl Buffer BR4 to the column. The column was then centrifuged for 3 minutes at 19,000 x g. The PAXgene RNA spin column was then transferred to a new 2 ml processing tube and was centrifuged for 1 minute at 19,000 x g to completely dry the column. The PAXgene spin column was then placed in a 1.5 ml microcentrifuge tube to which 40 µl elution buffer (Buffer BR5) was added and centrifuged for 1 minute at 19,000 x g. The elution

step was repeated with another 40 µl Buffer BR5. The RNA was aliquoted and stored at -80°C until required for further analysis.

2.2.6.4 RNA quantification and equalisation

The RNA yield and quantity of each sample was quantified based on optical density (OD) using the NanoDrop®ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific). On blanking the machine with either 1 µl RNase-free water for PBMC RNA, or 1 µl Buffer BR5 for whole blood RNA, 1 µl of each sample was placed on the spectrophotometer and the RNA concentration was measured at absorbance wavelengths of 260 nm. The quality of the RNA was assessed using the ratio of A260/280. To prevent contamination, the spectrophotometer was wiped with tissue paper after each individual sample. RNA from whole blood was equalised to 10 ng/µl and RNA isolated from PBMCs was equalised to 2 ng/µl using RNase-free water as diluent.

2.2.6.5 cDNA synthesis

cDNA synthesis was carried out using a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems).

Table 2.12 cDNA master mix solution

	Volume (µl/sample)
RT Buffer (10X)	2
dNTP mix (25X)	0.8
Random primers (10X)	2
Multiscribe™ Reverse Transcriptase	1
RNase-free water	4.2
Total	10

A master mix solution was made up as per Table 2.10. 10 µl RNA sample was then added to 10 µl master mix. The samples were vortexed and centrifuged briefly before being placed in the thermal cycler (Peltier Thermal Cycler PTC-200) and the program was set to protocol as follows: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 seconds. Upon completion, the samples were centrifuged for 30 seconds and diluted 1 in 5 with molecular grade water before being stored at -20°C.

2.2.6.6 Multiplex RT-PCR

The StepOnePlus™ Real-Time PCR system (Applied Biosystems) in the 96-well format was used for quantitative (Q)-PCR. To quantify expression of target genes of interest, Taqman® gene expression assays (Applied Biosystems) were used. Each gene expression assay contained a specific set of forward and reverse primers and a fluorescein amidite (FAM)-labelled minor groove binder (MGB) probe for the target of interest (Table 2.11). PCR reactions were in a duplex format also containing a Taqman® gene expression assay containing a VIC (Applied Biosystems proprietary dye) labelled probe for the endogenous control gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH).

Table 2.13 Contents of each well

	Volume (µl/sample)
Diluted cDNA (1 in 5)	4
FAM-labelled target gene assay (20X)	0.5
VIC-labelled GAPDH assay (60X)	0.167
Molecular grade water	0.333
Taqman® Fast Advanced Master Mix	5
Total	10

The 96-well PCR plate was loaded according to Table 2.11. Sample were assayed over 50 cycles using Applied Biosystems' universal cycling conditions using a fast protocol as follows: 50°C for 2 minutes, 95°C for 20 seconds for polymerase activation (holding stage), 95°C for 1 second for each cycle (denaturation) and 60°C for 20 seconds (annealing). At the end of the reaction, data analysis was performed with the StepOnePlus™ System Software (Applied Biosystems) and relative quantification (RQ) values ($2^{-\Delta\Delta CT}$, where CT is the threshold cycle) of the target genes relative to their own endogenous control were obtained. The RQ values were then used to assess differences between groups or converted into fold change values relative to control group for cross-sectional analysis.

With regard to the cycling conditions, a high temperature of 95°C for 1 second is initially applied to separate the target DNA strands from each other. As the temperature lowers to 60°C the complementary primers and a fluorogenic probe anneal to the target sequence of interest enabling Taq polymerase to specifically amplify the target sequence. During extension and synthesis of the complimentary DNA, the 5' exonuclease activity of the Taq polymerase cleaves the probe with the subsequent release of the reporter molecule from the quencher,

resulting in a fluorescent emission. Consequently, at 60°C the exponential accumulation of PCR product over 50 cycles was measured cycle by cycle on the fluorescent emission.

Table 2.14 List of genes used with the GenBank sequence reference numbers

Target Symbol	Target Name	Taqman Gene ID
CD3E	Cluster of differentiation 3 epsilon	Hs01062241_m1
CD4	Cluster of differentiation 4	Hs01058407_m1
CD69	Cluster of differentiation 69	Hs00934033_m1
CD8A	Cluster of differentiation 8A	Hs00233520_m1
CD8B	Cluster of differentiation 8B	Hs00174762_m1
FKBP5	FK506 binding protein 5	Hs01561006_m1
FOXP3	Forkhead box P3	Hs01085834_m1
IDO1	Indolamine 2,3 dioxygenase 1	Hs00984148_m1
IFN-γ	Interferon gamma	Hs00989291_m1
IL-10	Interleukin-10	Hs00961622_m1
IL-1β	Interleukin-1beta	Hs01555410_m1
IL-4	Interleukin-4	Hs00174122_m1
IL-6	Interleukin-6	Hs00985639_m1
	inducible protein A	Hs00152928_m1
NGFI-A	Nerve growth factor	
NR3C1/GR	Glucocorticoid receptor	Hs00353740_m1
NR3C2/MR	Mineralocorticoid receptor	Hs01031809_m1
SLC6A4/SERT	Serotonin transporter	Hs00169010_m1

2.2.6.7 RT-PCR analysis

The $\Delta\Delta CT$ method (Applied Biosystems RQ Software, Applied Biosystems) was used to assess gene expression for all real-time PCR analyses. This method is used to assess relative gene expression by comparing gene expression of treated/experimental samples to a normal control or untreated control sample, rather than quantifying the exact copy number of the target gene. In this manner, the fold difference (increase or decrease) can be assessed between treated and control samples. The fold difference is assessed using cycle number (CT) difference between samples. Briefly, a threshold fluorescence is set, against which the CT is measured. To accurately assess differences between gene expression, the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence greater gene expression. When a PCR is 100% efficient a one-cycle difference in samples means a 2-fold difference in copy number (2^1), similarly a five-cycle

difference is a 32-fold difference (2^5). To measure this fold-difference relative to control, the CT of the endogenous control (GAPDH) is subtracted from the CT of the target gene for each sample, thus accounting for any difference in cDNA quantity that may exist. This normalised CT value is called the Δ CT. The Δ CT of the control is subtracted from itself to give 0, and subtracted from all other samples; this is the $\Delta\Delta$ CT value. The $\Delta\Delta$ CT (cycle difference corrected for GAPDH) is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of the $\Delta\Delta$ CT (difference in control and sample CT corrected for GAPDH) gives the fold difference in gene expression between the control and treated samples. The control sample always has a $\Delta\Delta$ CT value of 0, thus 0^{-2} gives a $2^{-\Delta\Delta$ CT of 1, against which all other samples are referenced.

2.2.7 Enzyme-linked immunosorbent assay (ELISA)

2.2.7.1 Blood collection for plasma

Blood samples were taken from depressed patients and healthy controls into K₂ EDTA tubes. The samples were centrifuged for 10 minutes at 3,500 rpm and room temperature to separate plasma from blood cells. The plasma was aliquoted and stored at -80°C until required for cytokine and LC-MS analysis.

2.2.7.2 ELISA for IL-6 and IL-10

Human IL-6 and IL-10 were quantified by sandwich ELISA using Biolegend ELISA MAX™ Deluxe kits as per the manufacturer's recommended protocol. The capture antibodies for IL-6 and IL-10 were diluted 1:200 in 1X Coating Buffer. 100 µl of the appropriate capture antibody was added to the wells of the 96-well maxisorb ELISA plates and incubated overnight at 4°C. Plates were then washed 4 times with 300 µl/well Wash Buffer (PBS + 0.05% Tween 20) and were subsequently blocked with 200 µl/well 1X assay diluent for 1 hour at RT with shaking at 200 rpm, to prevent non-specific protein binding. As before, plates were washed 4 times and 100 µl sample/standards were added to the appropriate wells and incubated for 2 hours at RT with shaking.

Plasma samples for both IL-6 and IL-10 were run neat. Standards were prepared according to the kit instructions; six two-fold serial dilutions of the top 500 pg/ml top standard were carried out with 1X assay diluent for IL-6. This was repeated for IL-10 with a top standard of 250 pg/ml.

Following 4 washes, 100 µl detection antibody, diluted 1:200 in 1X assay diluent, was added to each well and incubated for 1 hour at RT with shaking. This was followed by washes as before and the addition of 100 µl/well Avidin-Horseradish peroxidase (HRP), diluted 1:10,000 in 1X assay diluent, which was incubated for 30 minutes at RT with shaking. 5 washes were completed and 100 µl of freshly mixed TMB substrate solution was added to each well and incubated in the dark until the wells turned blue (approximately 20 minutes). The addition of 100 µl/well sulfuric acid (2N H₂SO₄) stopped the reaction. The absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and was then recalculated as a concentration (pg/ml) using standard curves derived using GraphPad Prism Software Version 6.00 (GraphPad Software, Inc).

2.2.7.3 ELISA for CRP

Human CRP was quantified by sandwich ELISA using R&D Systems Quantikine® ELISA kits as per the manufacturer's recommended protocol. The 96-well plates come coated with a mouse monoclonal antibody against CRP. Plasma samples were diluted 1:100 in 1X Calibrator Diluent RD5P. Standards were prepared according to the kit instructions; six two-fold serial dilutions of the top 50 ng/ml were carried out with 1X Calibrator Diluent RD5P.

100 µl Assay Diluent RD1F was added to each well before the addition of 50 µl standard or sample to the appropriate wells. The plate was covered and incubated at room temperature for 2 hours. Plates were then washed 4 times with 400 µl/well Wash Buffer. 200 µl CRP Conjugate was added to each well and was left to incubate at room temperature for 2 hours. Following 4 washes as previously mentioned, 200 µl Substrate Solution was added to each well and was left to incubate for 30 minutes at room temperature, protected from light. The addition of 50 µl/well Stop Solution stopped the reaction. The absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and was then recalculated as a concentration (pg/ml) using standard curves derived using GraphPad Prism Software Version 6.00 (GraphPad Software, Inc).

2.2.8 Salivary Stress Hormone Analysis

2.2.8.1 Saliva sample collection

Saliva samples were collected in Salivette® tubes (Sarstedt) by the study participant at five time points throughout the course of the day. These time points were 0, 30 and 60 minutes after waking, and 12 and 12.5 hours after waking. The participants were given detailed collection instructions in person and also via an information sheet (Appendix 3a). The tubes were either collected in person or returned by post as soon as possible. Once returned to the lab, the tubes were centrifuged at 1,000 x g for 2 minutes and room temperature. Saliva was aliquoted and stored in microtubes at -80°C until all samples had been collected.

2.2.8.2 Liquid chromatography-mass spectrometry (LC-MS) for salivary cortisol and cortisone

LC-MS is a technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry and was performed at the University of Manchester in the Department of Clinical Biochemistry. The work was performed by members of Prof. Brian Keevil's laboratory at the University Hospital of South Manchester using a previously described methodology (Owen and Keevil, 2007). Calculation of all cortisol awakening response (CAR) parameters were derived by Dr. Kelly Doolin. These parameters were Area Under the Curve (AUC), AUC with respect to increase from baseline (AUC_i), AUC with respect to ground (AUC_g) where ground is equal to zero, reactivity, peak, time from baseline to peak (TBP), slope of a regression line fitted through the morning time points (Slope) and the intercept of that regression line (Intercept). All CAR parameters were calculated using data from the first three saliva collection time points at 0, 30 and 60 minutes after waking.

2.2.9 Measurement of Kynurenine Pathway metabolites

Plasma was obtained from whole blood as described in Section 2.2.6.1. 50 µl plasma was analysed by LC-MS for measurement of kynurenine pathway metabolites in the laboratory of Dr. Andre Liesener in Boehringer Ingelheim. Tryptophan, 3-hydroxykynurenine (3-HK), quinolinic acid and kynurenic acid was quantified by validated assays based on liquid chromatography tandem mass spectrometry (LC-MS/MS). Tryptophan, 3-HK, quinolinic acid and kynurenic acid are quantified together in one assay, while kynurenine is quantified separately. Both assays comprised sample clean-up protein precipitation followed by reversed-phase chromatography and mass spectrometric detection in the positive ion multiple reaction monitoring (MRM) mode using the deuterated analogues of the analytes: tryptophan, 3-hydroxykynurenine, kynurenic acid, kynurenine and quinolinic acid as internal standards.

2.2.10 Statistical Analysis

Data are presented as mean with standard error of the mean (SEM) or standard deviation (SD) where appropriate. Categorical variables were tested using the Chi-Square (χ^2) test. All data were tested for normality using the Shapiro-Wilk test. Normally distributed data were analysed using the student's t-test/general linear model/two-way ANOVA, where appropriate. Non-parametric data were analysed using the Mann-Whitney U test for independent sample comparisons. Correlational analysis was carried out using Spearman rho statistics. All statistical analysis were considered significant when $p \leq 0.05$. Graphs were generated using GraphPad Prism Software Version 6.00 (GraphPad Software, Inc). All data were analysed using SPSS (Version 22, IBM).

Chapter 3

**Hypothalamic pituitary adrenal (HPA)
axis alterations in depression – DNA
methylation and early life adversity**

3.1 Introduction

Major depressive disorder (MDD) is a debilitating mood disorder characterised by intense feelings of sadness and/or a diminished interest or pleasure in almost all activities (American Psychiatric Association, 2013). It is estimated that approximately 10% of the population suffer from depression (Kessler et al., 1994) and the World Health Organisation predicts that it will be the largest contributor to disease burden by 2030 (WHO, 2008). While the precise biological basis of depression is unknown, a robust and consistent body of literature implicates a dysregulated stress response system in the pathophysiology of the disorder (Anacker et al., 2011).

The hypothalamic-pituitary-adrenal (HPA) axis is the body's stress response centre, the physiology of which is outlined in Chapter 1. Cortisol, the end product of the HPA axis, binds to the high-affinity, low capacity mineralocorticoid receptor (MR) and to the low-affinity, high capacity glucocorticoid receptor (GR) (Young et al., 2003). MR and GR can form heterodimers resulting in the regulation of target genes (Trapp and Holsboer, 1996). Glucocorticoids feed-back to the hippocampus, the PVN of the hypothalamus and the anterior pituitary gland resulting in receptor-mediated negative feedback inhibition of the HPA axis. This negative feedback loop ensures the maintenance of relatively low levels of cortisol during normal physiological conditions (Anacker et al., 2011).

FK506 binding protein 5 (FKBP5) is a GR co-chaperone protein that is crucial to effective HPA axis regulation. It regulates glucocorticoid function by binding to the receptor complex, and when so bound, cortisol binds with lower affinity (Menke et al., 2013b). FKBP5 expression is induced by cortisol, as part of an intracellular ultra-short negative feedback loop for glucocorticoid activity (Binder, 2009).

It is likely that depression has multiple etiologies. However, one of the most consistent findings in the literature to date is the association of depression with hyperactivity of the HPA axis (Calfa et al., 2003), and it is thought to be causal in at least some forms of depression. There is mounting evidence to suggest that this hyperactivity is caused by CRH hypersecretion. It has been hypothesised that the dysfunctional feedback results from a reduced efficacy of central, limbic system, glucocorticoid and/or glucocorticoid receptor function (O'Keane et al., 2012a). Impaired function of the glucocorticoid receptor causes glucocorticoid resistance (i.e. the inability of glucocorticoids to exert their effects on target tissues), that in turn results in compensatory increases in circulating CRH (Chrousos et al., 1993). Both increases in CRH and cortisol have been observed in individuals with depression (Gold and Chrousos, 2002;

Bhagwagar et al., 2005b). The cortisol awakening response (CAR) refers to a surge in cortisol levels upon waking. It is believed to reflect the sensitivity of the HPA axis to a challenge (i.e. waking), but may also play a role in processes including restoration of consciousness, changes in other hormones and the balance of the immune system (Dedovic and Ngiam, 2015). Increased salivary cortisol levels have been reported in depression (Bhagwagar et al., 2005b) and are believed to represent a dysregulated HPA axis.

Exposure to early life adversity (ELA) has been identified as a major risk factor in the development of depression (Kessler and Magee, 1993) and numerous studies have revealed that childhood maltreatment is associated with an increased HPA response to stress (Anacker et al., 2014). Epigenetic modifications, including DNA methylation, provide a mechanism by which adverse environmental stimuli result in lasting changes in gene expression (Dalton et al., 2014). In relation to MDD and hyperactivity of the HPA axis, two of the most promising candidates are a nerve growth factor inducible protein A (NGFI-A) binding site within the glucocorticoid receptor gene (NR3C1), exon 1F, and a region of the FKBP5 gene, intron 7. Alterations in methylation at these sites have been observed in depression (Perroud et al., 2011b; Klengel et al., 2013b) and have been associated with increased HPA axis activity, as measured by increased salivary cortisol measures (Oberlander et al., 2008b).

The aims of this study were to:

- (1) determine if DNA methylation at the NR3C1 exon 1F promoter and/or FKBP5 intron 7 were altered in individuals with depression, and if so, whether these alterations were associated with subsequent changes in gene expression in mRNA isolated from
 - (a) whole blood;
 - (b) peripheral blood mononuclear cells;at baseline and following stimulation with the synthetic glucocorticoid dexamethasone;
- (2) explore associations of DNA methylation with cortisol output, as well as with changes in cortisol dynamics;
- (3) specifically investigate the effects of early life adversity on DNA methylation and HPA axis measures.

3.2 Methods

A detailed description of the study participants, recruitment process, clinical rating scales and sample collection and processing are outlined in Chapter 2.

3.2.1 DNA methylation analysis of HPA axis markers in whole blood

DNA methylation of the glucocorticoid receptor gene (*NR3C1*) exon 1F and FK506 binding protein 5 (*FKBP5*) intron 7 was measured in whole blood by pyrosequencing, performed on bisulfite-treated DNA.

For *NR3C1* exon 1F, NR3C1F_LR_F1-biotin (5Biosg/GTTTTTAATTTTTTAGGAAAAAGGGT) and NR3C1F_LR_R1 (ACCCCTTCCAAATAACACACTTC) were the forward and reverse primers, respectively, for the PCR reaction. NR3C1F_LR_S3 (AACTCCCAATAAATCTAAAC) and NR3C1F_LR_S4 (AAAAAACTCCATAACCCTCT) were the sequencing primers used in the pyrosequencing reaction.

```
GTTTTTAATTTTTTAGGAAAAAGGGTGGCGGCGGTAGCGGCGGGGGTCGATTGGTTTTTTGGGGC
GGCG41TTAAGAGGGTTATCG40AGTTTTTTTAGTTTTTTTTTCG39TTATTTTTTTTCG38TTTTTCG37TTT
AGTTTCG36CG35GTTTTAGATTTATTCGGGAGTTCGTTTTGTTTTTGGCGGTAAGCGTCGTTAGTGTT
TTTGGGGTGATAGCGGGCGGGTTATAAGAGTCGGGGCGTTTTTCACGGTTGAGTTGCGTGAGTGG
TTCGCGTCGACGTCGTCGGGTCGAGTTGCGTGAAGTGTTATTTGAAAGGGGT
```

Figure 3.1 319 bp PCR amplicon of *NR3C1* exon 1F, lower strand, converted sequence. Measured CG sites are in bold text. NGFI-A binding site is underlined.

For *FKBP5* intron 7, FKBP5_in7_F2 (TGGGATAATAATTTGGAGTTATAGTGTAGG) and FKBP5_in7_R2-biotin (5Biosg/AAATTTATCTTACCTCCAACACTAC) were the forward and reverse primers respectively, for the PCR reaction. FKBP5_S4 (GTTGATATATAGGAATAAAATAAGA) was the sequencing primer used in the pyrosequencing reaction.

```
TGGGATAATAATTTGGAGTTATAGTGTAGGTTTTTTCGTGATTTTTGTGAAGGGTATAATTCGTTTAG
TTTTGAAAAGTTGTATTTTATTTTTTAAGGAGTTATTTGGTAGAACGTGAATTTTTTTGTTTTTAATTT
AGGAAAAAAAAGTATAAAAAGAATAAGTTTAGGAATAAATAAGGGAATAAGTTTTGGATTTTATTT
AAAAAAGTTAAAAAAGTTGATATATAGGAATAAAATAAGAATACG6GAGTTTTTTTCG7
TTGTATATTAGTTGTGTTATGTTAGTTGTTTTATTTTTTAGTAGTAGTGTGGAGGTAAGAGATAAAT
TT
```

Figure 3.2 341 bp PCR amplicon of *FKBP5* intron 7, upper strand, converted sequence. Measured CpG sites are in bold text.

In total, percentage methylation was measured at seven CG sites: five in *NR3C1* exon 1F and two in *FKBP5* intron 7. Pyrosequencing was performed using the Q96 platform (Qiagen). Percentage methylation levels and quality control were analysed using PyroMark CpG Software (Version 1.0.11, Qiagen). Detailed methodology of the pyrosequencing procedure is outlined in Chapter 2 – section 2.2.3.

3.2.2 Real-time PCR analysis of HPA axis mRNA markers in whole blood

Total RNA was extracted from whole blood collected into PAXgene Blood RNA tubes using the PAXgene Blood RNA kit (Qiagen) as per the manufacturer's recommended protocol. Following quantification, all RNA samples were equalised to 10 ng/μl and cDNA was synthesised using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Gene expression analysis was performed using fluorescein amidite (FAM)-labelled probes for the genes of interest (Taqman Gene Expression assays, Applied Biosystems) [Table 3.1] with a VIC-labelled probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control (see Chapter 2 – section 2.2.5).

Table 3.1 List of genes used with GenBank sequence ref numbers

Target Symbol	Target Name	Taqman Gene Assay ID
FKBP5	FK506 binding protein 5	Hs01561006_m1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Hs02758991_g1
NGFI-A	Nerve growth factor inducible protein A	Hs00152928_m1
NR3C1 (GR)	Nuclear receptor subfamily 3 group C member 1 (glucocorticoid receptor)	Hs00353740_m1
NR3C2 (MR)	Nuclear receptor subfamily 3 group C member 2 (mineralocorticoid receptor)	Hs01031809_m1

3.2.3 Real-time PCR analysis of HPA axis mRNA markers in peripheral blood mononuclear cells (PBMCs)

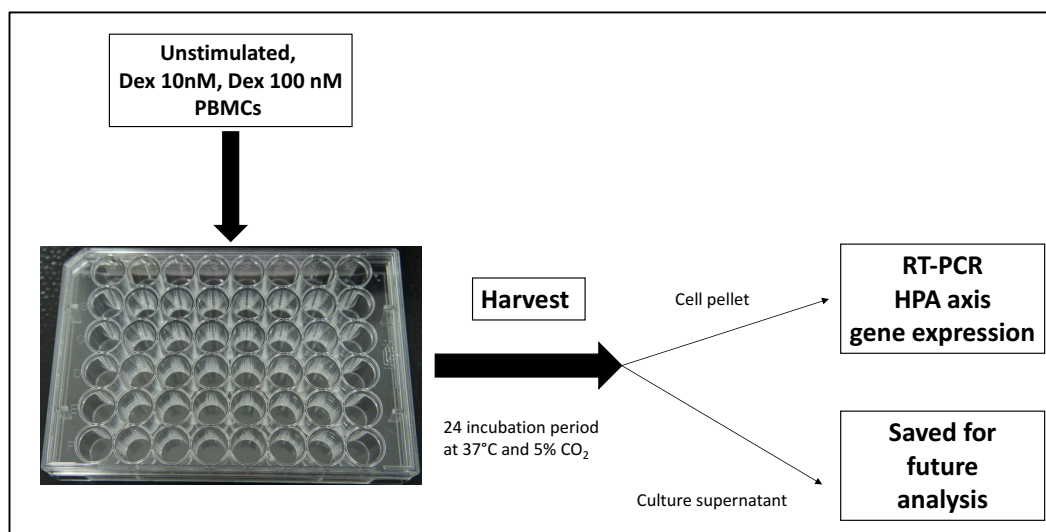


Figure 3.3 Diagrammatic representation of the experimental design

Culture media and dexamethasone (Dex) was prepared as outlined in Chapter 2 – Section 2.2.4.2. 450 µl cell suspension was added to each well and following this, 50 µl Dex (10 nM and 100 nM; final concentration) was added to the appropriate wells. The cells were left to incubate for 24 hours at 37°C and 5% CO₂ and were harvested as per Chapter 2 – section 2.2.4.3.5.

Total RNA was extracted from the PBMCs using the NucleoSpin® RNA Isolation kit (Macherey-Nagel) as per the manufacturer's recommended protocol. Following quantification, all RNA samples were equalised to 2 ng/µl and cDNA was synthesised using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Gene expression analysis was performed using FAM-labelled probes for the genes of interest (Taqman Gene Expression assays, Applied Biosystems) [Table 3.1] with a VIC-labelled probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control (see Chapter 2 – section 2.2.5).

3.2.4 Liquid chromatography-mass spectrometry (LC-MS) for salivary cortisol

Saliva samples were collected from all participants at five time-points throughout the day to measure the cortisol awakening response (CAR) and to assess potential diurnal variations in cortisol levels. Salivary cortisol levels were measured by liquid chromatography-mass spectrometry (LC-MS), performed at the University of Manchester in the Department of

Clinical Biochemistry. The work was performed by members of Prof. Brian Keevil's laboratory at the University Hospital of South Manchester. Calculation of CAR parameters was performed by Dr. Kelly Doolin from the REDEEM group. These parameters were Area Under the Curve (AUC) with respect to (1) increase from baseline (AUC_i), (2) to ground (AUC_g) where ground is equal to zero, reactivity, slope and morning intercept.

3.2.5 FKBP5 DNA Genotyping

The functional SNP rs1360780 was genotyped from DNA extracted from whole blood. A Taqman SNP Genotyping Assay was performed on a 7900HT Sequence Detection System (Applied Biosystems). Along with the patient samples, a number of HapMap CEU DNA sample positive controls (www.hapmap.com) and non-template controls (NTC) were genotyped for quality controls purposes. The genotyping was kindly performed by Dr. Derek Morris and Ms. Laura Whitton, National University of Ireland. T is the minor allele in this SNP and because homozygous TT samples were rare in this group, they were grouped with heterozygous TC samples for analysis.

3.2.6 Statistical Analysis

Data are presented as mean with standard deviation (SD) and standard error of the mean (SEM) where appropriate. Data normality was assessed using the Shapiro-Wilk test of normality. Differences in demographic variables were tested using Mann Whitney U test and Chi-square (χ^2) test. Following Shapiro-Wilk test for normality, it was revealed that the clinical PBMC data were not normally distributed. As there is no equivalent to a non-parametric Two-Way ANOVA, the data were log transformed. However, as this did not significantly improve the distribution of the data, statistical analysis was performed using a Kruskal-Wallis One-Way ANOVA followed by a Mann-Whitney U test. Spearman's rho correlation was used to assess associations between biological variables. All statistical analyses were considered significant when $p \leq 0.05$. Statistics were performed using SPSS (Version 22, IBM) and all graphs were generated using GraphPad Prism Software (Version 6, GraphPad Inc.).

3.3 Results

3.3.1 Demographic data for patients with depression and healthy controls whose DNA methylation levels were measured

DNA methylation analysis was performed on 69 participants: 36 depressed patients and 33 healthy controls. The difference in sample size was largely the result of needle phobia in some participants and the fact that the DNA methylation analysis protocol took considerable time to optimise. There were no significant differences in age, gender or BMI between the groups. Analysis revealed that the depressed group had a higher prevalence of smokers ($p=0.048$) and a lower duration of education, according to the International Standard Classification of Education (ISCED) scale ($p<0.001$), compared to that of the control group [Table 3.2].

Table 3.2 Demographic data for patients with depression and healthy controls whose DNA methylation levels were measured

	Patients (n=36)	Controls (n=33)	Statistic (p-value)
Age (years)	28.58 (9.01)	28.55 (7.32)	$Z=-0.13$, $p=0.899$
Gender (M/F)	10/26	13/20	$\chi^2=1.05$, $p=0.322$
BMI	27.45 (9.51)	23.29 (3.86)	$Z=-1.76$, $p=0.078$
Smoking (Y/N)	12/24	4/29	$\chi^2=4.35$, $p=0.048^*$
ISCED Education	3.00 (1.60)	6.12 (1.87)	$Z=-5.32$, $p<0.001^{**}$

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education. * $p\leq 0.05$ vs. control, ** $p\leq 0.001$ vs. control.

3.3.2 Clinical data for patients with depression and healthy controls whose DNA methylation levels were measured

Consistent with a diagnosis of depression, patients with Major Depressive Disorder (MDD) had significantly higher HAM-D and CES-D scores compared to healthy controls, and poorer sleep quality, as measured by higher overall PSQI scores. The depressed group had a higher prevalence of individuals who had experienced early life adversity (ELA) (Patients: 44.4% vs. Controls: 9.1%), with significantly higher CTQ Global scores compared to the control group. Within the depressed cohort, 22 individuals (61.1%) were receiving pharmacological treatment for depression at the time of recruitment. Of these 22 patients receiving pharmacological treatment, 15 were taking selective serotonin reuptake inhibitors (SSRIs), three were receiving serotonin-noradrenaline reuptake inhibitors (SNRIs) and four were receiving other antidepressant treatments (i.e. atypical antidepressants, melatonergic antidepressants) [Table 3.3].

Table 3.3 Clinical data for patients with depression and healthy controls whose DNA methylation levels were measured

	Patients (n=36)	Controls (n=33)	Statistic (p-value)
HAM-D	22.19 (5.39)	1.77 (1.98)	Z=-6.99, p<0.001***
CES-D	38.76 (9.58)	5.66 (5.78)	Z=-6.92, p<0.001***
PSQI	12.62 (3.71)	3.45 (1.96)	Z=-6.77, p<0.001***
CTQ Global Score	42.03 (15.15)	29.48 (7.89)	Z=-3.86, p<0.001***
ELA (Y/N)	16/20	3/30	$\chi^2=10.8$, p=0.001***
Antidepressant (Y/N)	22/14	0/33	

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (ELA). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. *p≤0.05 vs. control, ***p≤0.001 vs. control.

3.3.3 Mean DNA methylation levels of *NR3C1* exon 1F promoter in depressed patients and healthy controls

Mean *NR3C1* exon 1F methylation levels were calculated (5 CG sites). Smoking was selected as a covariate in the analysis as its prevalence was significantly different between the two groups and correlated with DNA methylation at a number of CG sites measured. Education was also significantly different between the two groups, however, it was not included as a covariate because it did not correlate with DNA methylation levels at any CG site examined. A univariate analysis of variance performed on log transformed *NR3C1* methylation data using smoking as a covariate revealed significantly higher DNA methylation in the depressed patients ($p=0.009$) relative to healthy controls [Figure 3.4].

Table 3.4 *NR3C1* exon 1F methylation data for depressed patients and healthy controls

	Patients (n=36)	Controls (n=33)	Statistic (p-value)
mean<i>NR3C1F</i>	4.07 (0.10)	3.68 (0.07)	F=7.26, $p=0.009^{**}$

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance, with smoking as a covariate, on log transformed data. $^{**}p \leq 0.01$ vs. controls.

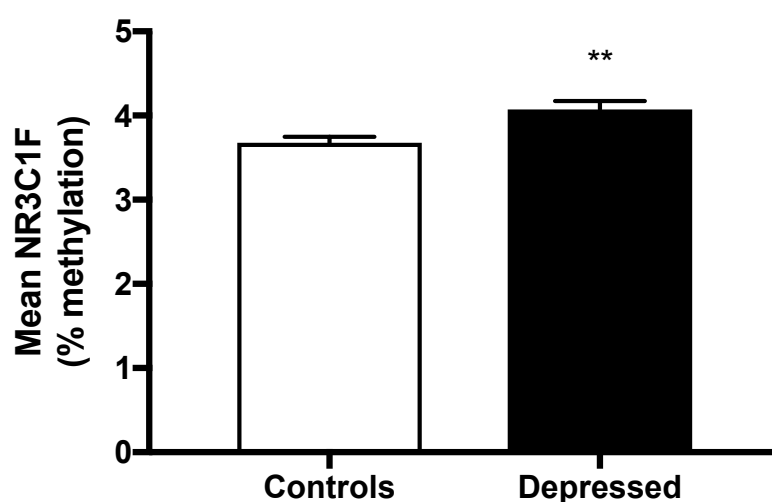


Figure 3.4 *NR3C1* exon 1F methylation in depressed patients and healthy controls. Mean *NR3C1* exon 1F DNA methylation in depressed patients and healthy controls. Data expressed as mean methylation percentage with SEM, Univariate Analysis of Variance, controlling for smoking. $^{**}p \leq 0.01$ vs. controls.

3.3.4 DNA methylation levels of specific CG sites in the *NR3C1* exon 1F promoter in depressed patients and healthy controls

Analysis revealed no significant differences in DNA methylation at the any of the specific CG sites measured [Table 3.5].

Table 3.5 Site-specific *NR3C1* exon 1F methylation data for depressed patients and healthy controls

	Patients (n=36)	Controls (n=33)	Statistic (p-value)
<i>NR3C1F</i> CG36	2.70 (0.21)	2.44 (0.17)	F=0.80, p=0.374
<i>NR3C1F</i> CG37	3.19 (0.19)	3.27 (0.21)	F=0.02, p=0.895
<i>NR3C1F</i> CG38	5.59 (0.16)	5.49 (0.16)	F=0.01, p=0.957
<i>NR3C1F</i> CG39	5.39 (0.13)	5.28 (0.16)	F=0.40, p=0.530
<i>NR3C1F</i> CG41	3.55 (0.30)	2.92 (0.17)	F=3.28, p=0.075

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance, with smoking as a covariate, on log transformed data.

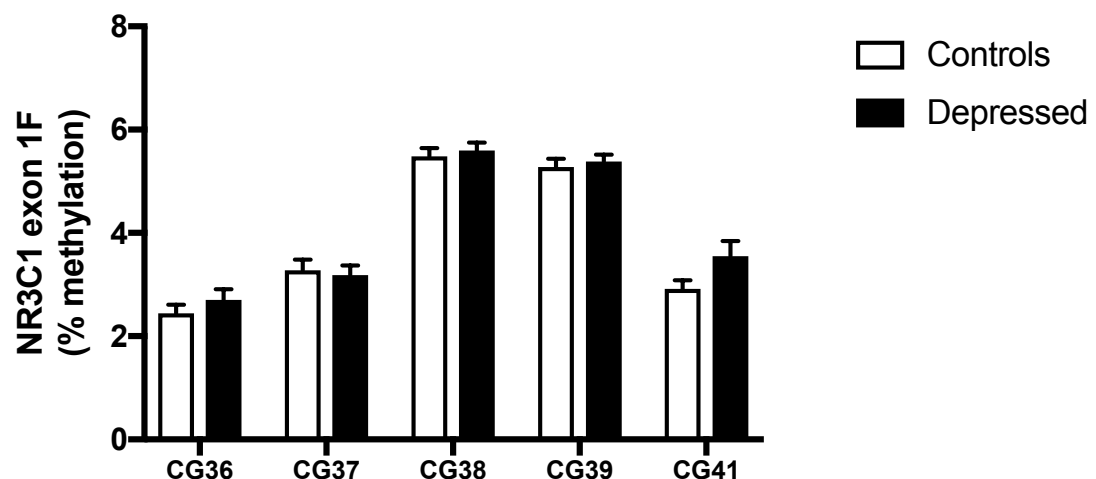


Figure 3.5 DNA methylation levels of selected *NR3C1* exon 1F CG sites in depressed patients and healthy controls. Site-specific *NR3C1* exon 1F DNA methylation in depressed patients and healthy controls. Data expressed as mean methylation percentage with SEM, Univariate Analysis of Variance, controlling for smoking.

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

Mean *FKBP5* intron 7 methylation levels were calculated (2 CG sites). A univariate analysis of variance performed on log transformed *FKBP5* methylation data using smoking as a covariate revealed no significant differences in DNA methylation between the two groups ($p=0.76$) [Table 3.6].

Table 3.6 *FKBP5* intron 7 methylation data for depressed patients and healthy controls

	Patients (n=36)	Controls (n=33)	Statistic (p-value)
mean<i>FKBP5</i>	59.79 (0.78)	60.03 (1.04)	F=0.09, p=0.760

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance, with smoking as a covariate, on log transformed data.

3.3.6 DNA methylation levels of specific CG sites in the *FKBP5* intron 7 promoter in depressed patients compared to healthy controls

A univariate analysis of variance performed on log transformed *FKBP5* methylation data using smoking as a covariate revealed no significant differences in DNA methylation at CG6 or CG7 between depressed patients and healthy controls [Table 3.7].

Table 3.7 Site-specific *FKBP5* intron 7 methylation data for depressed patients and healthy controls

	Patients (n=36)	Controls (n=33)	Statistic (p-value)
<i>FKBP5</i> CG6	62.09 (0.69)	62.32 (0.90)	F=0.25, p=0.621
<i>FKBP5</i> CG7	57.49 (0.98)	57.74 (1.48)	F=0.05, p=0.830

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance, with smoking as a covariate, on log transformed data.

3.3.7 Demographic data for patients with depression and healthy controls whose *FKBP5* rs1360780 single-nucleotide polymorphism (SNP) was genotyped

The *FKBP5* rs1360780 SNP was genotyped in 73 participants: 45 depressed patients and 28 healthy controls. The difference in sample size was largely the result of needle phobia in some participants and the fact that not all blood samples provided a sufficient DNA yield. There were no significant differences in age, gender or BMI between the groups. Analysis revealed that the depressed group had a higher prevalence of smokers ($p=0.015$) and a lower duration of education, according to the International Standard Classification of Education (ISCED) scale ($p<0.001$), compared to that of the control group [Table 3.8].

Table 3.8 Demographic data for patients with depression and healthy controls whose *FKBP5* rs1360780 SNP was genotyped

	Patients (n=45)	Controls (n=28)	Statistic (p-value)
Age (years)	29.27 (9.07)	28.71 (6.95)	$Z=-0.18$, $p=0.860$
Gender (M/F)	10/35	11/17	$\chi^2=2.45$, $p=0.183$
BMI	26.34 (9.12)	23.34 (3.64)	$Z=-0.90$, $p=0.368$
Smoking (Y/N)	17/28	3/25	$\chi^2=6.36$, $p=0.015^*$
ISCED Education	3.24 (1.82)	6.14 (1.84)	$Z=-5.31$, $p<0.001^{**}$

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education. * $p\leq 0.05$ vs. control, *** $p\leq 0.001$ vs. control.

3.3.8 Clinical data for patients with depression and healthy controls whose *FKBP5* rs1360780 SNP was genotyped

Consistent with a diagnosis of depression, patients with Major Depressive Disorder (MDD) had significantly higher HAM-D and CES-D scores compared to healthy controls, and poorer sleep quality, as measured by higher overall PSQI scores. The depressed group had a higher prevalence of individuals who had experienced early life adversity (ELA) (Patients: 51.1% vs. Controls: 7.1%), with significantly higher CTQ Global scores compared to the control group. Within the depressed cohort, 29 individuals (64.4%) were receiving pharmacological treatment for depression at the time of recruitment. Of these 29 patients receiving pharmacological treatment, 20 were taking selective serotonin reuptake inhibitors (SSRIs), five were receiving serotonin-noradrenaline reuptake inhibitors (SNRIs) and four were receiving other antidepressant treatments (i.e. atypical antidepressants, melatonergic antidepressants) [Table 3.9].

Table 3.9 Clinical data for patients with depression and healthy controls whose *FKBP5* rs1360780 SNP was genotyped

	Patients (n=45)	Controls (n=28)	Statistic (p-value)
HAM-D	22.27 (5.07)	2.00 (1.91)	Z=-6.91, p<0.001***
CES-D	39.49 (10.14)	5.70 (6.01)	Z=-6.93, p<0.001***
PSQI	13.12 (3.88)	3.81 (1.88)	Z=-6.81, p<0.001***
CTQ Global Score	44.02 (15.06)	29.57 (7.92)	Z=-4.35, p<0.001***
ELA (Y/N)	23/22	2/26	$\chi^2=14.8$, p<0.001***
Antidepressant (Y/N)	29/16	0/28	

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (ELA). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. control.

3.3.9 FKBP5 SNP rs1360780 prevalence in depressed patients and healthy controls

The FKBP5 T-allele variant at rs1360780 is considered a risk allele. As TT homozygotes are relatively rare, with only 4/73 or 5.5% of the cohort for whom polymorphism data was available, CT or TT were considered risk allele carriers and CC were labelled as protective allele carriers. Chi-squared (χ^2) test revealed no difference in polymorphism frequency between the two groups [Table 3.10]. Because of the equal divisions of the polymorphisms within groups the possible impact of FKBP5 SNP rs1360780 allele on FKBP5 methylation in depressed patients and healthy controls was taken to be almost nil.

Table 3.10 FKBP5 SNP rs1360780 prevalence in depressed patients and healthy controls

	Patients (n=45)	Controls (n=28)	Statistics (p-value)
CC/CT or TT	22/23	14/14	$\chi^2=0.01$, p=0.926

Statistical analysis was performed using a Chi squared (χ^2) test

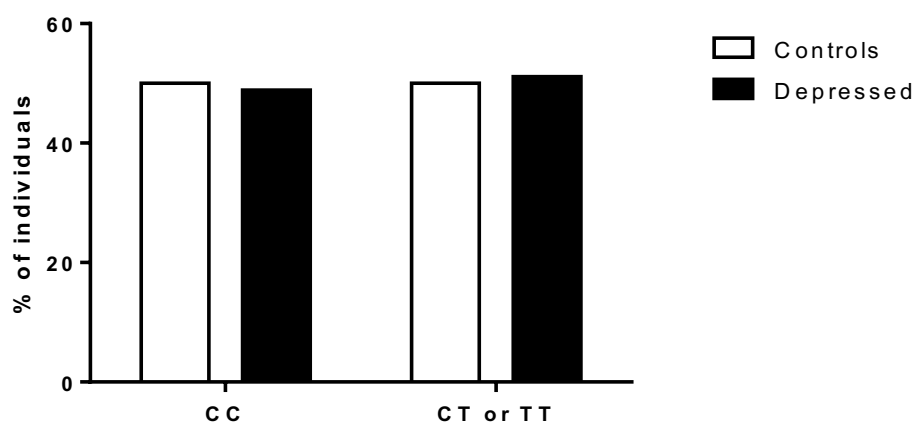


Figure 3.6 FKBP5 SNP rs1360780 prevalence in depressed patients and healthy controls.
FKBP5 SNP rs1360780 prevalence in depressed patients and healthy controls.

3.3.10 Demographic data for patients with depression and healthy controls whose whole blood mRNA was analysed by PCR

Gene expression analysis was performed on 87 participants: 50 depressed patients and 37 healthy controls. The difference in sample size here was the result of needle phobia in some participants and due to the fact that not all blood samples provided a sufficient RNA yield for analysis. There were no significant differences in age, gender or BMI between the groups. Analysis revealed that the depressed group had a higher prevalence of smokers ($p=0.008$) and a lower duration of education, according to the ISCED scale ($p<0.001$) compared to that of the control group [Table 3.11].

Table 3.11 Demographic data for patients with depression and healthy controls whose whole blood mRNA was analysed by PCR

	Patients (n=50)	Controls (n=37)	Statistic (p-value)
Age (years)	28.96 (8.73)	28.54 (7.19)	$Z=-0.14$, $p=0.887$
Gender (M/F)	13/37	14/23	$\chi^2=1.39$, $p=0.252$
BMI	26.85 (8.92)	23.19 (3.71)	$Z=-1.73$, $p=0.083$
Smoking (Y/N)	20/30	5/32	$\chi^2=7.29$, $p=0.008^{**}$
ISCED Education	3.18 (1.83)	6.08 (1.92)	$Z=-5.52$, $p<0.001^{***}$

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education. $^{**}p\leq 0.01$ vs. control, $^{***}p\leq 0.001$ vs. control.

3.3.11 Clinical data for patients with depression and healthy controls whose whole blood mRNA was analysed by PCR

Consistent with a diagnosis of depression, patients with Major Depressive Disorder (MDD) had significantly higher HAM-D and CES-D scores compared to healthy controls, and poorer sleep quality, as measured by higher overall PSQI scores. The depressed group had a higher prevalence of individuals who had experienced early life adversity (ELA) (Patients: 52.0% vs. Controls: 5.4%), with significantly higher CTQ Global scores compared to the control group. Within the depressed cohort, 32 individuals (64.0%) were receiving pharmacological treatment for depression at the time of recruitment. Of these 32 patients receiving pharmacological treatment, 22 were taking selective serotonin reuptake inhibitors (SSRIs), five were receiving serotonin-noradrenaline reuptake inhibitors (SNRIs) and five were receiving other antidepressant treatments (i.e. atypical antidepressants, melatonergic antidepressants) [Table 3.12].

Table 3.12 Clinical data for patients with depression and healthy controls whose whole blood mRNA was analysed by PCR

	Patients (n=50)	Controls (n=37)	Statistic (p-value)
HAM-D	22.42 (5.03)	2.06 (2.87)	Z=-7.69, p<0.001***
CES-D	39.10 (9.74)	6.47 (8.55)	Z=-7.49, p<0.001***
PSQI	12.78 (3.88)	3.44 (1.88)	Z=-7.53, p<0.001***
CTQ Global Score	44.04 (15.31)	28.51 (5.71)	Z=-4.99, p<0.001***
ELA (Y/N)	26/24	2/37	$\chi^2=21.2$, p=0.001***
Antidepressant (Y/N)	32/18	0/37	

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (ELA). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. control.

3.3.12 HPA axis associated gene expression data for depressed patients and healthy controls

Gene expression of *NR3C1*, *NR3C2*, *NGFI-A* and *FKBP5* were measured in whole blood from depressed patients relative to healthy controls. The average threshold cycle (Ct) for the target genes and *GAPDH* were analysed to ensure minimal inter-assay variability [Table 3.13].

Table 3.13 Average threshold cycle (Ct) of target genes, GAPDH and ΔCt in control groups.

	Target Gene Ct	GAPDH Ct	ΔCt
<i>NR3C1</i>	27.8	25.2	2.6
<i>NR3C2</i>	32.4	25.2	7.2
<i>NGFI-A</i>	31.9	25.2	6.7
<i>FKBP5</i>	27.8	24.5	3.3

Ct=threshold cycles, ΔCt=delta Ct.

A univariate analysis of variance performed on log transformed mRNA data using smoking as a covariate revealed no significant differences in gene expression of *NR3C1*, *NR3C2*, *NGFI-A* or *FKBP5* in the depressed patients relative to healthy controls. Smoking was selected as a covariate in the analysis as its prevalence was significantly different between the two groups and correlated with mRNA levels at a number of genes measured. Education was also significantly different between the two groups, however, it was not included as a covariate because it did not correlate with mRNA levels at any gene measured. There was no significant difference in *NR3C2:NR3C1* (MR:GR) ratio in the depressed patients relative to healthy controls [Table 3.14].

Table 3.14 HPA axis gene expression data for depressed patients and healthy controls

	Patients (n=50)	Controls (n=37)	Statistic (p-value)
NR3C1 mRNA	1.01 (0.04)	1.00 (0.04)	F=0.01, p=0.968
NR3C2 mRNA	0.92 (0.05)	1.00 (0.05)	F=0.92, p=0.342
NGFI-A mRNA	1.20 (0.21)	1.00 (0.07)	F=1.28, p=0.262
FKBP5 mRNA	0.99 (0.08)	1.00 (0.09)	F=0.01, p=0.962
MR:GR ratio	0.80 (0.05)	1.00 (0.05)	F=0.71, p=0.403

Data are expressed as mean fold change with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance, with smoking as a covariate, on log transformed data.

3.3.13 Demographic data for patients with depression and healthy controls whose salivary cortisol was measured by LC-MS

Salivary cortisol was quantified by LC-MS in 94 participants: 52 depressed patients and 42 healthy controls. The difference in sample size here was the result of insufficient sample quantity provided by a number of participants for analysis. There were no significant differences in age, gender, BMI or smoking between the groups. Analysis revealed that the depressed group had a lower duration of education, according to the ISCED scale ($p < 0.001$) compared to that of the control group [Table 3.15].

Table 3.15 Demographic data for patients with depression and healthy controls whose salivary cortisol was measured by LC-MS

	Patients (n=52)	Controls (n=42)	Statistic (p-value)
Age (years)	27.89 (8.17)	27.57 (5.50)	$Z = -0.26$, $p = 0.796$
Gender (M/F)	18/34	14/28	$\chi^2 = 0.02$, $p = 1.000$
BMI	24.72 (6.21)	22.91 (3.42)	$Z = -1.09$, $p = 0.277$
Smoking (Y/N)	21/31	10/32	$\chi^2 = 3.13$, $p = 0.083$
ISCED Education	3.02 (1.77)	6.31 (1.42)	$Z = -6.68$, $p < 0.001^{***}$

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education. *** $p \leq 0.001$ vs. control.

3.3.14 Clinical data for patients with depression and healthy controls whose salivary cortisol was measured by LC-MS

Consistent with a diagnosis of depression, patients with Major Depressive Disorder (MDD) had significantly higher HAM-D and CES-D scores compared to healthy controls, and poorer sleep quality, as measured by higher overall PSQI scores. The depressed group had a higher prevalence of individuals who had experienced early life adversity (ELA) (Patients: 46.2% vs. Controls: 11.9%), with significantly higher CTQ Global scores compared to the control group. Within the depressed cohort, 27 individuals (51.9%) were receiving pharmacological treatment for depression at the time of recruitment. Of these 27 patients receiving pharmacological treatment, 19 were taking selective serotonin reuptake inhibitors (SSRIs), three were receiving serotonin-noradrenaline reuptake inhibitors (SNRIs) and five were receiving other antidepressant treatments (i.e. atypical antidepressants, melatonergic antidepressants) [Table 3.16].

Table 3.16 Clinical data for patients with depression and healthy controls whose salivary cortisol was measured by LC-MS

	Patients (n=52)	Controls (n=42)	Statistic (p-value)
HAM-D	22.88 (4.68)	2.83 (2.86)	Z=-7.95, p<0.001***
CES-D	38.37 (10.15)	6.73 (6.60)	Z=-8.05, p<0.001***
PSQI	12.81 (4.20)	4.05 (2.71)	Z=-7.16, p<0.001***
CTQ Global Score	45.33 (17.74)	30.30 (7.50)	Z=-4.34, p<0.001***
ELA (Y/N)	24/28	5/37	$\chi^2=13.3$, p<0.001***
Antidepressant (Y/N)	27/25	0/42	

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (ELA). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. control.

3.3.15 Salivary cortisol levels in depressed patients and healthy controls

A Student's t-test performed on log transformed cortisol data revealed a significant increase in morning (T0) cortisol levels in depressed patients ($p=0.007$) compared to healthy controls. There were no other significant differences in cortisol measurements at any other time-points throughout the day between depressed patients and healthy controls [Table 3.17].

Table 3.17 Morning and evening cortisol concentrations in depressed patients and healthy controls

	Patients (n=52)	Controls (n=42)	Statistic (p-value)
Cortisol T0	11.57 (1.41)	7.49 (0.63)	$t=-2.759$, $p=0.007^{**}$
Cortisol T30	12.84 (1.01)	11.17 (0.88)	$t=-0.936$, $p=0.352$
Cortisol T60	9.99 (1.18)	9.05 (0.83)	$t=0.401$, $p=0.689$
Cortisol T720	3.62 (1.06)	3.33 (1.09)	$t=-0.551$, $p=0.584$
Cortisol T750	3.81 (1.23)	3.33 (1.02)	$t=-0.016$, $p=0.987$

Data are expressed as mean cortisol concentrations (nMol/L) with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data. $^{**}p\leq 0.01$ vs. controls.

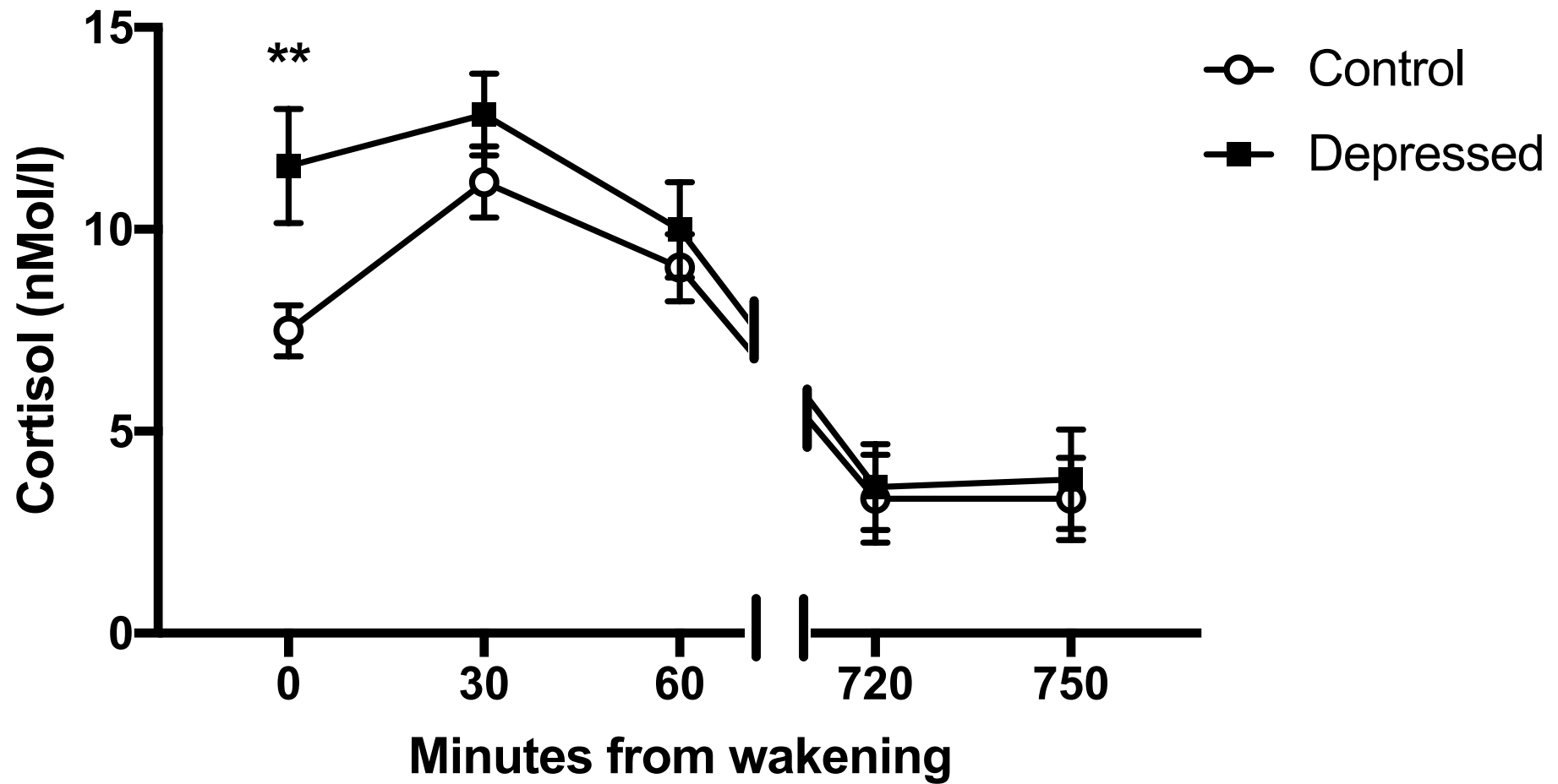


Figure 3.7 Morning and evening cortisol concentrations in depressed patients and healthy controls. Cortisol concentrations at three morning and two evening time points in depressed patients and healthy controls. Data are expressed as mean cortisol concentrations (nMol/L) with SEM, Student's t-test. **p<0.01 vs. control.

3.3.16 Salivary cortisol awakening response (CAR) parameters in depressed patients and healthy controls

Cortisol awakening response (CAR) parameters were calculated for all participants who had LC-MS data available. A univariate analysis of variance performed on log transformed cortisol data revealed a significant decrease in cortisol reactivity ($p=0.042$) in depressed patients relative to healthy controls. There were no significant differences between groups at any of the other CAR parameters examined [Table 3.18].

Table 3.18 CAR parameters for depressed patients and healthy controls.

	Patients (n=52)	Controls (n=42)	Statistic (p-value)
Cortisol AM average	10.85 (0.82)	9.23 (0.59)	$t=-1.56$, $p=0.122$
Cortisol PM average	2.48 (0.36)	3.18 (0.93)	$t=-0.27$, $p=0.786$
Cortisol AUCi	67.81 (38.9)	132.74 (42.7)	$t=1.72$, $p=0.089$
Cortisol AUCg	664.68 (59.2)	582.84 (37.5)	$t=-1.30$, $p=0.199$
Cortisol Reactivity	-0.59 (1.03)	1.35 (1.00)	$t=2.07$, $p=0.042^*$

Data are expressed as mean cortisol concentrations (nMol/L) with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data. AUCi=Area Under the Curve with respect to increase; AUCg=Areas Under the Curve with respect to ground, where ground is equal to zero. * $p\leq 0.05$ vs. controls.

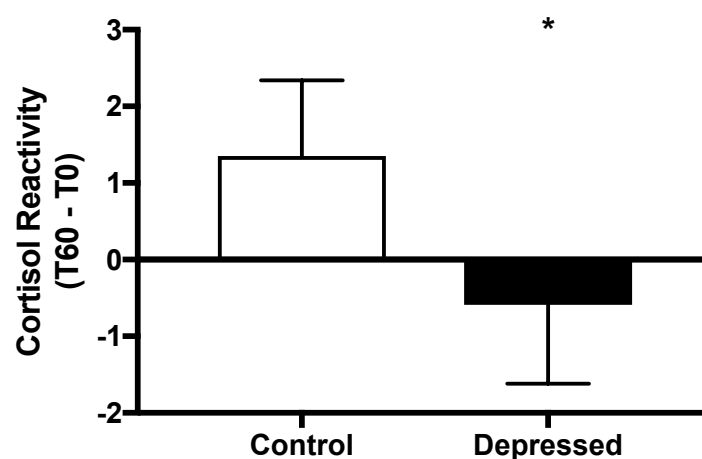


Figure 3.8 Cortisol awakening response (CAR) reactivity in depressed patients and healthy controls. CAR reactivity in depressed patients and healthy controls. Data are expressed as means with SEM, Student's t-test. * $p\leq 0.05$ vs. control.

3.3.17 Demographic data for patients with depression and healthy controls whose PBMC mRNA was analysed by PCR

Gene expression analysis was performed on 58 participants: 33 depressed patients and 25 healthy controls. The difference in sample size here was the result of needle phobia in some individuals as well as insufficient cell counts in a number of samples. There were no significant differences in age, gender or BMI between the groups. Analysis revealed that the depressed group had a higher prevalence of smokers ($p=0.008$) and lower duration of education, according to the ISCED scale ($p<0.001$) compared to that of the control group [Table 3.19].

Table 3.19 Demographic data for patients with depression and healthy controls whose PBMC mRNA was analysed by PCR

	Patients (n=33)	Controls (n=25)	Statistic (p-value)
Age (years)	27.94 (8.79)	27.52 (6.99)	$Z=-0.06$, $p=0.950$
Gender (M/F)	7/26	9/16	$\chi^2=1.56$, $p=0.247$
BMI	27.94 (9.92)	23.06 (3.51)	$Z=-1.89$, $p=0.058$
Smoking (Y/N)	13/20	2/23	$\chi^2=7.31$, $p=0.008^{**}$
ISCED Education	2.94 (1.72)	5.96 (2.11)	$Z=-4.48$, $p<0.001^{***}$

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education. $^{**}p\leq 0.01$ vs. control. $^{***}p\leq 0.001$ vs. control.

3.3.18 Clinical data for patients with depression and healthy controls whose PBMC mRNA was analysed by PCR

Consistent with a diagnosis of depression, patients with Major Depressive Disorder (MDD) had significantly higher HAM-D and CES-D scores compared to healthy controls, and poorer sleep quality, as measured by higher overall PSQI scores. The depressed group had a higher prevalence of individuals who had experienced early life adversity (ELA) (Patients: 48.5% vs. Controls: 4.0%), with significantly higher CTQ Global scores compared to the control group. Within the depressed cohort, 19 individuals (57.6%) were receiving pharmacological treatment for depression at the time of recruitment. Of these 19 patients receiving pharmacological treatment, 12 were taking selective serotonin reuptake inhibitors (SSRIs), three were receiving serotonin-noradrenaline reuptake inhibitors (SNRIs) and four were receiving other antidepressant treatments (i.e. atypical antidepressants, melatonergic antidepressants) [Table 3.20].

Table 3.20 Clinical data for patients with depression and healthy controls whose PBMC mRNA was analysed by PCR

	Patients (n=33)	Controls (n=25)	Statistic (p-value)
HAM-D	22.21 (5.78)	3.22 (3.41)	Z=-6.26, p<0.001***
CES-D	39.84 (9.52)	7.68 (9.27)	Z=-6.08, p<0.001***
PSQI	12.94 (3.82)	3.70 (1.96)	Z=-6.12, p<0.001***
CTQ Global Score	43.52 (16.12)	28.00 (4.58)	Z=-3.91, p<0.001***
ELA (Y/N)	16/17	1/24	$\chi^2=13.6$, p=0.001***
Antidepressant (Y/N)	19/14	0/25	

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (ELA). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. control.

3.3.19 PBMC gene expression of HPA axis genes in depressed patients and healthy controls

Basal levels of *NR3C1* and *FKBP5* mRNA was measured from PBMCs from depressed patients and healthy controls.

A Mann-Whitney U test performed on PBMC data revealed no significant difference in gene expression of *NR3C1* or *FKBP5* between depressed patients and healthy controls [Table 3.21].

Table 3.21 HPA axis-associated gene expression in PBMCs in depressed patients and healthy controls

	Patients (n=33)	Controls (n=25)	Statistic (p-value)
<i>NR3C1</i> mRNA (FC)	0.97 (0.10)	1.00 (0.07)	Z=-1.13, p=0.257
<i>FKBP5</i> mRNA (FC)	1.25 (0.18)	1.00 (0.08)	Z=-0.06, p=0.952

Data are expressed as mean fold change with SEM in parentheses. Statistical analysis was performed using a Mann-Whitney U test.

3.3.20 HPA axis associated gene expression profile following dexamethasone stimulation in depressed patients and healthy controls

A Kruskal-Wallis One-Way ANOVA revealed no significant differences in the expression of *NR3C1* mRNA in PBMCs isolated from depressed patients and healthy controls following stimulation with the synthetic glucocorticoid dexamethasone ($\chi^2=3.243$, $df=2$, $p=0.198$). There were no significant difference in the unstimulated expression ($Z=-1.418$, $p=0.156$), or following dexamethasone 10nM ($Z=-0.560$, $p=0.575$) or dexamethasone 100nM ($Z=-0.215$, $p=0.829$) of *NR3C1* between the depressed patients and controls [Figure 3.9a].

A Kruskal-Wallis One-Way ANOVA revealed a differential expression of *FKBP5* in PBMCs isolated from depressed patients and healthy controls following stimulation with the synthetic glucocorticoid dexamethasone ($\chi^2=96.024$, $df=2$, $p<0.001$). In the control samples dexamethasone at a concentration of 10nM ($Z=-4.254$, $p<0.001$) and at 100nM ($Z=-5.723$, $p<0.001$) induced a significant increase in *FKBP5* mRNA expression relative to unstimulated control levels. The dexamethasone 100nM induced increase in *FKBP5* mRNA expression was significantly greater than that observed by following dexamethasone 10nM stimulation ($Z=-3.240$, $p=0.001$). In the depressed group, dexamethasone at a concentration of 10nM ($Z=-5.454$, $p<0.001$) and 100nM ($Z=-6.210$, $p<0.001$) also induced a significant increase in *FKBP5* mRNA expression relative to unstimulated control levels. The dexamethasone 100nM induced increase in *FKBP5* mRNA expression was significantly greater than that observed by following dexamethasone 10nM stimulation ($Z=-4.347$, $p<0.001$). However, there was no significant difference in the unstimulated expression ($Z=-0.300$, $p=0.765$), dexamethasone 10nM ($Z=-0.679$, $p=0.497$) or dexamethasone 100nM ($Z=-0.456$, $p=0.648$) *FKBP5* between the depressed patients and healthy controls [Figure 3.9b].

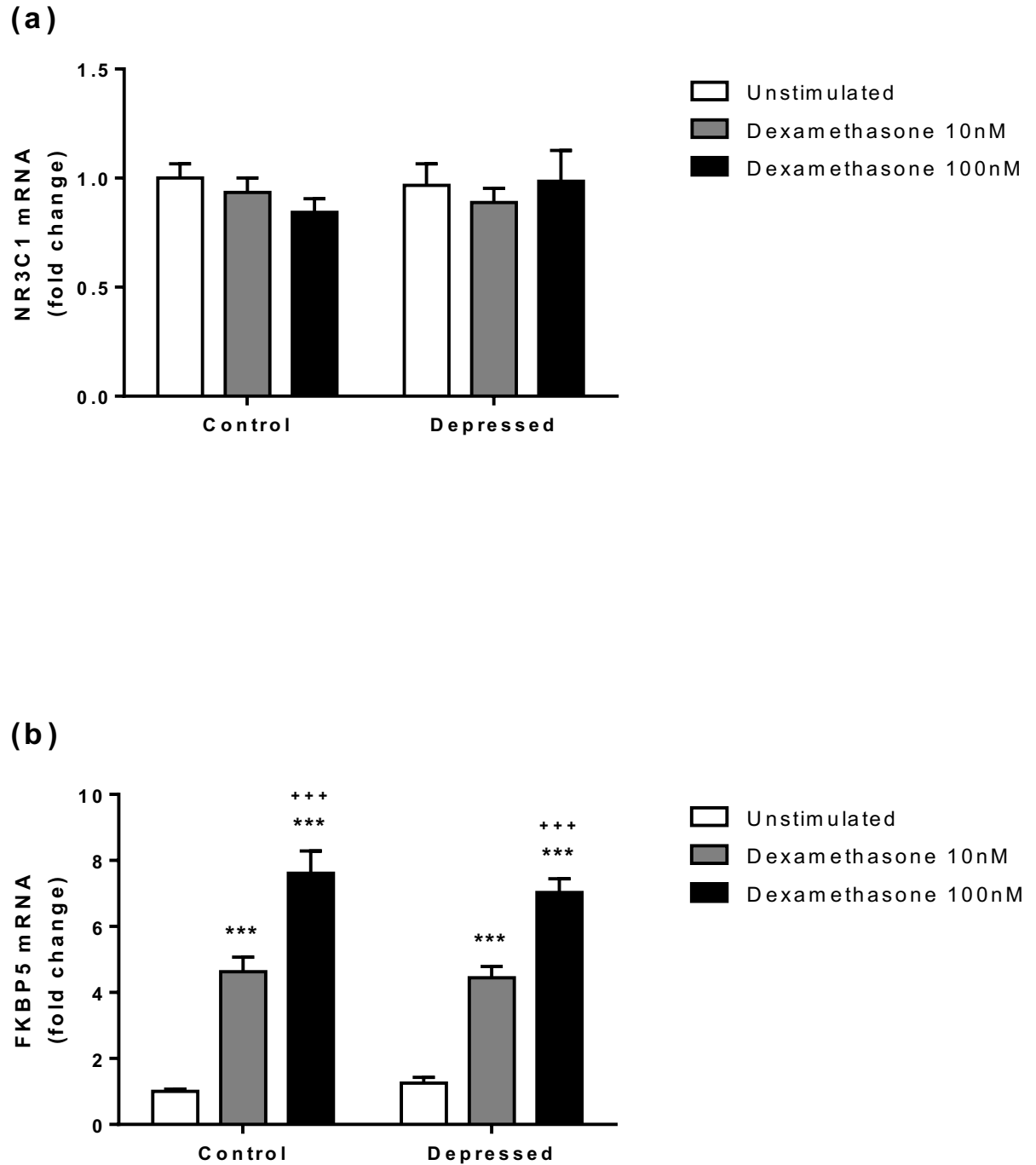


Figure 3.9 Dexamethasone 10nM and 100nM stimulated NR3C1 and FKBP5 expression in PBMCs from depressed patients and healthy controls. PBMCs isolated from depressed patients and healthy controls were incubated for 24 hours with dexamethasone at concentrations of 10nM and 100nM. Relative mRNA expression for (a) *NR3C1* and (b) *FKBP5* was quantified. Data are expressed as mean with SEM. *** $p \leq 0.001$ vs. unstimulated FKBP5 expression, +++ $p \leq 0.001$ vs. dexamethasone 10nM stimulated FKBP5 expression (Kruskal-Wallis One-Way ANOVA followed by Mann-Whitney U tests).

3.3.21 Evaluation of the relationships between *NR3C1* and *FKBP5* methylation with depression and trauma scores

Spearman's rho correlation analysis, performed on all participants, revealed no associations between *NR3C1* or *FKBP5* DNA methylation levels with depression or trauma scores.

3.3.22 Evaluation of the relationships between *NR3C1* methylation, cortisol and CAR parameters

Spearman's rho correlational analysis revealed a significant relationship between *NR3C1* exon 1F CG38 methylation and cortisol T0 and cortisol T30. A significant negative association was revealed between *NR3C1* exon 1F CG39 and cortisol T750 concentrations [Table 3.22].

Correlational analysis was then performed to assess potential relationships between *NR3C1* exon 1F DNA methylation and cortisol dynamics. Mean *NR3C1* exon 1F methylation levels, as well as methylation levels at CG36, were positively associated with average morning cortisol output. *NR3C1* exon 1F CG38 methylation levels were also positively correlated with average morning cortisol output as well as with cortisol AUCg. A significant relationship was observed between *NR3C1* exon 1F CG39 methylation and average evening cortisol output [Table 3.23].

Table 3.22 Correlational analysis of *NR3C1* exon 1F promoter DNA methylation and salivary cortisol levels in all participants.

	Cortisol T0	Cortisol T30	Cortisol T60	Cortisol T720	Cortisol T750
NR3C1 CG36	$r=0.294$ ($p=0.065$)	$r=0.173$ ($p=0.274$)	$r=0.197$ ($p=0.222$)	$r=0.066$ ($p=0.743$)	$r=-0.336$ ($p=0.137$)
NR3C1 CG37	$r=0.182$ ($p=0.260$)	$r=0.092$ ($p=0.563$)	$r=0.153$ ($p=0.347$)	$r=-0.119$ ($p=0.553$)	$r=0.120$ ($p=0.605$)
NR3C1 CG38	$r=0.320$ ($p=0.044$)*	$r=0.314$ ($p=0.043$)*	$r=0.265$ ($p=0.098$)	$r=0.130$ ($p=0.518$)	$r=0.062$ ($p=0.791$)
NR3C1 CG39	$r=-0.130$ ($p=0.465$)	$r=0.158$ ($p=0.358$)	$r=0.129$ ($p=0.466$)	$r=-0.129$ ($p=0.557$)	$r=-0.564$ ($p=0.018$)*
NR3C1 CG41	$r=-0.057$ ($p=0.728$)	$r=0.181$ ($p=0.257$)	$r=0.058$ ($p=0.725$)	$r=0.036$ ($p=0.859$)	$r=-0.103$ ($p=0.656$)
meanNR3C1F	$r=0.295$ ($p=0.096$)	$r=0.291$ ($p=0.090$)	$r=0.202$ ($p=0.259$)	$r=-0.029$ ($p=0.895$)	$r=-0.212$ ($p=0.413$)

Statistical analysis was performed using the Spearman's rho correlation, with * $p \leq 0.05$ considered significant. Bold type face represents significant findings.

Table 3.23 Correlational analysis of *NR3C1* exon 1F DNA methylation and CAR parameters in all participants.

	Cortisol AM	Cortisol PM	AUCg	AUCi	Reactivity
NR3C1 CG36	$r=0.339$ ($p=0.028$)*	$r=-0.110$ ($p=0.576$)	$r=0.202$ ($p=0.223$)	$r=-0.016$ ($p=0.926$)	$r=-0.051$ ($p=0.760$)
NR3C1 CG37	$r=0.157$ ($p=0.321$)	$r=-0.046$ ($p=0.817$)	$r=0.229$ ($p=0.166$)	$r=-0.108$ ($p=0.520$)	$r=-0.209$ ($p=0.208$)
NR3C1 CG38	$r=0.371$ ($p=0.016$)*	$r=0.120$ ($p=0.542$)	$r=0.325$ ($p=0.046$)*	$r=-0.042$ ($p=0.804$)	$r=-0.071$ ($p=0.673$)
NR3C1 CG39	$r=-0.022$ ($p=0.898$)	$r=-0.407$ ($p=0.049$)*	$r=0.104$ ($p=0.572$)	$r=0.277$ ($p=0.152$)	$r=0.093$ ($p=0.613$)
NR3C1 CG41	$r=0.025$ ($p=0.878$)	$r=-0.091$ ($p=0.646$)	$r=0.051$ ($p=0.763$)	$r=0.068$ ($p=0.689$)	$r=0.007$ ($p=0.965$)
meanNR3C1F	$r=0.350$ ($p=0.039$)*	$r=-0.150$ ($p=0.484$)	$r=0.321$ ($p=0.087$)	$r=-0.006$ ($p=0.976$)	$r=-0.168$ ($p=0.367$)

Statistical analysis was performed using the Spearman's rho correlation, with * $p \leq 0.05$ considered significant. Bold type face represents significant findings.

AUCi=Area Under the Curve with respect to increase; AUCg=Areas Under the Curve with respect to ground, where ground is equal to zero.

3.3.23 Evaluation of the relationships between *NR3C1* exon 1F DNA methylation and *NR3C1* mRNA expression

Spearman's rho correlation analysis revealed no association between *NR3C1* whole blood mRNA and DNA methylation at any of the CG sites measured at the *NR3C1* exon 1F promoter.

However, analysis did reveal significant associations between *NR3C1* PBMC mRNA and DNA methylation at *NR3C1* exon 1F CG35 and CG38 [Table 3.24].

Table 3.24 Correlational analysis of *NR3C1* exon 1F DNA methylation and *NR3C1* gene expression

	<i>NR3C1</i> PBMC mRNA expression
<i>NR3C1</i> CG36	r=-0.05 (p=0.765)
<i>NR3C1</i> CG37	r=-0.09 (p=0.603)
<i>NR3C1</i> CG38	r=-0.39 (p=0.018)*
<i>NR3C1</i> CG39	r=0.08 (p=0.681)
<i>NR3C1</i> CG41	r=0.01 (p=0.985)
mean<i>NR3C1</i>	r=-0.20 (p=0.296)

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

3.3.24 Evaluation of the relationships between HPA axis associated genes and CAR parameters

Correlational analysis was performed to assess whether there were relationships between HPA axis associated gene expression and cortisol concentrations and/or CAR parameters.

Whole blood *NR3C1* mRNA expression was positively associated with cortisol T30. Interestingly, PBMC *NR3C1* mRNA expression displayed an association with cortisol T30 of similar magnitude but it was in the opposite direction of the whole blood mRNA finding [Table 3.17]. PBMC *NR3C1* mRNA expression was also found to be negatively associated with cortisol morning average output and cortisol AUCg [Table 3.25].

Whole blood *FKBP5* mRNA expression was not associated with any cortisol or CAR measurements. However, PBMC *FKBP5* mRNA expression was negatively associated with cortisol T30 and cortisol AUCg [Table 3.25 and 3.26 respectively].

Whole blood *NGFI-A* mRNA expression was negatively associated with cortisol T30 and cortisol morning average output [Table 3.25 and 3.26 respectively].

Table 3.25 Correlational analysis of HPA axis associated genes and salivary cortisol levels in all participants.

	Cortisol T0	Cortisol T30	Cortisol T60	Cortisol T720	Cortisol T750
<i>NR3C1</i> WB mRNA	r=0.186 (p=0.264)	r=0.336 (p=0.030)*	r=0.131 (p=0.427)	r=0.044 (p=0.831)	r=0.065 (p=0.784)
<i>NR3C1</i> PBMC mRNA	r=-0.263 (p=0.177)	r=-0.367 (p=0.042)*	r=-0.216 (p=0.243)	r=0.043 (p=0.854)	r=0.128 (p=0.626)
<i>FKBP5</i> WB mRNA	r=-0.056 (p=0.701)	r=0.089 (p=0.526)	r=0.099 (p=0.491)	r=0.063 (p=0.719)	r=-0.135 (p=0.512)
<i>FKBP5</i> PBMC mRNA	r=-0.141 (p=0.474)	r=-0.375 (p=0.038)*	r=-0.207 (p=0.263)	r=0.155 (p=0.504)	r=0.163 (p=0.531)
<i>NR3C2</i> WB mRNA	r=0.193 (p=0.260)	r=0.075 (p=0.644)	r=0.048 (p=0.778)	r=-0.025 (p=0.907)	r=0.284 (p=0.238)
<i>NGFI-A</i> WB mRNA	r=-0.079 (p=0.643)	r=-0.393 (p=0.011)**	r=-0.259 (p=0.116)	r=0.093 (p=0.658)	r=0.341 (p=0.154)
MR:GR ratio	r=0.033 (p=0.848)	r=-0.180 (p=0.266)	r=-0.087 (p=0.607)	r=-0.093 (p=0.665)	r=0.199 (p=0.414)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. Bold type face represents significant findings. WB=whole blood; PBMC=peripheral blood mononuclear cells.

Table 3.26 Correlational analysis of HPA axis associated genes and CAR parameters in all participants.

	Cortisol AM	Cortisol PM	AUCi	AUCg	Reactivity
<i>NR3C1</i> WB mRNA	r=0.287 (p=0.062)	r=-0.116 (p=0.565)	r=0.191 (p=0.279)	r=0.294 (p=0.092)	r=-0.014 (p=0.936)
<i>NR3C1</i> PBMC mRNA	r=-0.354 (p=0.047)*	r=0.080 (p=0.725)	r=-0.210 (p=0.293)	r=-0.428 (p=0.026)*	r=-0.093 (p=0.645)
<i>FKBP5</i> WB mRNA	r=0.104 (p=0.450)	r=0.069 (p=0.688)	r=0.187 (p=0.217)	r=0.031 (p=0.839)	r=0.176 (p=0.241)
<i>FKBP5</i> PBMC mRNA	r=-0.258 (p=0.154)	r=0.142 (p=0.529)	r=-0.286 (p=0.148)	r=-0.413 (p=0.032)*	r=-0.158 (p=0.433)
<i>NR3C2</i> WB mRNA	r=0.099 (p=0.537)	r=-0.010 (p=0.962)	r=0.006 (p=0.973)	r=0.135 (p=0.460)	r=-0.037 (p=0.836)
<i>NGFI-A</i> WB mRNA	r=-0.361 (p=0.019)*	r=0.143 (p=0.485)	r=-0.225 (p=0.208)	r=-0.317 (p=0.072)	r=-0.159 (p=0.369)
MR:GR ratio	r=-0.54 (p=0.337)	r=0.038 (p=0.858)	r=-0.085 (p=0.644)	r=-0.084 (p=0.646)	r=0.000 (p=0.999)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. Bold type face represents significant findings. AUCi=Area Under the Curve with respect to increase; AUCg=Areas Under the Curve with respect to ground, where ground is equal to zero. WB=whole blood; PBMC=peripheral blood mononuclear cells.

3.3.25 Demographic data for depressed patients with and without exposure to early life adversity (ELA) whose DNA methylation levels were measured

In an attempt to separate effects of early life adversity from depression, analysis was conducted on the depressed group only while dividing groups based on their exposure to early life adversity (ELA+ or ELA-). There were no significant differences in age, gender, BMI, prevalence of smokers or duration of education between the groups. [Table 3.27].

Table 3.27 Demographic data for depressed patients with and without exposure to ELA whose DNA methylation levels were measured

	ELA+ (n=16)	ELA- (n=20)	Statistic (p-value)
Age (years)	31.44 (10.12)	26.30 (7.51)	Z=-1.68, p=0.095
Gender (M/F)	5/11	5/15	$\chi^2=0.17$, p=0.722
BMI	27.87 (12.82)	27.17 (6.86)	Z=-0.79, p=0.451
Smoking (Y/N)	6/10	6/14	$\chi^2=0.23$, p=0.729
ISCED Education	3.06 (1.65)	2.95 (1.61)	Z=-0.03, p=0.987

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education.

3.3.26 Clinical data for depressed patients with and without exposure to ELA whose DNA methylation levels were measured

There were no significant differences in HAM-D, CES-D, PSQI scores or antidepressant use between the groups [Table 3.28].

Table 3.28 Clinical data for depressed patients with and without exposure to ELA whose DNA methylation levels were measured

	ELA+ (n=16)	ELA- (n=20)	Statistic (p-value)
HAM-D	22.50 (7.50)	21.95 (2.98)	Z=-1.07, p=0.290
CES-D	40.47 (9.70)	37.42 (9.52)	Z=-0.75, p=0.471
PSQI	13.00 (3.91)	12.32 (4.24)	Z=-0.42, p=0.681
CTQ Global Score	56.50 (10.18)	30.45 (4.82)	Z=-5.10, p<0.001***
Antidepressant (Y/N)	11/5	11/9	$\chi^2=0.71$, p=0.501

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (Antidepressants). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. ELA-.

3.3.27 Mean DNA methylation levels of *NR3C1* exon 1F promoter in depressed patients with and without ELA

Mean *NR3C1* exon 1F methylation levels were calculated (5 CG sites). Student's t-test performed on log transformed *NR3C1* methylation data revealed no differences in overall DNA methylation between the groups [Table 3.29].

Table 3.29 *NR3C1* exon 1F methylation data for depressed patients with and without exposure to ELA

	ELA+ (n=16)	ELA- (n=20)	Statistic (p-value)
mean<i>NR3C1F</i>	4.07 (0.15)	4.08 (0.13)	t=-0.65, p=0.521

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data.

Neither were there any differences in DNA methylation at any of the specific CG sites measured between the groups [Table 3.30].

Table 3.30 Site-specific *NR3C1* exon 1F methylation data for depressed patients with and without exposure to ELA

	ELA+ (n=16)	ELA- (n=20)	Statistic (p-value)
<i>NR3C1F</i> CG36	2.39 (0.22)	2.98 (0.34)	t=1.21, p=0.236
<i>NR3C1F</i> CG37	3.41 (0.31)	2.98 (0.22)	t=-1.04, p=0.309
<i>NR3C1F</i> CG38	5.66 (0.24)	5.54 (0.22)	t=-0.37, p=0.717
<i>NR3C1F</i> CG39	5.16 (0.20)	5.57 (0.17)	t=-1.73, p=0.094
<i>NR3C1F</i> CG41	3.83 (0.46)	3.31 (0.38)	t=-0.97, p=0.340

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data.

3.3.28 Mean DNA methylation levels of *FKBP5* intron 7 promoter in depressed patients with and without ELA

There were no significant differences in overall *FKBP5* DNA methylation between the two groups. Neither were there any significant differences in DNA methylation at CG6 or CG7 between the two groups [Table 3.31].

Table 3.31 *FKBP5* intron 7 methylation data for depressed patients with and without exposure to ELA

	ELA+(n=16)	ELA-(n=20)	Statistic (p-value)
meanFKBP5	58.69 (1.19)	60.76 (1.01)	t=1.43, p=0.162
FKBP5 CG6	61.55 (0.93)	62.57 (1.03)	t=0.70, p=0.491
FKBP5 CG7	55.84 (1.60)	58.96 (1.10)	t=1.69, p=0.100

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data.

3.3.29 Impact of *FKBP5* SNP rs1360780 allele on *FKBP5* methylation in depressed patients with and without ELA

ELA+ and ELA- depressed patients were further divided based on their *FKBP5* rs1360780 variant status. A two-Way ANOVA performed on log transformed data revealed no allele-specific differences in DNA methylation at the region of *FKBP5* intron 7 measured [Table 3.32].

Table 3.32 *FKBP5* intron 7 methylation in depressed with and without ELA with *FKBP5* SNP rs1360780 variant groups

	ELA+ CC (n=6)	ELA+ CT/TT (n=10)	ELA- CC (n=10)	ELA- CT/TT (n=7)	Statistics (p-value)
FKBP5 CG6	62.92 (1.09)	60.73 (1.30)	62.49 (1.51)	62.47 (1.65)	F=0.49 (p=0.693)
FKBP5 CG7	58.65 (1.60)	54.15 (2.26)	58.33 (1.43)	60.11 (2.01)	F=2.04 (p=0.130)
meanFKBP5	60.87 (1.07)	57.44 (1.71)	60.41 (1.42)	61.29 (1.74)	F=1.48 (p=0.250)

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a Two-Way ANOVA on log transformed data.

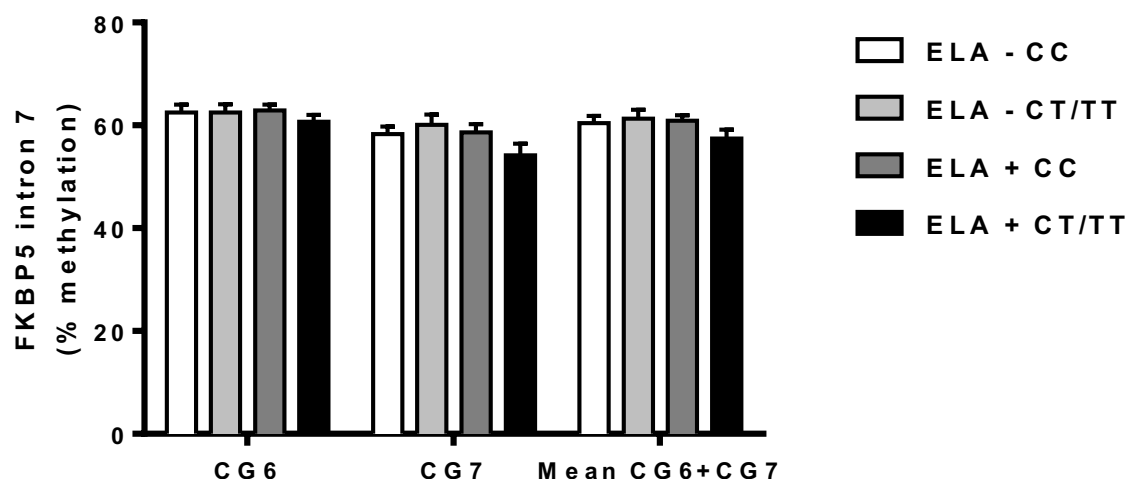


Figure 3.10 Allele-specific methylation status of *FKBP5* intron 7 in depressed patients with and without ELA. Data expressed as mean methylation percentage with SEM, Two-Way ANOVA.

3.3.30 Demographic data for depressed patients with and without exposure to early life adversity (ELA) whose whole blood mRNA was analysed by PCR

There were no significant differences in age, gender, BMI, prevalence of smokers or duration of education between the groups. [Table 3.33].

Table 3.33 Demographic data for depressed patients with and without exposure to ELA whose whole blood mRNA was analysed by PCR

	ELA+ (n=26)	ELA- (n=24)	Statistic (p-value)
Age (years)	30.77 (9.72)	27.00 (7.21)	Z=-1.50, p=0.134
Gender (M/F)	7/19	6/18	$\chi^2=0.24$, p=0.877
BMI	27.08 (11.07)	26.62 (6.58)	Z=-0.79, p=0.429
Smoking (Y/N)	12/14	8/16	$\chi^2=0.86$, p=0.355
ISCED Education	3.40 (1.96)	2.96 (1.71)	Z=-0.55, p=0.585

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education.

3.3.31 Clinical data for depressed patients with and without exposure to ELA whose whole blood mRNA was analysed by PCR

There were no significant differences in HAM-D, CES-D, PSQI scores or antidepressant use between the groups [Table 3.34].

Table 3.34 Clinical data for depressed patients with and without exposure to ELA whose whole blood mRNA was analysed by PCR

	ELA+ (n=26)	ELA- (n=24)	Statistic (p-value)
HAM-D	22.35 (7.24)	22.50 (3.14)	Z=-0.49, p=0.625
CES-D	39.04 (9.65)	39.17 (10.06)	Z=-0.07, p=0.942
PSQI	12.72 (3.96)	12.85 (3.87)	Z=-0.16, p=0.877
CTQ Global Score	56.65 (9.56)	30.38 (5.01)	Z=-6.07, p<0.001***
Antidepressant (Y/N)	17/9	15/9	$\chi^2=0.05$, p=0.832

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (Antidepressants). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. ELA-.

3.3.32 Whole blood gene expression of HPA axis associated genes in depressed patients with and without ELA

Gene expression of *NR3C1*, *NR3C2*, *NGFI-A* and *FKBP5* was measured in whole blood from ELA depressed patients compared to ELA depressed patients. A Student's t-test performed on log transformed data revealed no differences in whole blood gene expression of *NR3C1*, *NGFI-A* or *FKBP5* between groups. There was an almost significant difference ($p=0.052$) in *NR3C2* gene expression between the groups. There was a significant decrease in *NR3C2:NR3C1* (MR:GR) ratio between groups [Table 3.35] [Figure 3.11].

Table 3.35 HPA axis associated gene expression data for depressed patients with and without ELA

	ELA+ (n=26)	ELA- (n=24)	Statistic (p-value)
<i>NR3C1</i> mRNA	1.03 (0.06)	1.00 (0.05)	$t=-0.35$, $p=0.726$
<i>NR3C2</i> mRNA	0.82 (0.06)	1.00 (0.07)	$t=2.01$, $p=0.052$
<i>NGFI-A</i> mRNA	0.73 (0.06)	1.00 (0.29)	$t=0.74$, $p=0.466$
<i>FKBP5</i> mRNA	0.91 (0.10)	1.00 (0.11)	$t=0.68$, $p=0.466$
MR:GR ratio	0.71 (0.05)	0.88 (0.05)	$t=2.41$, $p=0.021^*$

Data are expressed as mean fold change or relative quantity with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data. $*p \leq 0.05$ vs. ELA-.

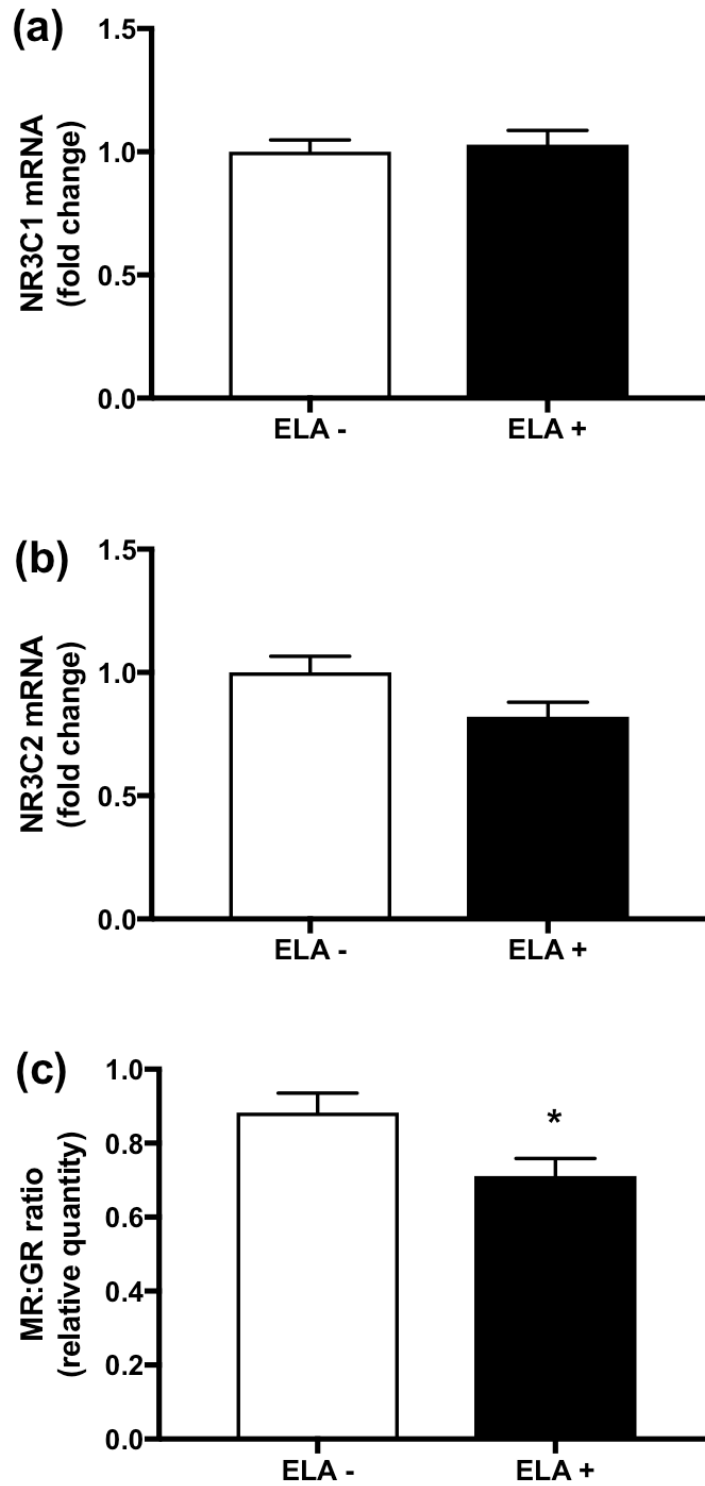


Figure 3.11 HPA axis associated gene expression in depressed patients with and without ELA. Whole blood mRNA expression of (a) glucocorticoid receptor, (b) mineralocorticoid receptor and (c) MR:GR ratio in depressed patients with and without ELA. Data expressed as mean fold change or relative quantity with SEM. * $p \leq 0.05$ vs. ELA- (Student's t-test).

3.3.33 Demographic data for depressed patients with and without exposure to early life adversity (ELA) whose salivary cortisol was measured by LC-MS

There were no significant differences in age, gender, BMI, prevalence of smokers or duration of education between the groups. [Table 3.36].

Table 3.36 Demographic data for depressed patients with and without exposure to ELA whose salivary cortisol was measured by LC-MS

	ELA+ (n=24)	ELA- (n=28)	Statistic (p-value)
Age (years)	28.79 (8.82)	26.80 (7.07)	Z=-0.84, p=0.400
Gender (M/F)	9/15	9/19	$\chi^2=0.50$, p=0.551
BMI	24.16 (6.65)	25.48 (6.03)	Z=-0.98, p=0.341
Smoking (Y/N)	10/14	8/20	$\chi^2=4.01$, p=0.078
ISCED Education	3.09 (1.76)	2.84 (1.82)	Z=-0.43, p=0.668

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education.

3.3.34 Clinical data for depressed patients with and without exposure to ELA whose salivary cortisol was measured by LC-MS

There were no significant differences in HAM-D, CES-D, PSQI scores or antidepressant use between the groups [Table 3.37].

Table 3.37 Clinical data for depressed patients with and without exposure to whose salivary cortisol was measured by LC-MS

	ELA+ (n=24)	ELA- (n=28)	Statistic (p-value)
HAM-D	23.88 (5.63)	22.12 (3.66)	Z=-1.74, p=0.081
CES-D	39.00 (9.35)	39.04 (11.01)	Z=-0.19, p=0.853
PSQI	12.88 (3.40)	13.35 (4.13)	Z=-0.70, p=0.481
CTQ Global Score	60.17 (13.11)	31.08 (5.77)	Z=-6.01, p<0.001***
Antidepressant (Y/N)	13/11	14/14	$\chi^2=0.02$, p=1.000

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (Antidepressants). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. ELA-.

3.3.35 Salivary cortisol levels in depressed patients with and without ELA

A Student's t-test revealed no differences in cortisol measurements at any time-points throughout the day between the groups [Table 3.38]. There were no significant differences between groups at any of the CAR parameters examined [Table 3.39].

Table 3.38 Morning and evening cortisol concentrations in depressed patients with and without ELA

	ELA+ (n=24)	ELA- (n=28)	Statistic (p-value)
Cortisol T0	11.32 (1.73)	11.89 (2.39)	t=-0.276, p=0.784
Cortisol T30	13.11 (1.46)	12.71 (1.54)	t=-0.413, p=0.682
Cortisol T60	11.80 (2.24)	8.20 (1.13)	t=-1.027, p=0.310
Cortisol T720	2.71 (0.582)	4.46 (2.29)	t=0.073, p=0.942
Cortisol T750	3.29 (1.11)	4.29 (2.17)	t=0.014, p=0.989

Data are expressed as mean cortisol concentrations (nMol/L) with SEM in parentheses. Statistical analysis was performed using Student t-test on log transformed data

Table 3.39 CAR parameters for depressed patients with and without ELA

	ELA+ (n=24)	ELA- (n=28)	Statistic (p-value)
Cortisol AM average	15.53 (4.07)	10.88 (1.34)	t=-0.841, p=0.405
Cortisol PM average	2.96 (0.59)	3.74 (1.79)	t=-0.243, p=0.810
Cortisol AUCg	709.45 (98.96)	674.28 (86.344)	t=-0.252, p=0.803
Cortisol AUCi	48.12 (63.85)	-24.81 (110.11)	t=-0.367, p=0.716
Cortisol Reactivity	0.442 (1.99)	-2.87 (2.211)	t=-0.333, p=0.741

Data are expressed as means with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data. AUCi=Area Under the Curve with respect to increase; AUCg=Areas Under the Curve with respect to ground, where ground is equal to zero.

3.3.36 Demographic data for depressed patients with and without exposure to early life adversity (ELA) whose PBMC mRNA was analysed by PCR

There were no significant differences in age, gender, BMI, prevalence of smokers or duration of education between the groups. [Table 3.40].

Table 3.40 Demographic data for depressed patients with and without exposure to ELA whose PBMC mRNA was analysed by PCR

	ELA+ (n=16)	ELA- (n=17)	Statistic (p-value)
Age (years)	29.19 (9.85)	26.76 (7.77)	Z=-0.72, p=0.488
Gender (M/F)	3/13	4/13	$\chi^2=0.11$, p=0.737
BMI	29.15 (13.01)	26.72 (5.77)	Z=-0.46, p=0.671
Smoking (Y/N)	7/9	6/11	$\chi^2=0.25$, p=0.728
ISCED Education	3.27 (1.58)	2.65 (1.84)	Z=-0.67, p=0.526

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education.

3.3.37 Clinical data for depressed patients with and without exposure to ELA whose PBMC mRNA was analysed by PCR

There were no significant differences in HAM-D, CES-D, PSQI scores or antidepressant use between the groups [Table 3.41].

Table 3.41 Clinical data for depressed patients with and without exposure to whose PBMC mRNA was analysed by PCR

	ELA+ (n=16)	ELA- (n=17)	Statistic (p-value)
HAM-D	22.06 (7.70)	22.35 (3.33)	Z=-0.29, p=0.079
CES-D	41.07 (8.73)	38.69 (10.35)	Z=-0.48, p=0.654
PSQI	12.20 (4.11)	13.63 (3.52)	Z=-1.07, p=0.299
CTQ Global Score	57.63 (10.67)	30.24 (5.07)	Z=-4.91, p<0.001***
Antidepressant (Y/N)	9/7	10/7	$\chi^2=0.02$, p=0.881

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (Antidepressants). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. ELA-.

3.3.38 PBMC gene expression of HPA axis associated genes in depressed patients with and without ELA

Basal levels of *NR3C1* and *FKBP5* mRNA were measured from PBMCs in these participants. A Mann-Whitney U test revealed no significant difference between the two groups [Table 3.42].

Table 3.42 HPA axis-associated gene expression in PBMCs in depressed patients with and without ELA

	ELA+ (n=16)	ELA- (n=17)	Statistic (p-value)
<i>NR3C1</i> mRNA (FC)	1.10 (0.19)	1.000 (0.10)	Z=-0.10, p=0.943
<i>FKBP5</i> mRNA (FC)	1.30 (0.29)	1.000 (0.15)	Z=-1.07, p=0.302

Data are expressed as mean fold change with SEM in parentheses. Statistical analysis was performed using a Mann-Whitney U test.

3.3.39 HPA axis associated gene expression profile following dexamethasone stimulation in depressed patients with and without ELA

A Kruskal-Wallis One-Way ANOVA revealed no differences in the expression of *NR3C1* in PBMCs isolated from ELA+ and ELA- depressed patients following stimulation with the synthetic glucocorticoid dexamethasone ($\chi^2=0.84$, $df=2$, $p=0.658$). Neither were there any differences in the unstimulated expression ($Z=-0.10$, $p=0.940$), dexamethasone 10nM ($Z=-1.897$, $p=0.060$) or dexamethasone 100nM ($Z=-0.04$, $p=0.984$) of *NR3C1* between groups.

A Kruskal-Wallis One-Way ANOVA revealed a differential expression of *FKBP5* in PBMCs isolated from ELA+ and ELA- depressed patients following stimulation from the synthetic glucocorticoid dexamethasone ($\chi^2=55.90$, $df=2$, $p<0.001$). Subsequent Mann-Whitney U tests revealed that in the ELA- samples dexamethasone at a concentration of 10nM ($Z=-4.27$, $p<0.001$) and 100nM ($Z=-4.353$, $p<0.001$) induced a significant increase in *FKBP5* mRNA expression relative to unstimulated control levels. In the ELA+ group, dexamethasone at a concentration of 10nM ($Z=-3.448$, $p<0.001$) and 100nM ($Z=-4.495$, $p<0.001$) also induced a significant increase in *FKBP5* mRNA expression relative to unstimulated control levels. There were no significant difference between the ELA+ and ELA- groups.

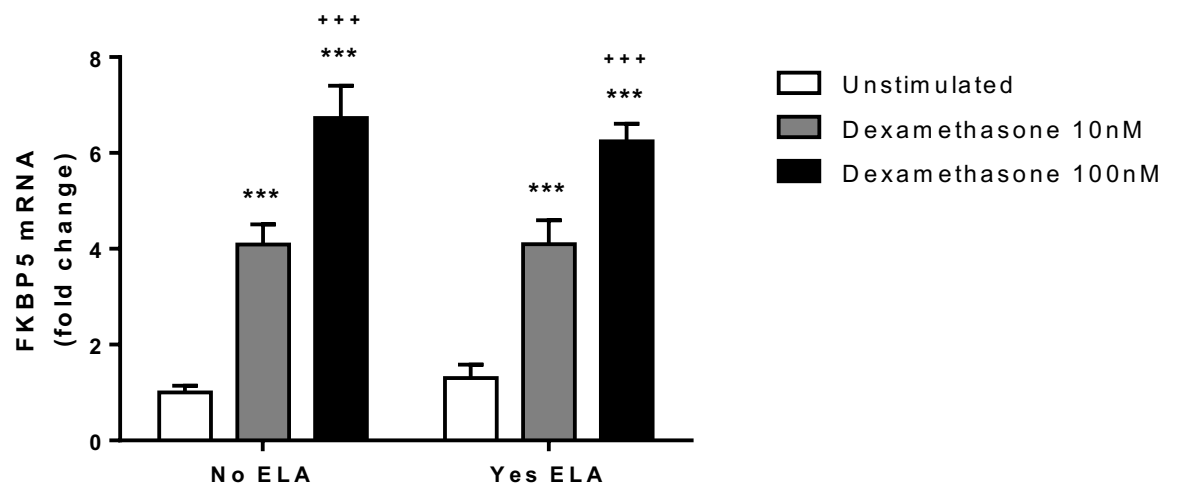
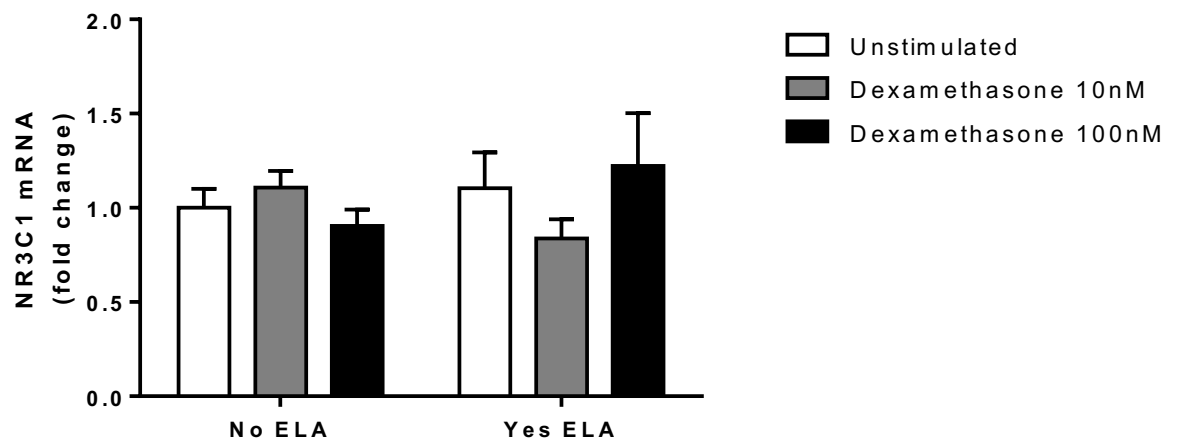


Figure 3.12 Dexamethasone 10nM and 100nM stimulated *NR3C1* and *FKBP5* expression in PBMCs from ELA+ and ELA- depressed patients. PBMCs isolated from ELA+ and ELA-depressed patients were incubated for 24 hours with dexamethasone at concentrations of 10nM and 100nM. Relative mRNA expression for (a) *NR3C1* and (b) *FKBP5* was quantified. Data are expressed as mean with SEM. *** $p \leq 0.001$ vs. unstimulated *FKBP5* expression, +++ $p \leq 0.001$ vs. dexamethasone 10nM stimulated *FKBP5* expression (Kruskal-Wallis One-Way ANOVA followed by Mann-Whitney U tests).

3.3.40 *NR3C1* exon 1F DNA methylation and childhood trauma severity in depressed patients with ELA

Spearman's rho correlation analysis revealed a significant positive association between early life emotional abuse severity and *NR3C1* exon 1F CG37 and CG38 DNA methylation [Figure 3.14]. Analysis also revealed a significant negative association between early life physical abuse severity and *NR3C1* exon 1F CG41 DNA methylation [Table 3.43].

Table 3.43 Correlational analysis of *NR3C1* exon 1F promoter DNA methylation and individual items on the childhood trauma questionnaire in depressed patients with ELA.

	Total CTQ	Emotional Abuse	Physical Abuse	Sexual Abuse	Emotional Neglect	Physical Neglect
<i>NR3C1</i> CG36	r=-0.339 (p=0.199)	r=0.114 (p=0.657)	r=-0.202 (p=0.452)	r=-0.349 (p=0.186)	r=-0.132 (p=0.626)	r=-0.233 (p=0.385)
<i>NR3C1</i> CG37	r=0.208 (p=0.440)	r=0.565 (p=0.023)*	r=0.233 (p=0.385)	r=0.096 (p=0.724)	r=0.009 (p=0.974)	r=0.072 (p=0.791)
<i>NR3C1</i> CG38	r=0.126 (p=0.642)	r=0.582 (p=0.018)*	r=0.128 (p=0.636)	r=-0.042 (p=0.878)	r=-0.194 (p=0.472)	r=-0.106 (p=0.696)
<i>NR3C1</i> CG39	r=-0.404 (p=0.135)	r=-0.028 (p=0.921)	r=-0.149 (p=0.596)	r=-0.299 (p=0.279)	r=-0.114 (p=0.686)	r=-0.262 (p=0.345)
<i>NR3C1</i> CG41	r=-0.496 (p=0.051)	r=-0.060 (p=0.825)	r=-0.555 (p=0.026)*	r=-0.300 (p=0.260)	r=0.112 (p=0.680)	r=0.249 (p=0.352)
mean<i>NR3C1F</i>	r=-0.164 (p=0.362)	r=-0.036 (p=0.843)	r=-0.141 (p=0.440)	r=-0.100 (p=0.586)	r=0.042 (p=0.818)	r=-0.072 (p=0.696)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. Bold type face represents significant findings. HAM-D=Hamilton Rating Scale for Depression; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire.

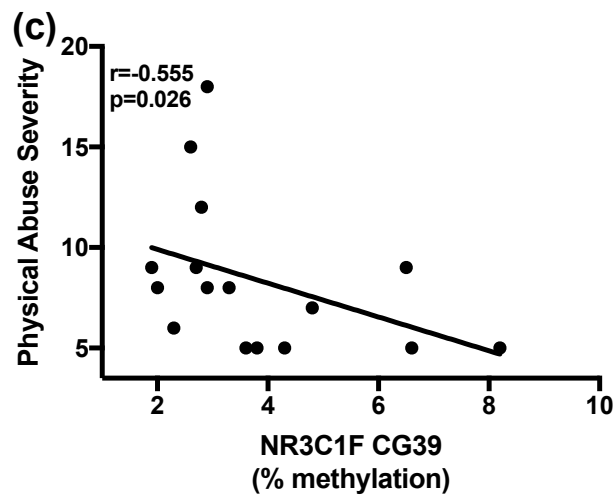
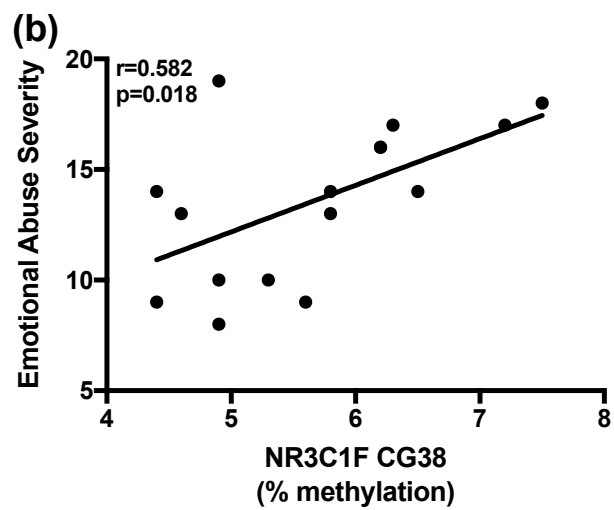
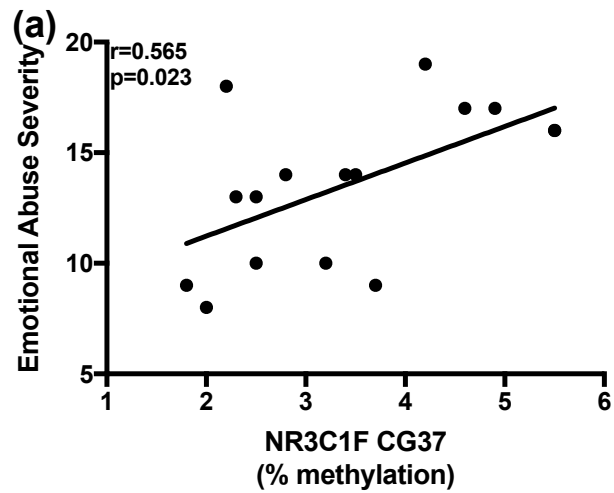


Figure 3.13 Relationships between *NR3C1* exon 1F DNA methylation and early life abuse severity in depressed patients with ELA. A significant relationship exists between (a) *NR3C1* exon 1F CG37 DNA methylation and emotional abuse, (b) *NR3C1* exon 1F CG38 DNA methylation and emotional abuse and (c) *NR3C1* exon 1F CG39 DNA methylation and physical abuse.

3.4 Discussion

This study sought to determine whether alterations in DNA methylation levels at the glucocorticoid receptor gene (*NR3C1*) exon 1F promoter and *FKBP5* intron 7 were present in depressed individuals, and if so, whether these alterations were associated with subsequent changes in gene expression, cell function and HPA axis measures. The overall percentage of methylation at the *NR3C1* exon 1F promoter region was significantly increased in the depressed patients relative to healthy controls. This was related to increased cortisol concentrations upon waking as well as decreased cortisol reactivity. No significant differences in *FKBP5* at the DNA or gene expression level were observed.

3.4.1 Increased *NR3C1* exon 1F promoter DNA methylation in depression

Although no significant differences in methylation at specific CG sites within the *NR3C1* exon 1F, including the NGFI-A binding site, were revealed, the overall percentage of methylation at the *NR3C1* exon 1F promoter region measured was significantly increased in the depressed patients relative to healthy controls.

As increased DNA methylation is typically associated with gene silencing (Newell-Price et al., 2000), correlational analysis was conducted to assess the relationship between *NR3C1* DNA methylation and gene expression in our cohort. DNA methylation levels at the *NR3C1* exon 1F promoter, however, were not associated with *NR3C1* mRNA expression in whole blood. In contrast significant associations between *NR3C1* exon 1F promoter methylation were found with *NR3C1* mRNA in PBMCs. Methylation at CG38, which is part of the NGFI-A binding site, was negatively associated with *NR3C1* gene expression. This indicates that higher methylation at this site is associated with reduced GR mRNA and is consistent with previous studies (Palma-Gudiel et al., 2015).

A previous study has shown that gene expression profiles in blood-based samples are strongly influenced by the predominant cell type constituent, and that PAXgene samples show a decrease in the number of expressed genes with higher variability when compared with PBMC mRNA samples (Min et al., 2010). Our study supports this finding and suggests that mRNA source should be carefully considered in future studies.

3.4.2 Altered cortisol awakening responses and association with DNA methylation in depression

The depressed group had increased waking cortisol concentrations, and a decrease in cortisol reactivity. This high baseline and flattened cortisol reactivity to stress has been found in most previous studies examining cortisol patterns in depression (Suzuki et al., 2013). My results support the hypothesis that these cortisol differences result from glucocorticoid receptor (GR) resistance. There was a significant and positive relationship between DNA methylation at the NGFI-A binding site, CG38, and morning cortisol concentrations. Furthermore CG38 methylation was negatively associated with *NR3C1* mRNA expression. Put together, there was a significant association between increased *NR3C1* exon 1F methylation, decreased *NR3C1* mRNA expression and subsequent morning cortisol increases. These findings provide strong evidence for DNA methylation-mediated alterations in HPA axis function.

3.4.3 *NR3C1* exon 1F methylation and trauma

As increased methylation has been associated with exposure to ELA (McGowan et al., 2009), analysis was performed to assess whether ELA may be driving the differences in DNA methylation at the *NR3C1* exon 1F promoter between the depressed and control groups. There was a significant positive association between childhood emotional abuse severity and DNA methylation at CG37 and CG38. This effect was not present when assessing all participants, suggesting an effect of ELA severity, rather than depression per se, on methylation status of the *NR3C1* exon 1F.

3.4.4 *FKBP5* intron 7 DNA methylation in depression

As *FKBP5* intron 7 methylation alterations have previously been observed in individuals with a history of early life adversity and suffering with post-traumatic stress disorder (PTSD) (Klengel et al., 2013b), we sought to assess differences in methylation at the same region in our depressed cohort. Analysis revealed no 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 was observed in depressed patients relative to healthy controls.

The same analysis was conducted on the depressed individuals only, to assess whether exposure to ELA had any impact and/or interaction with genotype to alter *FKBP5* methylation status. There appeared to be a trend towards decreased methylation in the depressed individuals who were exposed to ELA and had the risk T-allele, however, statistical significance was not reached. Significance may have been reached if more participants were included in the study, as the samples size in this analysis was small (n=6-10 per group).

Overall, this study did not reveal any differences in *FKBP5* intron 7 promoter DNA methylation between depressed individuals and healthy controls or between ELA+ and ELA- depressed individuals, even when considering the presence of a risk polymorphism.

3.4.5 Mineralocorticoid receptor alterations and exposure to ELA

mRNA levels of genes associated with the HPA axis were measured in whole blood of depressed patients and healthy controls. Analysis revealed no significant differences between the groups. However, when the depressed group was split into those who were exposed to ELA and those who had not been exposed to ELA, there was an almost significant difference ($p=0.052$) in mineralocorticoid receptor (MR) mRNA levels and a subsequent significant decrease in MR:GR ratio in ELA+ depressed patients relative to ELA- depressed patients. MR levels are important to examine as these receptors have a high affinity for cortisol; cortisol initially binding to MR, to which it has a higher affinity, prior to GR. Mineralocorticoid receptors are known work in a heterodimer fashion to regulate HPA axis activity (Trapp and Holsboer, 1996). The fact that depressed individuals who have been exposed to ELA have an altered MR:GR ratio may in itself lead to a dysregulated HPA axis. This effect was not present in analysis examining differences between depressed and control individuals, suggesting again an effect of ELA, rather than depression per se, on the HPA axis. The MR finding merits further investigation.

3.4.6 Effects of stimulation of peripheral blood mononuclear cells (PBMCs) by dexamethasone on HPA axis associated gene expression

The results of the PBMC stimulation work revealed a dose related effect of dexamethasone on *FKBP5* gene expression, but not on *NR3C1* gene expression. There were no significant differences in gene expression of *NR3C1* or *FKBP5* between groups. It did appear, albeit non-significant, that dexamethasone did reduce *NR3C1* expression in healthy controls, as would be expected, but not at all in depressed patients – it may be the case that the sample size was too small to reveal significant differences and that increased power is required to achieve significance.

Dexamethasone has a higher affinity and potency for the glucocorticoid receptor, compared to the endogenous hormone cortisol (Ponec et al., 1986), and it may be more useful to perform future stimulations with cortisol to more appropriately mirror the in vivo environment.

3.4.7. Limitations

As is often the case with clinical research the study would have benefited from a larger sample size. It is important to note the relatively small sample size as a limitation in this study as it could result in the statistics being underpowered. Underpowered statistical analyses run the risk of Type II errors (i.e. false negative results) and this should be considered when interpreting these results.

As mentioned in Section 1.8 (Aims), given the novelty of DNA methylation analysis in psychiatric research, much of the work presented throughout the thesis 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 of this Chapter.

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.

3.4.8 Conclusion

These data replicate findings of hyperactivity of the HPA axis in individuals suffering with major depressive disorder: the pattern being high baseline levels and blunted responses to stress. My studies provide evidence that this hyperactivity is, at least partly, driven by alterations at the glucocorticoid receptor gene (*NR3C1*) exon 1F promoter and that regions of the gene may be particularly sensitive to exposure to early life adversity.

Chapter 4

**Serotonin neurotransmission in
depression: methylation of the SLC6A4
gene, tryptophan levels and the HPA axis**

4.1 Introduction

Major depressive disorder (MDD) is a serious psychiatric illness with a lifetime prevalence of approximately 17% (Kessler, 2005). However, despite its high rate of occurrence, the biological basis of the disorder remains elusive.

The monoamine hypothesis of depression states that the underlying pathophysiology in depression is a reduction in serotonin (5-hydroxytryptamine /5-HT), norepinephrine (NA), and or dopamine (DA) neurotransmission in the brain (Delgado, 2000). This has been one the most investigated mechanisms in the literature to date, with the majority of the work focusing on 5-HT. Alterations in serotonin neurotransmission have been consistently implicated in depression research. One mechanism to explain alterations in 5-HT function is dysregulation of the 5-HT transporter (5-HTT). The 5-HTT is responsible for terminating the action of 5-HT in the synaptic space, in the brain as well as other tissues, via reuptake of released 5-HT. 5-HTT terminates the action of 5-HT by transporting it back into the pre-synaptic neuron where it is transported into vesicles using a sodium-dependent mechanism, thereby reducing post-synaptic 5-HT neurotransmission. The 5-HTT protein is encoded by the solute carrier family 6, member 4 (*SLC6A4*) gene.

A functional polymorphism in the promoter region of *SLC6A4* gene exists, referred to as 5-HTTLPR, effects the transcriptional activity and subsequent 5-HT reuptake. Insertion or deletion of a 44bp long region gives rise to short (s) and long (l) forms of the promoter region, the s form bring associated with reduced 5-HTT expression (Taylor et al., 2010). Given the hypothesised role of 5-HT/5-HTT in depression, and an increasing body of literature suggesting epigenetic changes contributing to various psychiatric disorders (Nestler, 2009), investigations into *SLC6A4* promoter DNA methylation changes in depression are of particular interest.

Due to the interest in 5-HT alterations in the pathophysiology of depression, a significant body of research has examined the potential role of the essential amino acid tryptophan in the pathogenesis of depression. It has been hypothesised that individuals with depression have decreased tryptophan concentrations resulting in reduced availability for 5-HT synthesis. It has been proposed that the decreased tryptophan concentrations observed in depressed patients could result from an increase in the metabolism of tryptophan in the kynurenine pathway, possibly induced by altered inflammatory and stress processes (Muller and Schwarz, 2007).

Whether the well replicated finding of increased HPA axis output, with high basal secretion of cortisol and blunted cortisol stress responses, found in depression may be related to the altered 5-HT function also seen in depression remains a moot point. Dysregulation of the HPA axis and 5-HT system has been implicated in the pathophysiology of disease states such as affective disorders, anxiety disorders and obesity (Heisler, 1998; Kelly et al., 1980; Porter et al., 2004). Depressed patients are often characterised by a reduction in central 5-HT function and accompanying hypercortisolemia (O'Keane, 1991). This may be why treatment with SSRIs are generally associated with alterations in HPA axis dynamics. Second, many early studies found that 5-HT mediated endocrine responses were inversely related to HPA axis function (Cleare et al., 1995). For example, conditions characterised by low HPA axis output, such as the Chronic Fatigue Syndrome and Fibromyalgia, have been reported to be associated with increased 5-HT mediated responses (Cleare et al., 2004). Depression, conversely, is associated with increased HPA axis output, and with reduced 5-HT mediated responses (Cowen, 2002).

Therefore the aims of this study were to:

1. Investigate *SLC6A4* promoter DNA methylation status and subsequent mRNA expression in depressed patients compared to healthy controls
2. Assess the *SLC6A4* promoter DNA methylation in relation to the HPA axis and circulating tryptophan/kynurenine levels.

4.2 Methods

A detailed description of the study participants, recruitment process, clinical rating scales and sample collection and processing are outlined in chapter 2.

4.2.1 DNA methylation analysis of the serotonin transporter gene promoter in whole blood

DNA methylation of the serotonin transporter gene (*SLC6A4*) promoter was measured in whole blood by pyrosequencing, performed on bisulfite-treated DNA. SLC6A4_N2_F(TTGTTAGGTTTTAGGAAGAAAGAGAGA) and SLC6A4_N2_R-biotin (5Biosg/AAAAAAACTACACAAAAAAACAAATATAC) were the forward and reverse primers, respectively, for the PCR reaction. SLC6A4_N2_Seq_1 (ATCGATGGTATCGATGGAGTAGTCGAGCTAGAGATGTCGTGATCGAG) and SLC6A4_N2_Seq_2 (GTCGTTGTAGTCAGTCGGTCGCTAGGATGTCGTA) were the sequencing primers used in the pyrosequencing reaction. In total, percentage methylation was measured at eight CG sites. Pyrosequencing was performed using the Q96 platform (Qiagen). Percentage methylation levels and quality control were analysed using PyroMark CpG Software (Version 1.0.11, Qiagen). Detailed methodology of the pyrosequencing procedure is outlined in Chapter 2 – Section 2.2.3.

TTGTTAGGTTTTAGGAAGAAAGAGAGAGTAGTTTT**CG**¹GGATGGGG**ACG**²ATGGGGAGGTGTT**CG**³AGGTTAAGAGAAAGCG⁴GTACG⁵AGTAGATTTTTGTGTGT**CG**⁶TTTTGTGGG**CG**⁷**CG**⁸GGG**CG**⁹GTAGGGAGGG**CG**¹⁰TATATTTGTTTTTTGTGTAGTTTTTTT

Figure 4.1 Region encompassing *SLC6A4* promoter DNA sequence measured. 162 bp nested PCR amplicon of *SLC6A4* promoter, upper strand, converted sequence. Measured CG sites are in bold font.

4.2.2 Real-time PCR analysis of *SLC6A4* in whole blood

Total RNA was extracted from whole blood collected into PAXgene Blood RNA tubes using the PAXgene Blood RNA kit (Qiagen) as per the manufacturer's recommended protocol. Following quantification, all RNA samples were equalised to 10 ng/μl and cDNA was synthesised using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Gene expression analysis was performed using fluorescein amidite (FAM)-labelled probes for the genes of interest (Taqman Gene Expression assays, Applied Biosystems) [Table 4.1] with a VIC-labelled probe for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the endogenous control (see Chapter 2 – Section 2.2.5).

Table 4.1 List of genes used with GenBank sequence ref numbers

Target Symbol	Target Name	Taqman Gene Assay ID
<i>SLC6A4/SERT</i>	Serotonin transporter	Hs00169010_m1
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	Hs02758991_g1

4.2.3 Measurement of tryptophan and kynurenine in plasma

Plasma was obtained from whole blood as described in Section 2.2.6.1. 50 μl plasma was analysed by liquid chromatography tandem mass spectroscopy (LC-MS/MS) for the measurement of tryptophan and kynurenine pathway metabolites in the laboratory of Dr. Andre Liesener in Boehringer Ingelheim. Tryptophan and kynurenine concentrations were quantified by validated assays based on liquid chromatography tandem mass spectrometry (LC-MS/MS). Both assays comprised sample clean-up protein precipitation followed by reversed-phase chromatography and mass spectrometric detection in the positive ion multiple reaction monitoring (MRM) mode using the deuterated analogues of the analytes, tryptophan and kynurenine as internal standards.

4.2.5 Liquid chromatography-mass spectrometry (LC-MS) for salivary cortisol

Saliva samples were collected from all participants at five time-points throughout the day to measure the cortisol awakening response (CAR) and to assess potential diurnal variations in cortisol levels. Salivary cortisol and cortisone levels were measured by LC-MS, performed at the University of Manchester in the Department of Clinical Biochemistry. The work was

performed by members of Prof. Brian Keevil's laboratory at the University Hospital of South Manchester. Calculation of all CAR parameters were derived by Dr. Kelly Doolin. These parameters were Area Under the Curve (AUC) with respect to (1) increase from baseline (AUCi), (2) to ground (AUCg) where ground is equal to zero, and cortisol reactivity.

4.2.6 Statistical Analysis

Data are presented as mean with standard deviation (SD) and standard error of the mean (SEM) where appropriate. Data normality was assessed using the Shapiro-Wilk test of normality. Differences in demographic variables were tested using Mann Whitney U test and Chi-square (χ^2) test. Differences in biological variables were tested using a Univariate General Linear Model or Student's t-test where appropriate. Spearman's rho correlation was used to assess correlations between biological variables. All statistical analyses were considered significant when $p \leq 0.05$. Statistics were performed using SPSS (Version 22, IBM) and all graphs were generated using GraphPad Prism Software (Version 6, GraphPad Inc.).

4.3 Results

4.3.1 Mean DNA methylation levels of the *SLC6A4* promoter in depressed patients and healthy controls

SLC6A4 promoter DNA methylation analysis was performed on 69 participants: 36 depressed patients and 33 healthy controls. The demographic and clinical profile of these 69 participants can be found in Section 3.3.1 and 3.3.2 respectively. Mean *SLC6A4* promoter methylation levels were calculated (8 CG sites). A univariate analysis of variance performed on log transformed *SLC6A4* methylation data, using smoking as a covariate, revealed no differences in overall methylation status between the groups [Table 4.2].

Table 4.2 Mean *SLC6A4* promoter methylation data for depressed patients and healthy controls

	Patients (n=36)	Controls (n=33)	Statistic (p-value)
mean <i>SLC6A4</i>	4.04 (0.11)	4.24 (0.14)	F=0.13, p=0.723

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance on log transformed data with smoking as a covariate.

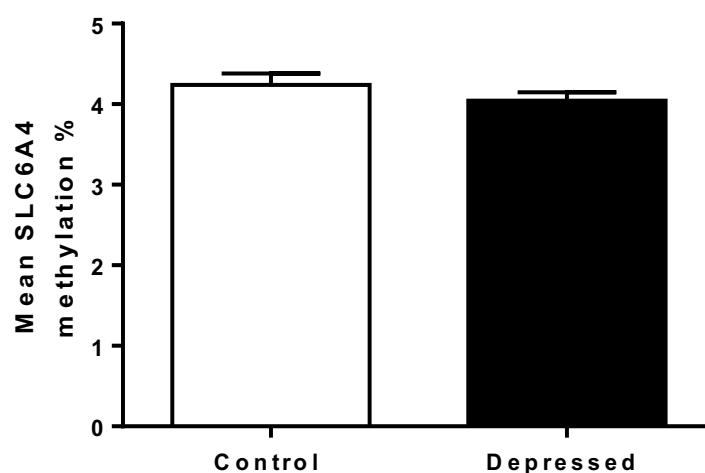


Figure 4.2 *SLC6A4* promoter methylation in depressed patients and healthy controls. Mean *SLC6A4* DNA methylation in depressed patients and healthy controls. Data expressed as mean methylation with SEM.

4.3.2 DNA methylation levels of specific CG sites in the *SLC6A4* promoter in depressed patients and healthy controls

There was significantly decreased DNA methylation levels at *SLC6A4* promoter CG8 in the depressed patients relative to healthy controls [Figure 4.3]; and no significant differences in DNA methylation at any other sites measured [Table 4.3].

Table 4.3 *SLC6A4* methylation data for depressed patients and healthy controls

	Patients (n=36)	Controls (n=33)	Statistic (p-value)
<i>SLC6A4</i> CG1	7.90 (0.27)	8.08 (0.28)	F=0.01, p=0.951
<i>SLC6A4</i> CG2	4.36 (0.13)	4.39 (0.13)	F=0.01, p=0.987
<i>SLC6A4</i> CG3	5.12 (0.11)	5.17 (0.13)	F=0.03, p=0.869
<i>SLC6A4</i> CG6	2.30 (0.17)	2.23 (0.06)	F=0.03, p=0.863
<i>SLC6A4</i> CG7	4.61 (0.19)	4.48 (0.09)	F=0.32, p=0.576
<i>SLC6A4</i> CG8	3.22 (0.16)	3.75 (0.14)	F=5.41, p=0.023*
<i>SLC6A4</i> CG9	1.56 (0.09)	1.73 (0.11)	F=1.72, p=0.196
<i>SLC6A4</i> CG10	2.50 (0.14)	2.60 (0.08)	F=0.01, p=0.959

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance on log transformed data with smoking as a covariate. *p≤0.05 vs controls.

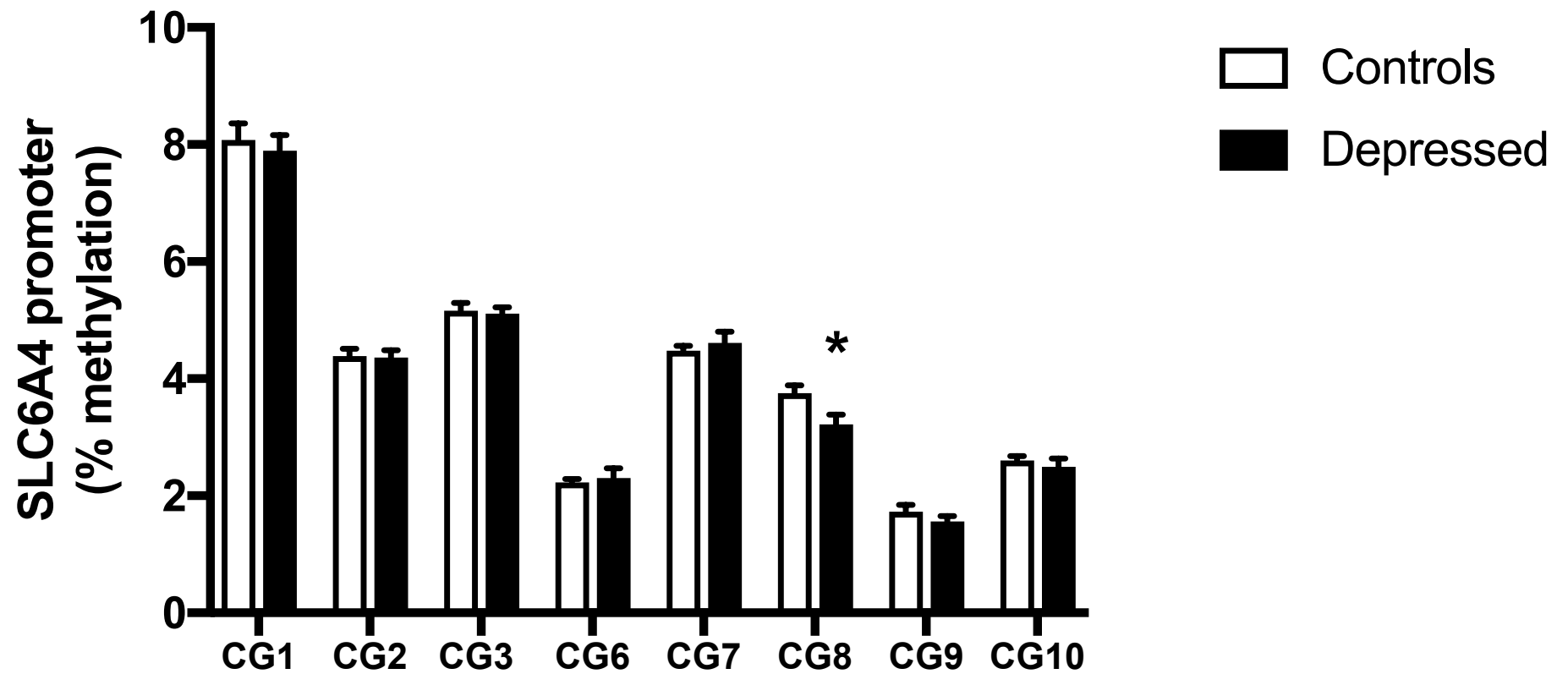


Figure 4.3 DNA methylation levels of selected *SLC6A4* promoter CG sites in depressed patients and healthy controls. DNA methylation of *SLC6A4* promoter at CG1, CG2, CG3, CG6, CG7, CG8, CG9 and CG10 in depressed patients compared to healthy controls. Data are expressed as mean methylation percentage with SEM, controlling for smoking. * $p \leq 0.05$ vs controls.

4.3.3 Whole blood gene expression of *SLC6A4* in depressed patients and healthy controls

Basal *SLC6A4* mRNA levels were measured the whole blood of 87 participants: 50 depressed patients and 37 healthy controls. The demographic and clinical profile of these 87 participants can be found in Section 3.3.10 and 3.3.11 respectively. There were no significant group differences [Table 4.4].

Table 4.4 *SLC6A4* gene expression for depressed patients and healthy controls

	Patients (n=50)	Controls (n=36)	Statistic (p-value)
<i>SLC6A4</i> mRNA	0.92 (0.12)	1.00 (0.13)	F=0.01, p=0.944

Data are expressed as mean fold change with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance, with smoking as a covariate, on log transformed data.

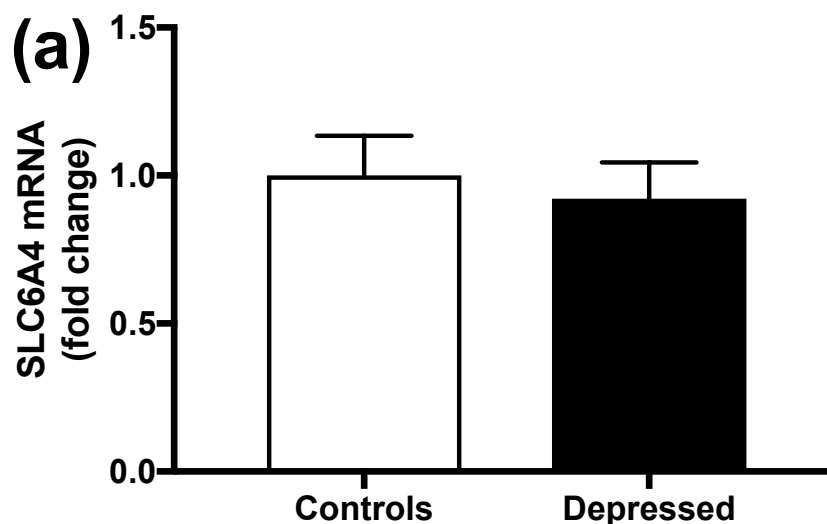


Figure 4.4 *SLC6A4* and gene expression in depressed patients and healthy controls. Whole blood mRNA expression of *SLC6A4* in depressed patients and healthy controls. Data expressed as mean fold change with SEM.

4.3.4 Demographic data for patients with depression and healthy controls whose circulating kynurenine pathway metabolite concentrations were measured

Circulating kynurenine pathway (KP) metabolite concentrations were measured in 90 participants: 57 depressed patients and 33 healthy controls. The difference in sample size here was the result of needle phobia in some participants. There were no significant differences in age, gender or BMI between the groups. Analysis revealed that the depressed group had a higher prevalence of smokers ($p=0.002$) and a lower duration of education, according to the ISCED scale ($p<0.001$) compared to that of the control group [Table 4.5].

Table 4.5 Demographic data for patients with depression and healthy controls whose circulating kynurenine pathway metabolite concentrations were measured

	Patients (n=57)	Controls (n=33)	Statistic (p-value)
Age (years)	27.91 (8.32)	28.54 (7.59)	$Z=-0.44$, $p=0.663$
Gender (M/F)	16/41	16/17	$\chi^2=3.80$, $p=0.068$
BMI	26.10 (8.53)	23.65 (3.74)	$Z=-0.79$, $p=0.431$
Smoking (Y/N)	23/34	3/30	$\chi^2=9.94$, $p=0.002^{**}$
ISCED Education	3.10 (1.80)	6.03 (1.94)	$Z=-5.56$, $p<0.001^{***}$

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education. $^{**}p\leq 0.01$ vs. control, $^{***}p\leq 0.001$ vs. control.

4.3.5 Clinical data for patients with depression and healthy controls whose circulating kynurenine pathway metabolite concentrations were measured

Consistent with a diagnosis of depression, patients with Major Depressive Disorder (MDD) had significantly higher HAM-D and CES-D scores compared to healthy controls, and poorer sleep quality, as measured by higher overall PSQI scores. The depressed group had a higher prevalence of individuals who had experienced early life adversity (ELA) (Patients: 47.4% vs. Controls: 9.1%), with significantly higher CTQ Global scores compared to the control group. Within the depressed cohort, 35 individuals (61.4%) were receiving pharmacological treatment for depression at the time of recruitment. Of these 35 patients receiving pharmacological treatment, 24 were taking selective serotonin reuptake inhibitors (SSRIs), six were receiving serotonin-noradrenaline reuptake inhibitors (SNRIs) and five were receiving other antidepressant treatments (i.e. atypical antidepressants, melatonergic antidepressants) [Table 4.6].

Table 4.6 Clinical data for patients with depression and healthy controls whose circulating kynurenine pathway metabolite concentrations were measured

	Patients (n=57)	Controls (n=33)	Statistic (p-value)
HAM-D	22.65 (5.12)	2.19 (2.34)	Z=-7.71, p<0.001***
CES-D	38.82 (9.62)	5.94 (5.95)	Z=-7.70, p<0.001***
PSQI	12.85 (4.34)	3.52 (1.79)	Z=-7.06, p<0.001***
CTQ Global Score	44.22 (15.15)	29.94 (7.92)	Z=-4.64, p<0.001***
ELA (Y/N)	27/30	3/30	$\chi^2=15.2$, p<0.001***
Antidepressant (Y/N)	35/22	0/33	

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (ELA). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. control.

4.3.6 Circulating kynurenine pathway metabolite concentrations in depressed patients compared to healthy controls

In addition to circulating KP metabolite concentrations, kynurenine/tryptophan (KYN/TRP) ratios were calculated for all participants. There was significantly decreased tryptophan and kynurenine concentrations in depressed patients compared to healthy controls [Figure 4.5]. There was no significant difference in KYN/TRP ratios between the two groups [Table 4.7].

Table 4.7 Kynurenine pathway metabolite concentrations for depressed patients and healthy controls

	Patients (n=57)	Controls (n=33)	Statistic (p-value)
Tryptophan (ng/ml)	46892.98 (1435.05)	59443.94 (2897.21)	F=18.23, p<0.001***
Kynurenine (ng/ml)	2460.19 (79.61)	2807.42 (153.35)	F=4.79, p=0.031*
KYN/TRP ratio	0.05 (0.01)	0.05 (0.01)	F=2.85, p=0.095

Data are expressed as means with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance on log transformed data with smoking as a covariate. *p≤0.05; ***p≤0.001.

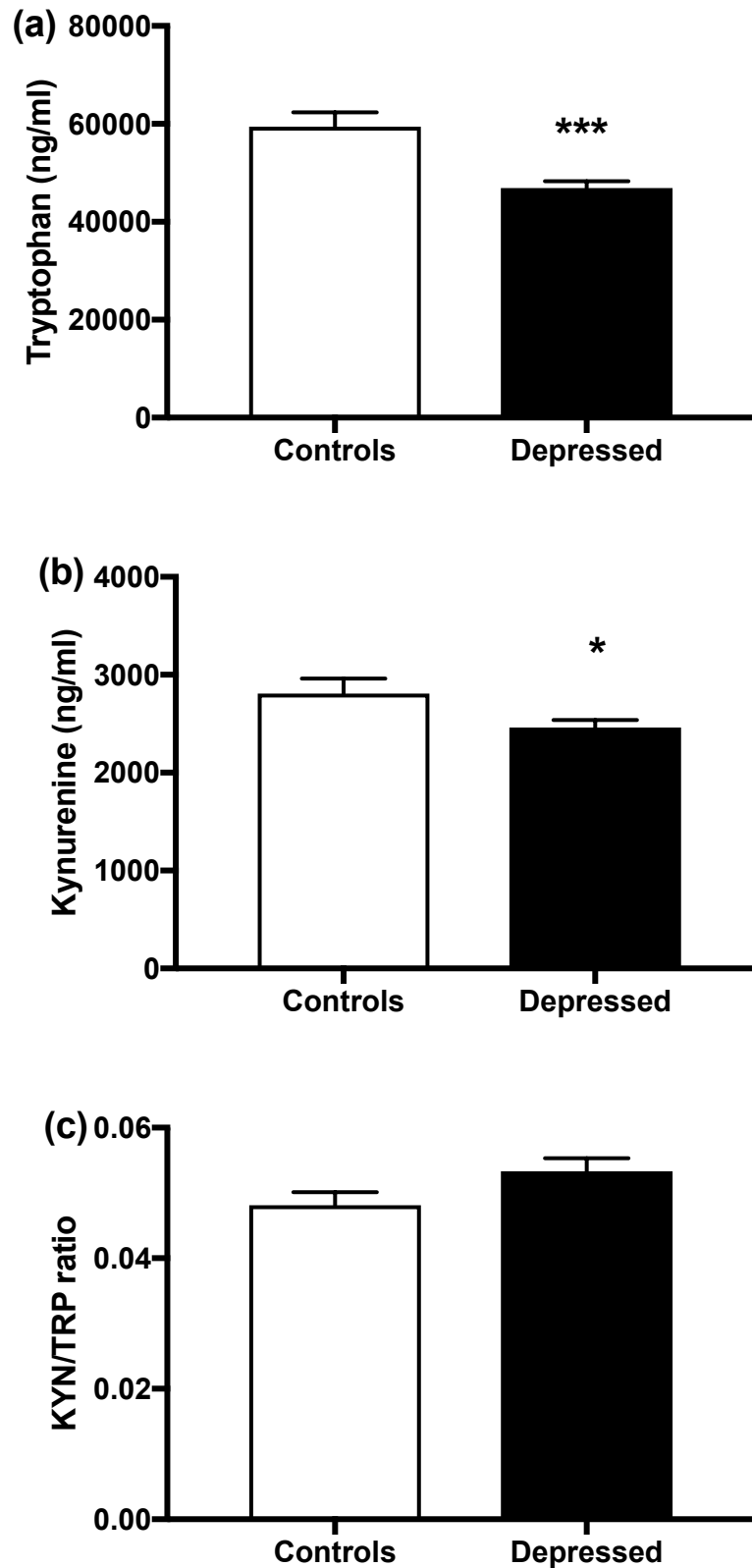


Figure 4.5 Kynurenine pathway metabolites in depressed patients and healthy controls. Plasma concentrations of (a) tryptophan, (b) kynurenine and (c) kynurenine/tryptophan (KYN/TRP) ratio in depressed patients compared with healthy controls. Data are expressed as mean with SEM. * $p \leq 0.05$ vs controls. *** $p \leq 0.001$ vs controls.

4.3.7 Associations between *SLC6A4* promoter DNA methylation with *SLC6A4* mRNA expression in the entire cohort

Correlational analysis was performed to assess whether a relationship existed between *SLC6A4* promoter DNA methylation levels and expression of *SLC6A4* mRNA. No statistical relationship existed between the degree of methylation at any CG site in the *SLC6A4* region measured and mRNA expression of *SLC6A4*.

4.3.8 Associations between *SLC6A4* promoter DNA methylation with clinical measures in the entire cohort

Correlational analysis was performed to assess whether associations existed between *SLC6A4* promoter DNA methylation levels and clinical measures. Spearman's rho analysis revealed significant negative associations between *SLC6A4* CG8 methylation levels and suicidality scores and sleep quality [Table 4.8]. There was a negative relationship between *SLC6A4* promoter CG6 DNA methylation levels and total CTQ scores. When further examining the relationship between *SLC6A4* CG6 methylation and specific abuse subtypes, a significant negative correlation was found between CG6 and emotional neglect [Table 4.9].

4.3.9 Associations between *SLC6A4* promoter DNA methylation and cortisol measures

There were significant negative associations between *SLC6A4* promoter CG8 DNA methylation levels and average morning cortisol output, cortisol T60 concentrations and cortisol AUCg measurements in the entire cohort [Table 4.10]. There were no significant associations between *SLC6A4* promoter DNA methylation and cortisol measurements within the depressed group alone.

4.3.10 Associations between *SLC6A4* DNA methylation and kynurenine pathway metabolites

When examining relationships between *SLC6A4* DNA methylation levels and kynurenine pathway metabolites, a significant negative relationship between *SLC6A4* CG6 and tryptophan concentrations ($r=-0.354$, $p=0.009$) was revealed (Figure 4.6). There were no significant relationships observed between *SLC6A4* DNA methylation at any specific CG site and kynurenine concentrations or KYN/TRP ratios.

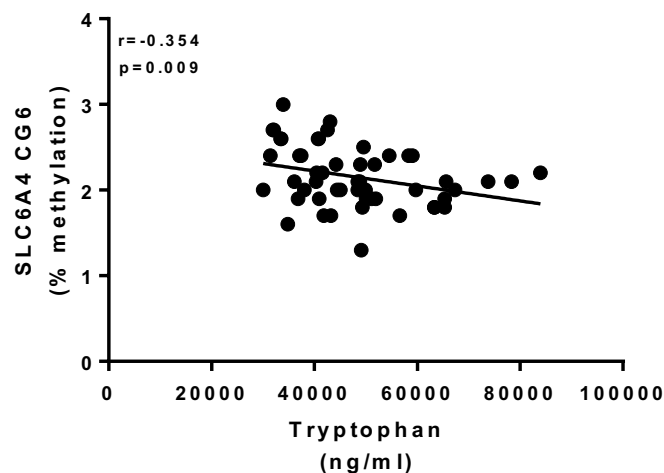


Figure 4.6 Relationships between *SLC6A4* DNA methylation and kynurenine pathway metabolites in the entire cohort. A significant relationships exists between DNA methylation levels at *SLC6A4* promoter CG6 and tryptophan concentrations in the entire cohort.

4.3.11 Evaluation of the associations between cortisol concentrations and kynurenine pathway metabolites

Cortisol T0 concentrations and AM average output were negatively associated with kynurenine concentrations. Cortisol T0, T30, T60, AM average, T720 and PM average concentrations were all negatively associated with KYN/TRP ratio in all participants [Table 4.11] [Figure 4.7]. In the depressed group, cortisol T0 concentrations were negatively associated with kynurenine concentrations. Cortisol T0, AM average and T750 concentrations were all negatively associated with KYN/TRP ratio [Table 4.12].

Table 4.8 Correlational analysis of SLC6A4 promoter DNA methylation and clinical rating scales in the entire cohort.

	HAM-D	CES-D	Suicidality	PSQI
<i>SLC6A4</i> CG1	r=-0.162 (p=0.209)	r=-0.139 (p=0.282)	r=-0.201 (p=0.163)	r=-0.211 (p=0.105)
<i>SLC6A4</i> CG2	r=-0.012 (p=0.928)	r=-0.085 (p=0.510)	r=-0.057 (p=0.693)	r=-0.111 (p=0.398)
<i>SLC6A4</i> CG3	r=-0.020 (p=0.880)	r=-0.097 (p=0.460)	r=0.032 (p=0.830)	r=0.018 (p=0.896)
<i>SLC6A4</i> CG6	r=-0.105 (p=0.422)	r=-0.095 (p=0.466)	r=0.016 (p=0.915)	r=0.048 (p=0.719)
<i>SLC6A4</i> CG7	r=0.164 (p=0.204)	r=0.112 (p=0.384)	r=0.126 (p=0.377)	r=0.073 (p=0.581)
<i>SLC6A4</i> CG8	r=-0.221 (p=0.085)	r=-0.201 (p=0.117)	r=-0.323 (p=0.021)*	r=-0.323 (p=0.012)*
<i>SLC6A4</i> CG9	r=-0.105 (p=0.477)	r=-0.180 (p=0.220)	r=-0.208 (p=0.223)	r=-0.242 (p=0.105)
<i>SLC6A4</i> CG10	r=-0.104 (p=0.483)	r=-0.194 (p=0.187)	r=-0.152 (p=0.378)	r=-0.092 (p=0.544)
Mean <i>SLC6A4</i>	r=-0.042 (p=0.739)	r=-0.134 (p=0.286)	r=-0.010 (p=0.942)	r=-0.137 (p=0.283)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. Bold font indicates significance. HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index.

Table 4.9 Correlational analysis of SLC6A4 promoter DNA methylation and childhood trauma in the entire cohort.

	Total CTQ	Emotional Abuse	Physical Abuse	Sexual Abuse	Emotional Neglect	Physical Neglect
<i>SLC6A4</i> CG1	r=-0.063 (p=0.618)	r=-0.082 (p=0.521)	r=-0.036 (p=0.778)	r=-0.041 (p=0.745)	r=-0.113 (p=0.374)	r=-0.097 (p=0.447)
<i>SLC6A4</i> CG2	r=0.060 (p=0.635)	r=-0.022 (p=0.866)	r=0.038 (p=0.764)	r=-0.027 (p=0.833)	r=0.058 (p=0.651)	r=-0.040 (p=0.752)
<i>SLC6A4</i> CG3	r=-0.137 (p=0.283)	r=-0.143 (p=0.267)	r=-0.115 (p=0.375)	r=-0.108 (p=0.404)	r=-0.124 (p=0.338)	r=-0.153 (p=0.236)
<i>SLC6A4</i> CG6	r=-0.273 (p=0.029)*	r=-0.127 (p=0.320)	r=-0.144 (p=0.259)	r=-0.198 (p=0.120)	r=-0.384 (p=0.002)**	r=-0.172 (p=0.177)
<i>SLC6A4</i> CG7	r=-0.014 (p=0.910)	r=0.054 (p=0.674)	r=-0.042 (p=0.377)	r=-0.215 (p=0.088)	r=0.061 (p=0.629)	r=0.083 (p=0.515)
<i>SLC6A4</i> CG8	r=-0.137 (p=0.275)	r=-0.117 (p=0.356)	r=-0.051 (p=0.686)	r=-0.202 (p=0.110)	r=-0.138 (p=0.278)	r=-0.011 (p=0.931)
<i>SLC6A4</i> CG9	r=-0.006 (p=0.965)	r=0.050 (p=0.733)	r=-0.010 (p=0.945)	r=-0.062 (p=0.671)	r=0.003 (p=0.983)	r=-0.023 (p=0.876)
<i>SLC6A4</i> CG10	r=0.024 (p=0.865)	r=0.023 (p=0.874)	r=0.075 (p=0.605)	r=-0.020 (p=0.892)	r=-0.024 (p=0.866)	r=-0.081 (p=0.574)
Mean <i>SLC6A4</i>	r=-0.045 (p=0.715)	r=-0.087 (p=0.483)	r=-0.185 (p=0.133)	r=0.007 (p=0.954)	r=0.055 (p=0.658)	r=-0.037 (p=0.768)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. Bold font indicates significance. CTQ=Childhood Trauma Questionnaire

Table 4.10 Correlational analysis of *SLC6A4* promoter DNA methylation and cortisol measurements in the entire cohort.

	Cortisol T0	Cortisol T30	Cortisol T60	Cortisol AM avg	Cortisol AUCg
<i>SLC6A4</i> CG1	r=-0.272 (p=0.103)	r=-0.154 (p=0.349)	r=-0.094 (p=0.578)	r=-0.310 (p=0.055)	r=-0.166 (p=0.349)
<i>SLC6A4</i> CG2	r=-0.134 (p=0.429)	r=-0.074 (p=0.655)	r=-0.126 (p=0.459)	r=-0.212 (p=0.195)	r=-0.095 (p=0.591)
<i>SLC6A4</i> CG3	r=0.095 (p=0.586)	r=0.056 (p=0.741)	r=-0.028 (p=0.873)	r=0.017 (p=0.920)	r=0.123 (p=0.504)
<i>SLC6A4</i> CG6	r=0.050 (p=0.766)	r=0.031 (p=0.850)	r=0.032 (p=0.850)	r=0.020 (p=0.902)	r=0.084 (p=0.630)
<i>SLC6A4</i> CG7	r=-0.030 (p=0.857)	r=0.039 (p=0.809)	r=-0.216 (p=0.194)	r=-0.118 (p=0.469)	r=-0.033 (p=0.853)
<i>SLC6A4</i> CG8	r=-0.281 (p=0.088)	r=-0.275 (p=0.086)	r=-0.421 (p=0.008)**	r=-0.429 (p=0.006)**	r=-0.478 (p=0.004)**
<i>SLC6A4</i> CG9	r=-0.107 (p=0.567)	r=-0.122 (p=0.505)	r=-0.202 (p=0.285)	r=-0.173 (p=0.345)	r=-0.156 (p=0.429)
<i>SLC6A4</i> CG10	r=-0.187 (p=0.314)	r=-0.092 (p=0.617)	r=0.050 (p=0.793)	r=-0.135 (p=0.461)	r=-0.111 (p=0.573)
mean <i>SLC6A4</i>	r=-0.274 (p=0.087)	r=-0.118 (p=0.458)	r=-0.060 (p=0.713)	r=-0.247 (p=0.115)	r=-0.174 (p=0.304)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. Bold font indicates significance. AUCg=Area Under the Curve (AUC) with respect to ground, where ground is equal to zero.

Table 4.11 Correlational analysis of kynurenine pathway metabolites and cortisol and pro-inflammatory markers in the entire cohort.

	Tryptophan	Kynurenine	KYN/TRP ratio
Cortisol T0	r=-0.144 (p=0.289)	r=-0.350 (p=0.010)**	r=-0.353 (p=0.010)**
Cortisol T30	r=0.087 (p=0.516)	r=-0.180 (p=0.188)	r=-0.331 (p=0.014)*
Cortisol T60	r=0.141 (p=0.294)	r=-0.079 (p=0.566)	r=-0.252 (p=0.063)
Cortisol AM Average	r=-0.045 (p=0.729)	r=-0.282 (p=0.032)*	r=-0.337 (p=0.010)**
Cortisol T720	r=0.070 (p=0.694)	r=-0.269 (p=0.143)	r=-0.311 (p=0.089)
Cortisol T750	r=0.350 (p=0.086)	r=0.097 (p=0.659)	r=-0.205 (p=0.348)
Cortisol PM Average	r=0.190 (p=0.267)	r=-0.187 (p=0.296)	r=-0.365 (p=0.037)*

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

Table 4.12 Correlational analysis of cortisol concentrations and kynurenine pathway metabolites in depressed patients

	Tryptophan	Kynurenine	KYN/TRP ratio
Cortisol T0	r=0.130 (p=0.444)	r=-0.376 (p=0.028)*	r=-0.567 (p<0.001)*
Cortisol T30	r=0.071 (p=0.668)	r=-0.139 (p=0.418)	r=-0.324 (p=0.054)
Cortisol T60	r=0.065 (p=0.700)	r=-0.048 (p=0.781)	r=-0.230 (p=0.178)
Cortisol AM Average	r=0.024 (p=0.879)	r=-0.221 (p=0.176)	r=-0.346 (p=0.031)*
Cortisol T720	r=-0.144 (p=0.533)	r=-0.402 (p=0.099)	r=-0.112 (p=0.657)
Cortisol T750	r=0.411 (p=0.128)	r=-0.176 (p=0.566)	r=-0.588 (p=0.035)*
Cortisol PM Average	r=0.147 (p=0.504)	r=-0.324 (p=0.163)	r=-0.347 (p=0.134)

Statistical analysis was performed using the Spearman's rho correlation with *p≤0.05 considered significant. Bold font indicates significance.

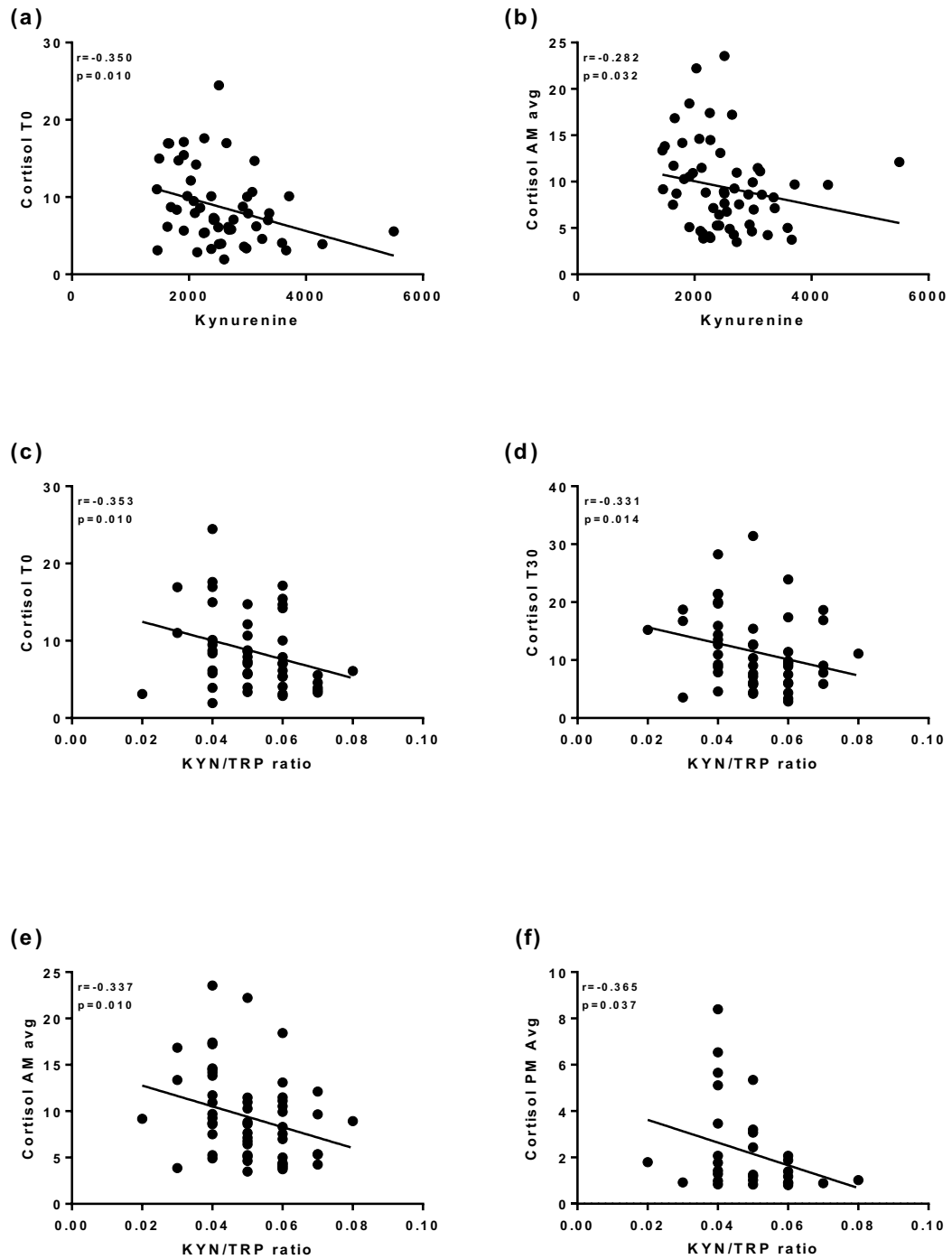


Figure 4.7 Relationships between cortisol concentrations and kynurenine pathway metabolites in the entire cohort. Significant relationships exist between kynurenine concentrations and (a) cortisol T0 concentrations and (b) cortisol morning average output. Significant relationships exist between KYN/TRP ratio and (c) cortisol T0 concentrations, (d) cortisol T30 concentrations, (e) cortisol morning average output and (f) cortisol evening average output.

4.3.12 Mean DNA methylation levels of the *SLC6A4* promoter in depressed patients with and without ELA

Similarly to Chapter 3, analysis was performed on the depressed group while dividing the groups based on their exposure to early life adversity (ELA+ or ELA-). *SLC6A4* promoter DNA methylation analysis was performed on 36 depressed participants: 16 ELA+ and 20 ELA- individuals. The demographic and clinical profile of these 36 participants can be found in Section 3.3.25 and 3.3.26 respectively. Mean *SLC6A4* promoter methylation levels were calculated (8 CG sites). A Student's t-test performed on log transformed *SLC6A4* methylation data revealed no differences in overall methylation status between the groups [Table 4.13].

Table 4.13 Mean *SLC6A4* promoter methylation data for depressed patients with and without ELA

	ELA+ (n=16)	ELA- (n=20)	Statistic (p-value)
mean <i>SLC6A4</i>	4.08 (0.16)	4.01 (0.15)	t=-0.73, p=0.473

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data.

4.3.13 DNA methylation levels of specific CG sites in the *SLC6A4* promoter in depressed patients with and without ELA

Student's t-test performed on log transformed *SLC6A4* methylation data revealed no differences in DNA methylation levels at any of the CG sites measured between the groups [Table 4.14].

Table 4.14 Site-specific *SLC6A4* promoter methylation data for depressed patients with and without ELA

	ELA+ (n=16)	ELA- (n=20)	Statistic (p-value)
<i>SLC6A4</i> CG1	7.99 (0.45)	7.82 (0.327)	t=-0.25, p=0.804
<i>SLC6A4</i> CG2	4.36 (0.19)	4.36 (0.174)	t=-0.02, p=0.988
<i>SLC6A4</i> CG3	4.95 (0.15)	5.24 (0.145)	t=1.37, p=0.182
<i>SLC6A4</i> CG6	2.08 (0.11)	2.19 (0.094)	t=-0.29, p=0.776
<i>SLC6A4</i> CG7	4.90 (0.37)	4.37 (0.143)	t=-1.45, p=0.158
<i>SLC6A4</i> CG8	3.21 (0.30)	3.23 (0.165)	t=-0.73, p=0.471
<i>SLC6A4</i> CG9	1.54 (0.15)	1.58 (0.107)	t=-0.74, p=0.470
<i>SLC6A4</i> CG10	2.32 (0.25)	2.67 (0.092)	t=1.07, p=0.298

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data.

4.3.14 Whole blood gene expression of SLC6A4 in depressed patients with and without ELA

Gene expression analysis was performed on 50 depressed patients: 26 ELA+ and 24 ELA- individuals. The demographic and clinical profile of these 50 participants be found in Section 3.3.30 and 3.3.31 respectively. Basal expression of *SLC6A4* mRNA was measured in whole blood samples. A Student's t-test performed on log transformed mRNA data revealed no significant differences in gene expression of *SLC6A4* between the two groups [Table 4.15].

Table 4.15 *SLC6A4* gene expression for depressed patients with and without ELA

	ELA+ (n=26)	ELA- (n=24)	Statistic (p-value)
<i>SLC6A4</i> mRNA (FC)	0.78 (0.17)	1.00 (0.17)	t=1.05, p=0.298

Data are expressed as mean fold change (FC) with SEM in parentheses. Statistical analysis was performed using a Student's t-test.

4.3.15 Demographic data for depressed patients with and without early life adversity whose circulating kynurenine pathway (KP) metabolite concentrations were measured

There were no significant differences in age, gender, BMI, prevalence of smokers or duration of education between the groups. [Table 4.16].

Table 4.16 Demographic data for depressed patients with and without exposure to ELA whose circulating KP metabolite concentrations were measured

	ELA+ (n=30)	ELA- (n=27)	Statistic (p-value)
Age (years)	29.74 (9.45)	26.78 (7.09)	Z=-1.22, p=0.222
Gender (M/F)	9/21	7/20	$\chi^2=0.09$, p=1.000
BMI	26.82 (10.81)	25.43 (5.81)	Z=-0.34, p=0.479
Smoking (Y/N)	14/16	8/19	$\chi^2=1.95$, p=0.264
ISCED Education	3.35 (1.94)	3.00 (1.66)	Z=-0.36, p=0.720

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education) and Chi squared (χ^2) test (Gender, Smoking). BMI=Body Mass Index; ISCED=International Standard Classification of Education.

4.3.16 Clinical data for depressed patients with and without exposure to ELA whose circulating kynurenine pathway metabolite concentrations were measured

There were no significant differences in HAM-D, CES-D, PSQI scores or antidepressant use between the groups. [Table 4.17].

Table 4.17 Clinical data for depressed patients with and without exposure to ELA whose circulating KP metabolite concentrations were measured

	ELA+ (n=30)	ELA- (n=27)	Statistic (p-value)
HAM-D	22.52 (6.20)	22.37 (3.66)	Z=-0.64, p=0.520
CES-D	38.92 (9.47)	39.73 (9.95)	Z=-0.45, p=0.654
PSQI	13.00 (3.94)	12.94 (4.20)	Z=-0.12, p=0.905
CTQ Global Score	56.81 (10.34)	31.63 (5.62)	Z=-6.31, p<0.001***
Antidepressant (Y/N)	18/12	17/10	$\chi^2=0.08$, p=1.000

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, CTQ Global Score) and Chi squared (χ^2) test (Antidepressants). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire. ***p≤0.001 vs. ELA-.

4.3.17 Kynurenine pathway metabolite concentrations in depressed patients with and without ELA

We found no differences in tryptophan concentration between the two groups. However, kynurenine concentrations were significantly increased in the ELA+ patients relative to the ELA- patients [Figure 4.8]. There was no significant difference in KYN/TRP ratios present between the two groups [Table 4.18].

Table 4.18 Kynurenine pathway metabolite concentrations for depressed patients with and without ELA

	ELA+ (n=30)	ELA- (n=27)	Statistic (p-value)
Tryptophan (ng/ml)	48207.41 (1995.41)	45092.59 (2023.52)	t=-1.15, p=0.256
Kynurenine (ng/ml)	2635.00 (137.36)	2282.40 (83.92)	t=-2.09, p=0.042*
KYN/TRP ratio	0.054 (0.01)	0.05 (0.01)	t=-0.58, p=0.574

Data are expressed as means with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data. *p≤0.05 vs ELA-.

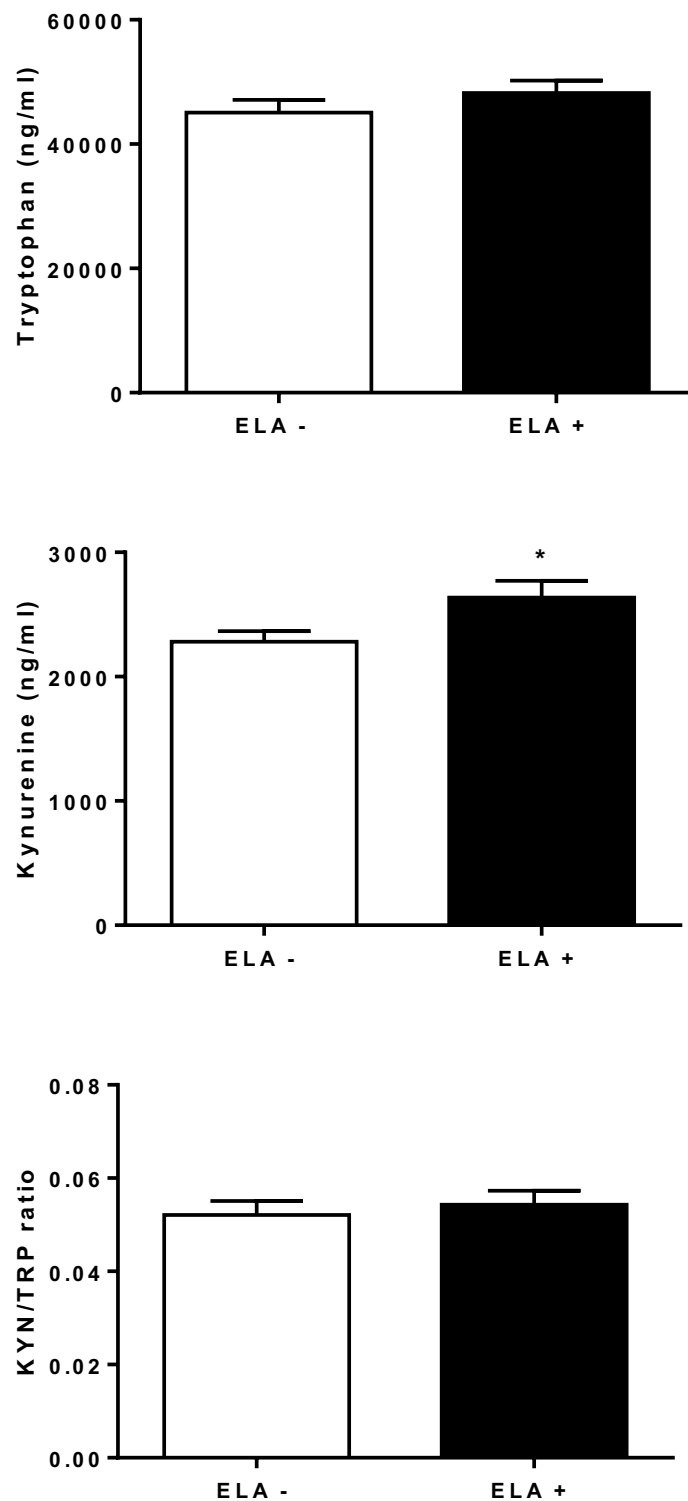


Figure 4.8 Kynurenine pathway metabolites in depressed patients with and without ELA. Plasma concentrations of (a) tryptophan, (b) kynurenine and (c) kynurenine/tryptophan (KYN/TRP) ratio in depressed patients with and without ELA. Data are expressed as mean with SEM. * $p \leq 0.05$ vs. ELA-.

4.4 Discussion

The present study sought to compare *SLC6A4* promoter DNA methylation and subsequent mRNA expression in depressed patients and healthy controls in relation to markers of peripheral 5-HT metabolism and HPA axis function. *SLC6A4* DNA methylation was significantly decreased at a CG site, CG8, within the promoter in the depressed compared to the control, group. There were decreased levels of tryptophan in the depressed, compared to the control, group. Interestingly, there was a linear relationship between *SLC6A4* DNA methylation and plasma tryptophan levels, with decreases in tryptophan concentrations associated with increases in DNA methylation at *SLC6A4* promoter CG6. Methylation at the CG8 site was also significantly associated with multiple cortisol measures in the whole group, as well as in the depressed group. Clinical rating scales, the most robust markers of the depressive state, were also associated with methylation levels. There were no differences in DNA methylation in the depressed when participants were separated on the basis of their exposure to ELA

4.4.1 *SLC6A4* promoter DNA methylation status in depression

DNA methylation levels at the *SLC6A4* promoter CG8 site were significantly decreased in the depressed patients when compared to healthy controls. A decrease in DNA methylation at the *SLC6A4* promoter may result in an increase in SERT expression/function, which could ultimately reduce the synaptic availability of 5-HT. Although no differences in SERT expression or relationships between SERT expression and DNA methylation were revealed, it is important to note the limitation of PAXgene blood RNA samples which were used in this study. Studies have reported that PAXgene samples show a decrease in the number of expressed genes with higher variability compared to PBMC samples (Min et al., 2010). This means that a subtle change in gene expression may go undetected. The *NR3C1* findings from Chapter 3, support this possible interpretation and suggest that RNA from cells may be a more appropriate medium to use in subsequent studies examining DNA methylation and gene expression.

These decreased methylation levels at CG8 were significantly correlated with increased morning cortisol concentrations. This is an interesting finding as a recent study has reported that allele-specific decreases in DNA methylation levels at *SLC6A4* are related to increased cortisol stress reactivity (Alexander et al., 2014). This mirrors previous work that has reported reduced 5-HT function and hypercortisolemia in depression (O'Keane, 1991) and provides a

mechanistic explanation for the longstanding observation of HPA axis and 5-HT systems, both of which are hypothesised to be dysregulated in depression.

As no differences in DNA methylation appeared in depressed individuals when separated on the basis of their exposure to ELA, one could hypothesise that DNA methylation changes at the *SLC6A4* promoter demonstrate a state marker of depression, rather than for developmental adversity. Furthermore, relationships between the CG8 site and sleep disturbances as well as suicidal ideation were revealed, strengthening the potential of *SLC6A4* DNA methylation levels as a state marker of depression. It would be of great interest to follow these patients up and see if these alterations are changed when depressive symptoms are alleviated. If so, methylation at this site could potentially be a marker used to indicate of treatment response in future investigations.

4.4.2 Tryptophan concentrations and kynurenine pathway profile in depression

In support of previous studies (Cowen et al., 1989b; Capuron et al., 2002), the depressed cohort displayed a significant reduction (21%) in plasma tryptophan concentrations relative to healthy controls. Furthermore, the decrease in tryptophan concentration was accompanied by a significant decrease (12%) in kynurenine concentrations and no significant differences in KYN/TRP ratio in the depressed group relative to healthy controls.

Interestingly, an opposing kynurenine pathway profile was observed upon examination of the depressed group when they were divided on whether or not they had been exposed to early life adversity (ELA). There were no significant differences in circulating plasma tryptophan in ELA+ depressed patients relative to ELA- patients. Moreover, kynurenine concentrations were significantly elevated (15%) in ELA+ patients. However, it is important to note that despite this observed increase when examining the depressed cohort only, the mean kynurenine concentration in ELA+ depressed individuals was still lower (6%) than the control group.

4.4.3 Limitations

A limitation of this study that should be noted was the inability of the pyrosequencing reaction to measure *SLC6A4* promoter DNA methylation levels at CG4 and CG5. The absence of these sites in the analysis could impact reported findings.

A further limitation to this study is the absence of 5-HTTLPR genotype data. As a recent study has reported an influence of stress on the methylation status of the SLC6A4 promoter in (II) carriers of the polymorphism (Duman and Canli, 2015), the addition of that information to this study could have helped to further explain the relationship between HPA axis alterations and 5-HT neurotransmission.

As is often the case with clinical research the study would have benefited from a larger sample size. It is important to note the relatively small sample size as a limitation in this study as it could result in the statistics being underpowered. Underpowered statistical analyses run the risk of Type II errors (i.e. false negative results) and this should be considered when interpreting these results.

As mentioned in Section 1.8 (Aims), given the novelty of DNA methylation analysis in psychiatric research, much of the work presented throughout the thesis 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 of this Chapter.

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.

4.4.4 Conclusion

These data suggest that decreased levels of DNA methylation at specific sites within the *SLC6A4* promoter are present in depression and may represent a marker for the depressive state. The data also support the finding of tryptophan depletion in depression and demonstrate a strong relationship between methylation at this 5-HTT site and plasma tryptophan levels. Lastly, an inverse relationship between HPA axis overdrive and reduced 5-HT function was found that supports a functional relationship between increased HPA axis output and reduced 5-HT neurotransmission. This could explain, at least in part, the large body of evidence indicating reduced 5-HT neurotransmission in depression.

Chapter 5

**Adaptive immunity in depression:
methylation of the CD3E gene and the
contribution of early life adversity**

5.1 Introduction

There is a large body of research that links an activated innate immune system and inflammation to the development of MDD (Miller, 2010). Numerous meta-analyses have revealed positive associations between pro-inflammatory cytokines, including C-reactive protein, IL-1 β , IL-6, and depression (Dowlati et al., 2010; Howren et al., 2009; Valkanova et al., 2013). It has been demonstrated that when pro-inflammatory cytokines (IFN- α and IL-2 immunotherapy) are administered to non-depressed individuals, depressive symptoms worsen (Capuron et al., 2001). The anti-inflammatory effects of antidepressants have also been examined. Tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and serotonin-noradrenaline reuptake inhibitors (SNRIs) have been shown to display anti-inflammatory properties (Kubera et al., 2001). Taken together, these findings suggest a probable link between an altered innate immune system and depression.

However, despite the expanding literature available on innate immune responses and depression, far less attention has been focused on the potential role of adaptive immune responses, including the role of T cells, in the development of MDD. It is important to examine the role of T cells in depression as meta-analyses and *in vivo* work to date suggest decreased T cell activity in depressed patients (Miller, 2010).

A number of possible mechanisms by which T cell function is altered in stress and depression have been proposed. One potential mechanism to explain this reduced function is decreased circulating tryptophan concentrations. Tryptophan is essential for the successful proliferation of T cells and T cells will undergo apoptosis in a tryptophan depleted environment (Fallarino et al., 2002b). As decreased tryptophan concentrations have consistently been reported in depression (Capuron et al., 2002; Hughes et al., 2012), this decrease could somewhat explain the reduced T cell function observed in depression.

Another possible mechanism by which T cell function is attenuated in depression is via inhibition by glucocorticoids. Glucocorticoids have profound effects on immune system function and can induce T cell apoptosis, especially at developing T cells in the thymus. Increased secretion of cortisol often accompanies depression (Knorr et al., 2010b) and this is believed, in some cases, to result in glucocorticoid resistance (Anacker et al., 2011). Studies have demonstrated that both glucocorticoid-induced T cell proliferation and redistribution is decreased in depression (Bauer et al., 2003b; Bauer et al., 2002b). It has been suggested that this failure of T cells to respond to neuroendocrine trafficking signals could impact the ability

of T cells to mobilise to the brain and transmit neuroprotective functions during stress (Miller, 2010).

Regulatory T cells (CD25⁺ CD4⁺Treg cells) are a subpopulation of T cells which are crucial in the maintenance of immune system homeostasis. One of their main effector molecules is the anti-inflammatory cytokine interleukin-10 (IL-10) (Dong and Martinez, 2010). Decreased IL-10 levels have been reported in depression (Dhabhar et al., 2009b) and as Treg cells produce IL-10, a decrease in their function could contribute to the pathophysiology of depression. Treg cells are identified by the presence of the cell surface glycoprotein CD25 and by the expression of the transcription factor Forkhead box P3 (FoxP3), which is crucial in establishing Treg cell function during differentiation and its maintenance in the periphery (Rudensky, 2011). Changes in FoxP3 expression have been observed in depressed individuals (Li et al., 2010), further supporting a role of Treg dysfunction in depression.

Finally the dysregulation of the pro/anti-inflammatory Th1/Th2 cytokine balance has been implicated in the pathophysiology of depression (Pavón et al., 2006). In support of a pro-inflammatory signature in depressed patients, Th1 pro-inflammatory cytokines IFN- γ and IL-2 have been reported to be increased in depression (Maes et al., 1994; Bonaccorso et al., 2001), whereas the Th2 anti-inflammatory cytokines IL-4 and IL-10 have reported to be decreased in depression (Sutcgil et al., 2007; Dhabhar et al., 2009b).

T cells are characterised by the presence of a T cell receptor (TCR) on their cell surface (Janis Kuby et al., 2007). The TCR is a multi-subunit complex composed of the TCR α , TCR β , CD3 γ , CD3 δ , CD3 ϵ , CD3 ζ and CD3 η units (TCR-CD3 complex) (A.-M. K. Wegener et al., 1992). The CD3 ϵ polypeptide, which plays an essential role in T cell development, is encoded by the *CD3E* gene. This gene is located at chromosome 11q23.3 and spans 11,631 base pairs (GeneCards, 2017b). Defects in this gene result in immunodeficiency (Le Deist et al., 1991), so it is a useful gene to examine in the context of T cell activity alterations.

DNA methylation levels at cg06164961 (Illumina® CpG Loci ID), which is located in the body of the *CD3E* gene, have been used in various studies as a CD3⁺ T cell marker in clinical samples (Cao-Lei et al., 2015; Price et al., 2013), with increased methylation at this site associated with decreased *CD3E* mRNA expression (Guénard et al., 2013).

Activation of T cells in vitro is often induced through stimulation using the antibody anti-CD3. Stimulation of the TCR-CD3 complex results in the initiation of multiple signalling pathways

leading to T cell expansion, increased expression of T cell activation markers, T cell differentiation and cytokine production (Hughes, 2014b).

In light of this, the aims of the study were to:

1. Evaluate differences in DNA methylation at cg06164961 in the *CD3E* gene between depressed patients and healthy controls;
2. Assess differences in the expression of T cell activation markers, surface glycoproteins and effector molecules in depressed patients and healthy controls;
3. Evaluate the ability of peripheral blood mononuclear cells (PBMCs) to respond to the T cell mitogen, anti-CD3 through assessment of gene expression in depressed patients and healthy controls.

5.2 Methods

A detailed description of the study participants, recruitment process, clinical rating scales and sample collection and processing are outlined in Chapter 2.

5.2.1 DNA methylation analysis of CD3E gene in whole blood

DNA methylation of cg06164961 within the cluster of differentiation 3 epsilon (*CD3E*) gene was measured in whole blood by pyrosequencing, performed on bisulfite-treated DNA. CD3E_F1 (ATGGTTTtagggTTTTATTTTtagtagat) and CD3E_R2-biotin (5Biosg/ACATAACACMAACAATTCCTTACAT) were the forward and reverse primers, respectively, for the PCR reaction and CD3E_S3B (AGTTTtATGAAATAAAGATGTAGT) was the sequencing primer used in the pyrosequencing reaction. Pyrosequencing was performed using the Q96 platform (Qiagen). Percentage methylation levels and quality control were analysed using PyroMark CpG Software (Version 1.0.11, Qiagen). Detailed methodology of the pyrosequencing procedure is outlined in Chapter 2 –Section 2.2.3.

5.2.2 Real-time PCR analysis of adaptive immune mRNA markers in whole blood

Total RNA was extracted from whole blood collected into PAXgene Blood RNA tubes using the PAXgene Blood RNA kit (Qiagen) as per the manufacturer's recommended protocol. Following quantification, all RNA samples were equalised to 10 ng/μl and cDNA was synthesised using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Gene expression analysis was performed using FAM-labelled probes for the genes of interest (Taqman Gene Expression assays, Applied Biosystems) [Table 3.1] with a VIC-labelled probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control (see Chapter 2 – Section 2.2.5).

Table 5.1 List of genes used with GenBank sequence ref numbers

Target Symbol	Target Name	Taqman Gene Assay ID
<i>CD3E</i>	Cluster of differentiation 3 epsilon	Hs01062241_m1
<i>CD4</i>	Cluster of differentiation 4	Hs01058407_m1
<i>CD8A</i>	Cluster of differentiation 8A	Hs00233520_m1
<i>CD8B</i>	Cluster of differentiation 8B	Hs00174762_m1
<i>CD69</i>	Cluster of differentiation 69	Hs00934033_m1
<i>IL-10</i>	Interleukin 10	Hs00961622_m1
<i>IL-4</i>	Interleukin 4	Hs00174122_m1
<i>FOXP3</i>	Forehead box P3	Hs01085834_m1

5.2.3 Real-time PCR analysis of adaptive immune mRNA markers in peripheral blood mononuclear cells (PBMCs)

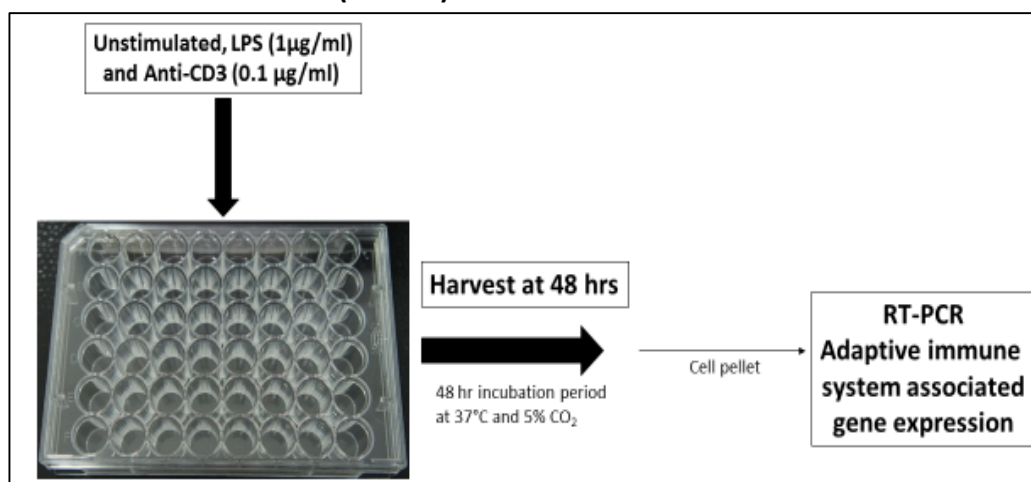


Figure 5.1 Diagrammatic representation of the experimental design

Culture media and treatments (LPS and Anti-CD3) were prepared as outlined in Chapter 2 – Section 2.2.4.2. 450 µl cell suspension was added to each well and following this, 50 µl 1µg/ml LPS or 0.1µg/ml Anti-CD3 was added to the appropriate wells. The cells were left to incubate for 48 hours at 37°C and 5% CO₂ and were harvested as per Chapter 2 – Section 2.2.4.3.5.

Total RNA was extracted from the PBMCs using the NucleoSpin® RNA Isolation kit (Macherey-Nagel) as per the manufacturer’s recommended protocol. Following quantification, all RNA samples were equalised to 2 ng/µl and cDNA was synthesised using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Gene expression analysis was performed using FAM-labelled probes for the genes of interest (Taqman Gene Expression assays, Applied Biosystems) [Table 5.1] with a VIC-labelled probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control (see Chapter 2 – section 2.2.5).

5.2.5 Statistical Analysis

Data are presented as mean with standard deviation (SD) and standard error of the mean (SEM) where appropriate. Data normality was assessed using the Shapiro-Wilk test of normality. Differences in demographic variables were tested using Mann Whitney U test and Chi-square (χ^2) test. Following Shapiro-Wilk test for normality, it was revealed that the clinical PBMC data were not normally distributed. As there is no equivalent to a non-parametric Two-Way ANOVA, the data were log transformed. However, as this did not significantly improve the distribution of the data, statistical analysis was performed using a Kruskal-Wallis One-Way ANOVA followed by a Mann-Whitney U test. Spearman's rho correlation was used to assess correlations between biological variables. All statistical analyses were considered significant when $p \leq 0.05$. Statistics were performed using SPSS (Version 22, IBM) and all graphs were generated using GraphPad Prism Software (Version 6, GraphPad Inc.).

5.3 Results

5.3.1 DNA methylation of CD3E (cg06164961) in depressed patients and healthy controls

CD3E (cg06164961) DNA methylation analysis was performed on 69 participants: 36 depressed patients and 33 healthy controls. The demographic and clinical profile of these 69 participants can be found in Section 3.3.1 and 3.3.2 respectively. A univariate analysis of variance performed on log transformed *CD3E* methylation data including smoking as a covariate revealed a significant increase ($p=0.005$) in DNA methylation at cg06164961 in depressed patients, when compared to healthy controls [Table 5.2].

Table 5.2 *CD3E* DNA methylation data for depressed patients and healthy controls

	Patients (n=36)	Controls (n=33)	Statistics (p-value)
<i>CD3E</i>	71.88 (1.33)	65.36 (1.50)	F=8.43, $p=0.005^{**}$

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance on log transformed data with smoking as a covariate. $^{**}p \leq 0.01$ vs controls

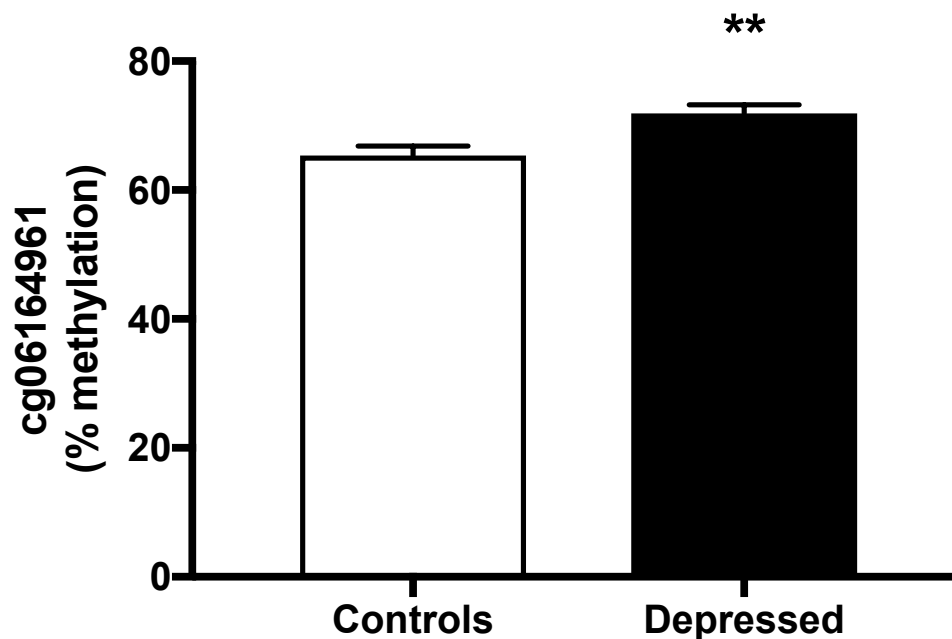


Figure 5.2 DNA methylation levels of cg06164961 in depressed patients and healthy controls. DNA methylation of *CD3E* gene in depressed patients compared to healthy controls. Data are expressed as means with SEM. $^{**}p \leq 0.01$ vs. control.

5.3.2 Whole blood gene expression of T cell surface glycoprotein genes in depressed patients and healthy controls

Basal gene expression of the T cell surface glycoproteins *CD3E*, *CD4*, *CD8A* and *CD8B* were measured the whole blood of 87 participants: 50 depressed patients and 37 healthy controls. The demographic and clinical profile of these 87 participants can be found in Section 3.3.10 and 3.3.11 respectively. The average threshold cycle (Ct) for the target genes and GAPDH were reported to ensure minimal inter-assay variability [Table 5.3].

Table 5.3 Average threshold cycle (Ct) of target genes, GAPDH and ΔCt in control groups.

	Target Gene Ct	GAPDH Ct	ΔCt
<i>CD3E</i>	26.4	24.7	1.7
<i>CD4</i>	28.3	22.9	5.4
<i>CD8A</i>	26.8	22.5	4.3
<i>CD8B</i>	28.8	22.5	6.3

A univariate analysis of variance performed on log transformed mRNA data using smoking as a covariate revealed no significant differences in gene expression of *CD3E*, *CD4*, *CD8A* or *CD8B* in the depressed patients relative to healthy controls [Table 5.4].

Table 5.4 T cell surface glycoprotein gene expression data for depressed patients and healthy controls

	Patients (n=50)	Controls (n=37)	Statistics (p-value)
<i>CD3E</i> mRNA (FC)	0.87 (0.04)	1.00 (0.06)	F=1.79, p=0.186
<i>CD4</i> mRNA (FC)	0.87 (0.05)	1.00 (0.05)	F=1.31, p=0.257
<i>CD8A</i> mRNA (FC)	0.86 (0.05)	1.00 (0.08)	F=2.10, p=0.152
<i>CD8B</i> mRNA (FC)	1.02 (0.06)	1.00 (0.06)	F=0.03, p=0.860

Data are expressed as mean fold change (FC) with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance on log transformed data with smoking as a covariate.

5.3.3 Whole blood gene expression of T cell associated genes in depressed patients and healthy controls

Basal gene expression of the T cell associated genes *CD69*, *IL-4*, *IL-10* and *FOXP3* were measured the whole blood of 87 participants: 50 depressed patients and 37 healthy controls. The demographic and clinical profile of these 87 participants can be found in Section 3.3.10 and 3.3.11 respectively. The average threshold cycle (Ct) for the target genes and GAPDH were reported to ensure minimal inter-assay variability [Table 5.5].

Table 5.5 Average threshold cycle (Ct) of target genes, GAPDH and Δ Ct in control groups.

	Target Gene Ct	GAPDH Ct	Δ Ct
<i>CD69</i>	31.0	24.8	6.2
<i>IL-4</i>	40.6	24.8	15.8
<i>IL-10</i>	35.9	25.0	10.9
<i>FOXP3</i>	31.8	24.8	7.0

A univariate analysis of variance performed on log transformed mRNA data using smoking as a covariate revealed no significant differences in gene expression of *CD69*, *IL-4*, *IL-10* and *FOXP3* in the depressed patients relative to healthy controls [Table 5.6].

Table 5.6 T cell associated gene expression data for depressed patients and healthy controls

	Patients (n=50)	Controls (n=37)	Statistics (p-value)
<i>CD69</i> mRNA (FC)	0.60 (0.16)	1.00 (0.27)	F=2.68, p=0.105
<i>IL-4</i> mRNA (FC)	1.40 (0.28)	1.00 (0.20)	F=1.63, p=0.205
<i>IL-10</i> mRNA (FC)	0.96 (0.09)	1.00 (0.08)	F=1.79, p=0.185
<i>FOXP3</i> mRNA (FC)	0.84 (0.05)	1.00 (0.08)	F=2.00, p=0.161

Data are expressed as mean fold change (FC) with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance on log transformed data with smoking as a covariate.

5.3.4 *CD3E* mRNA expression profile following adaptive immune cell stimulation in depressed patients and healthy controls

CD3E gene expression was measured in PBMC mRNA of 58 participants: 33 depressed patients and 25 healthy controls. The demographic and clinical profile of these 58 participants can be found in Section 3.3.17 and 3.3.18 respectively. A Kruskal-Wallis One-Way ANOVA revealed differential *CD3E* expression in PBMCs isolated from depressed patients and healthy controls following stimulation with the T-cell stimulus anti-CD3 ($Z=16.865$, $df=1$, $p<001$). Subsequent Mann-Whitney U tests revealed that in the control samples, anti-CD3 ($Z=-1.882$, $p=0.061$) did not induce a significant difference in *CD3E* mRNA expression relative to unstimulated control levels. In the depressed cohort, anti-CD3 ($Z=-3.828$, $p<0.001$) induced a significant decrease in the expression of *CD3E* mRNA relative to unstimulated control cells. While no significant difference in the unstimulated expression of *CD3E* between the depressed patients and controls was observed ($Z=-0.402$, $p=0.688$), an almost significant decrease in anti-CD3 stimulated *CD3E* mRNA expression ($Z=-1.951$, $p=0.051$) was observed in the depressed patients relative to the healthy controls (Figure 5.3).

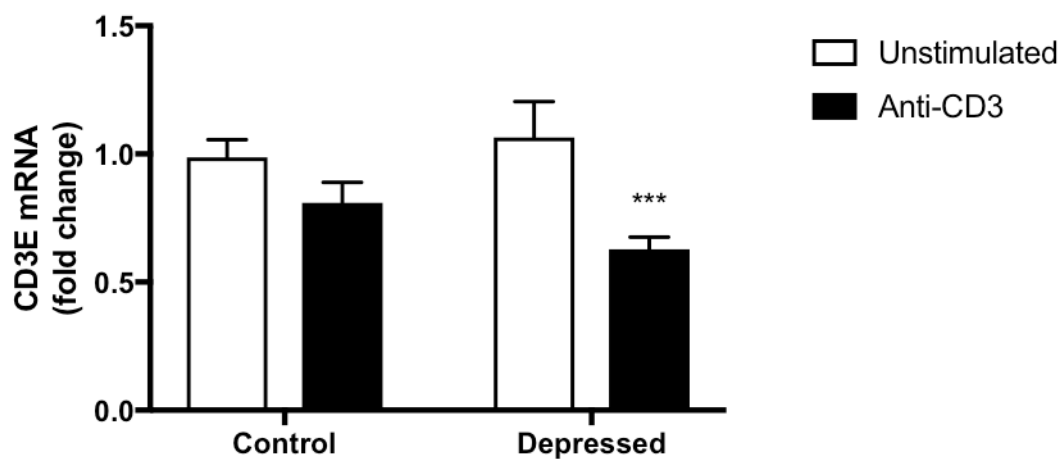


Figure 5.3 Anti-CD3 stimulated *CD3E* expression in PBMCs from depressed patients and healthy controls. PBMCs isolated from depressed patients and healthy controls were incubated for 48 hours with anti-CD3 at a concentration of 0.1 $\mu\text{g/ml}$. Relative mRNA expression for *CD3E* was quantified. Data are expressed as mean fold change with SEM. *** $p\leq 0.001$ vs. unstimulated *CD3E* expression (Kruskal-Wallis One-Way ANOVA followed by Mann-Whitney U tests).

5.3.5 IFN- γ mRNA expression profile following innate or adaptive immune cell stimulation in depressed patients and healthy controls

IFNG gene expression was measured in PBMC mRNA of 58 participants: 33 depressed patients and 25 healthy controls. The demographic and clinical profile of these 58 participants can be found in Section 3.3.17 and 3.3.18 respectively. A Kruskal-Wallis One-Way ANOVA revealed differential *IFNG* expression in PBMCs isolated from depressed patients and healthy controls following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 ($\chi^2=11.559$, $df=2$, $p=0.003$). Subsequent Mann-Whitney U test revealed that in the control group LPS ($Z=-1.704$, $p=0.093$) did not induce a significant difference in the expression of *IFNG* mRNA, however, anti-CD3 ($Z=-2.676$, $p=0.007$) induced a significant increase in *IFNG* mRNA expression relative to unstimulated control levels. The anti-CD3 induced increase in *IFNG* transcriptional expression was not significantly greater than that observed following LPS stimulation ($Z=-1.247$, $p=0.224$). In the depressed cohort, LPS ($Z=0.684$, $p=0.512$) and anti-CD3 ($Z=-1.856$, $p=0.065$) did not induce a significant increase in *IFNG* mRNA expression relative to unstimulated control levels. There were no significant differences in *IFNG* expression between LPS and anti-CD3 stimulated cells ($Z=-1.609$, $p=0.112$). There were no significant differences in the unstimulated ($Z=-0.316$, $p=0.770$), LPS stimulated ($Z=-0.786$, $p=0.451$) or anti-CD3 stimulated ($Z=-0.118$, $p=0.919$) expression of *IFNG* between depressed patients and healthy controls (Figure 5.4).

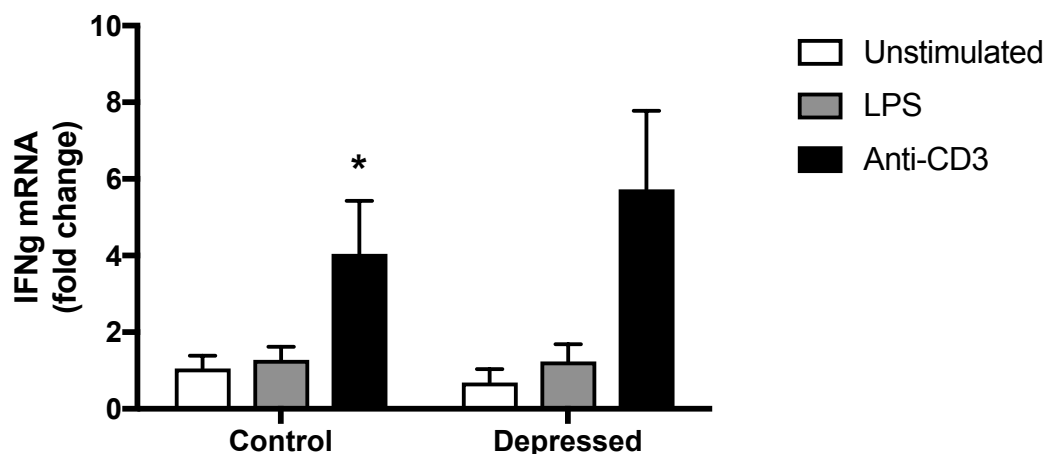


Figure 5.4 Basal, LPS and Anti-CD3 stimulated *IFN- γ* expression in PBMCs from depressed patients and healthy controls. PBMCs isolated from depressed patients and healthy controls were incubated for 48 hours with LPS at a concentration of 1 $\mu\text{g/ml}$ or anti-CD3 at a concentration of 0.1 $\mu\text{g/ml}$. Relative mRNA expression for *IFNG* was quantified. Data are expressed as mean fold change with SEM. *** $p \leq 0.001$ vs. unstimulated CD3E expression (Kruskal-Wallis One-Way ANOVA followed by Mann-Whitney U tests).

5.3.6 Evaluation of relationships between CD3E (cg06164961) DNA methylation and the expression of T cell surface glycoproteins

Spearman's rho correlation analysis revealed significant strong negative associations between CD3E DNA methylation and relative CD3E, CD4, CD8A, CD8B mRNA expression in all the participants. Taken together, these findings suggest that methylation at the CD3E gene (cg06164961) is strongly associated with mRNA expression of T-cell associated genes [Table 5.7][Figure 5.5].

Table 5.7 Correlational analysis of CD3E DNA methylation and T cell surface glycoprotein gene expression

	CD3E methylation (cg06164961)
CD3E mRNA (RQ)	r=-0.567, p<0.001***
CD4 mRNA (RQ)	r=-0.368, p=0.008***
CD8A mRNA (RQ)	r=-0.477, p=0.001***
CD8B mRNA (RQ)	r=-0.418, p=0.003***

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

5.3.7 Evaluation of relationships between CD3E (cg06164961) DNA methylation and kynurenine pathway metabolites

Spearman's rho correlation analysis revealed significant negative association between CD3E DNA methylation and circulating tryptophan concentrations in all participants [Table 5.8][Figure 5.6].

Table 5.8 Correlational analysis of CD3E DNA methylation and T cell surface glycoprotein gene expression

	CD3E methylation (cg06164961)
Tryptophan	r=-0.306, p=0.018*
Kynurenine	r=-0.252, p=0.066
KYN/TRP ratio	r=0.077, p=0.581

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

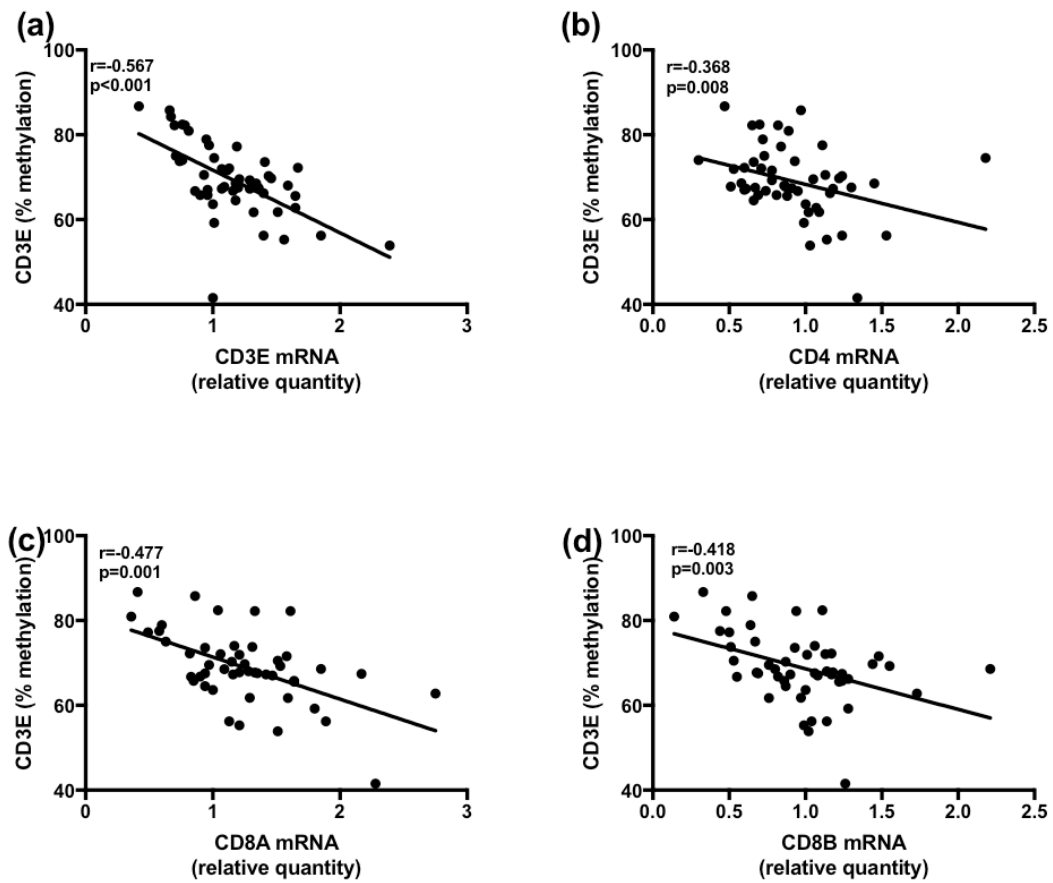


Figure 5.5 Relationships between *CD3E* (cg06164961) DNA methylation and the expression of T cell surface glycoproteins. Statistical analysis was performed using the Spearman's rho correlation, with * $p \leq 0.05$ considered significant.

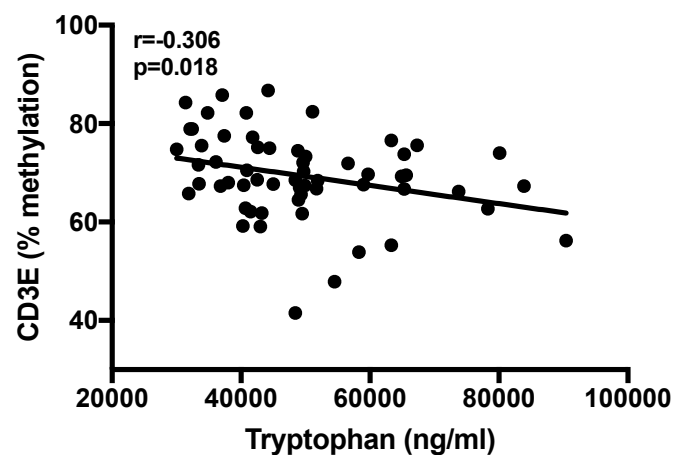


Figure 5.6 Relationships between *CD3E* (cg06164961) DNA methylation and tryptophan concentrations. Statistical analysis was performed using the Spearman's rho correlation, with * $p \leq 0.05$ considered significant.

5.3.8 Evaluation of relationships between *CD3E* DNA methylation and depression and sleep disturbances in all participants

Spearman's rho analysis revealed that *CD3E* DNA methylation at cg06164961 was positively correlated with HAM-D, CES-D and PSQI scores in all participants [Table 5.9].

5.3.9 Evaluation of relationships between T cell surface glycoprotein and T cell associated gene mRNA expression and depression and sleep disturbances in all participants

CD4 mRNA expression was negatively associated with HAM-D, CES-D and PSQI scores in all participants. No correlations existed between depression and sleep rating scales and gene expression of *CD3E*, *CD8A* or *CD8B* mRNA. *IL-10* mRNA expression was negatively associated with PSQI scores in all participants. *FOXP3* mRNA expression was negatively associated with CES-D scores in all participants. No correlations existed between depression and sleep rating scales and gene expression of *CD69* or *IL-4* [Table 5.9].

5.3.10 Evaluation of relationships between *CD3E* DNA methylation and childhood trauma severity in all participants

Spearman's rho analysis revealed that *CD3E* DNA methylation at cg06164961 was positively correlated with global CTQ scores, specifically emotional and physical neglect and emotional and physical abuse, in all participants [Table 5.10].

5.3.11 Evaluation of relationships between T cell surface glycoprotein and T cell associated gene mRNA expression and childhood trauma severity in all participants

CD3E mRNA expression was negatively associated with sexual abuse severity. *CD4* mRNA expression was negatively associated with total CTQ score, specifically emotional abuse scores, in all participants. No correlations existed between trauma severity and gene expression of *CD8A* or *CD8B* mRNA. *CD69* mRNA expression was negatively associated with total CTQ scores, specifically sexual abuse. *IL-10* and *IL-4* mRNA expression was negatively associated with physical abuse and physical neglect, respectively, in all participants. No correlations existed between trauma severity and gene expression of *FOXP3* [Table 5.10].

Table 5.9 Correlational analysis between specific immune markers and measures of depression severity and sleep disturbances

	Total HAM-D	Total CES-D	Total PSQI
<i>CD3E</i> methylation (%)	$r=0.431$ ($p<0.001$)***	$r=0.387$ ($p=0.001$)***	$r=0.359$ ($p=0.003$)***
<i>CD3E</i> mRNA	$r=-0.194$ ($p=0.121$)	$r=-0.240$ ($p=0.056$)	$r=-0.226$ ($p=0.077$)
<i>CD4</i> mRNA	$r=-0.333$ ($p=0.007$)**	$r=-0.258$ ($p=0.041$)*	$r=-0.273$ ($p=0.033$)*
<i>CD8A</i> mRNA	$r=-0.230$ ($p=0.077$)	$r=-0.246$ ($p=0.058$)	$r=-0.184$ ($p=0.167$)
<i>CD8B</i> mRNA	$r=-0.042$ ($p=0.749$)	$r=-0.106$ ($p=0.413$)	$r=-0.028$ ($p=0.833$)
<i>CD69</i> mRNA	$r=-0.086$ ($p=0.437$)	$r=-0.112$ ($p=0.311$)	$r=-0.056$ ($p=0.645$)
<i>IL-4</i> mRNA	$r=0.050$ ($p=0.661$)	$r=-0.004$ ($p=0.973$)	$r=0.039$ ($p=0.736$)
<i>IL-10</i> mRNA	$r=-0.106$ ($p=0.343$)	$r=-0.101$ ($p=0.365$)	$r=-0.231$ ($p=0.038$)*
<i>FOXP3</i> mRNA	$r=-0.106$ ($p=0.063$)	$r=-0.263$ ($p=0.017$)*	$r=-0.176$ ($p=0.117$)

Statistical analysis was performed using Spearman rho correlation, $p \leq 0.05$ was considered significant. Bold type face represents significant findings.

Table 5.10 Correlational analysis between specific immune markers and early life adversity

	Total CTQ	Emotional Abuse	Physical Abuse	Sexual Abuse	Emotional Neglect	Physical Neglect
<i>CD3E</i> meth	$r=0.312$ ($p=0.009$)**	$r=0.300$ ($p=0.012$)*	$r=0.294$ ($p=0.014$)*	$r=0.221$ ($p=0.068$)	$r=0.255$ ($p=0.034$)*	$r=0.274$ ($p=0.023$)*
<i>CD3E</i> mRNA	$r=-0.175$ ($p=0.157$)	$r=-0.074$ ($p=0.556$)	$r=-0.171$ ($p=0.172$)	$r=-0.248$ ($p=0.046$)*	$r=-0.157$ ($p=0.213$)	$r=-0.260$ ($p=0.037$)
<i>CD4</i> mRNA	$r=-0.243$ ($p=0.050$)*	$r=-0.280$ ($p=0.025$)*	$r=-0.164$ ($p=0.195$)	$r=0.003$ ($p=0.979$)	$r=-0.238$ ($p=0.058$)	$r=-0.212$ ($p=0.092$)
<i>CD8A</i> mRNA	$r=-0.232$ ($p=0.069$)	$r=-0.159$ ($p=0.226$)	$r=-0.225$ ($p=0.084$)	$r=-0.163$ ($p=0.213$)	$r=-0.217$ ($p=0.096$)	$r=-0.237$ ($p=0.068$)
<i>CD8B</i> mRNA	$r=-0.088$ ($p=0.490$)	$r=0.046$ ($p=0.720$)	$r=-0.149$ ($p=0.248$)	$r=-0.171$ ($p=0.184$)	$r=-0.086$ ($p=0.580$)	$r=-0.116$ ($p=0.370$)
<i>CD69</i> mRNA	$r=-0.225$ ($p=0.036$)*	$r=-0.177$ ($p=0.105$)	$r=-0.101$ ($p=0.356$)	$r=-0.364$ ($p=0.001$)***	$r=-0.195$ ($p=0.073$)	$r=-0.171$ ($p=0.119$)
<i>IL-10</i> mRNA	$r=-0.140$ ($p=0.198$)	$r=-0.084$ ($p=0.448$)	$r=-0.253$ ($p=0.020$)*	$r=-0.165$ ($p=0.134$)	$r=-0.131$ ($p=0.234$)	$r=-0.029$ ($p=0.790$)
<i>IL-4</i> mRNA	$r=-0.128$ ($p=0.250$)	$r=-0.072$ ($p=0.527$)	$r=-0.107$ ($p=0.343$)	$r=-0.087$ ($p=0.442$)	$r=-0.079$ ($p=0.486$)	$r=-0.225$ ($p=0.045$)*
<i>FOXP3</i> mRNA	$r=-0.182$ ($p=0.095$)	$r=-0.182$ ($p=0.099$)	$r=0.000$ ($p=0.997$)	$r=-0.071$ ($p=0.522$)	$r=-0.141$ ($p=0.204$)	$r=-0.165$ ($p=0.136$)

Statistical analysis was performed using Spearman rho correlation, $p \leq 0.05$ was considered significant. Bold type face represents significant findings.

5.3.12 DNA methylation of CD3E gene in depressed patients based on their ELA status

CD3E (cg06164961) DNA methylation analysis was performed on 36 depressed patients: 16 ELA+ depressed patients and 20 ELA- depressed patients. The demographic and clinical profile of these 36 participants can be found in Section 3.3.25 and 3.3.26 respectively. A Student's t-test performed on log transformed methylation data revealed a significant increase ($p=0.014$) in DNA methylation in the ELA+ group compared to ELA- group [Table 5.11].

Table 5.11 *CD3E* DNA methylation data for depressed patients with and without ELA

	ELA+ (n=16)	ELA- (n=20)	Statistics (p-value)
<i>CD3E</i>	75.56 (1.83)	68.94 (1.64)	$Z=-2.58$, $p=0.014^*$

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed data. $^*p \leq 0.05$ vs ELA-.

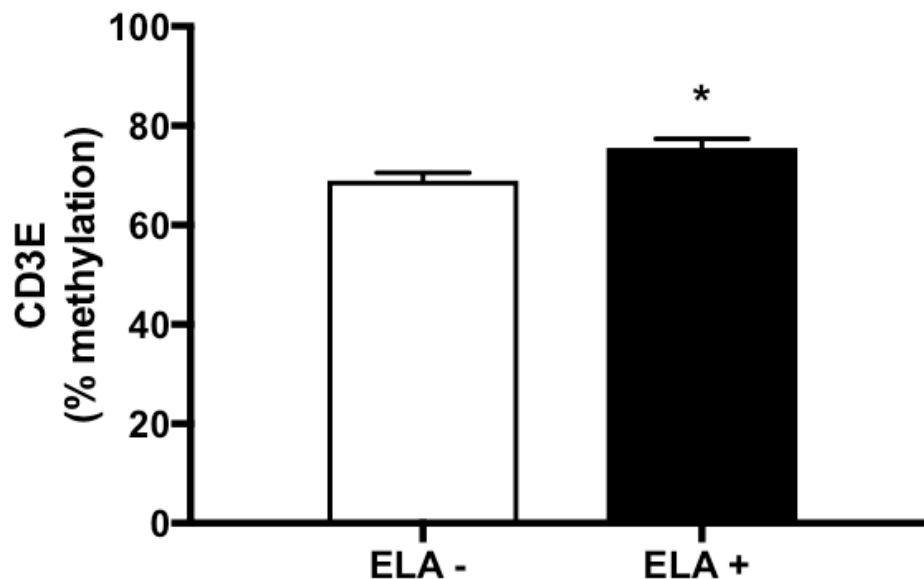


Figure 5.7 DNA methylation levels of cg06164961 in depressed patients. DNA methylation of *CD3E* gene in depressed patients compared to healthy controls. Data are expressed as means with SEM. $^*p \leq 0.05$ vs. ELA-.

5.3.13 Whole blood gene expression of T cell surface glycoprotein genes in depressed patients with and without ELA

Gene expression of *CD3E*, *CD4*, *CD8A* and *CD8B* were measured in whole blood from 50 depressed patients: 26 ELA+ depressed patients and 24 ELA- depressed patients. The demographic and clinical profile of these 50 participants can be found in Section 3.3.30 and 3.3.31 respectively. A Student's t-test performed on log transformed mRNA data revealed a significant decrease in *CD3E* and *CD8A* mRNA expression in the ELA+ group compared to the ELA- group. Analysis revealed no significant differences in gene expression of *CD4* or *CD8B* between the groups [Table 5.12].

Table 5.12 T cell surface glycoprotein gene expression data for depressed patients with and without ELA

	ELA+ (n=26)	ELA- (n=24)	Statistics (p-value)
<i>CD3E</i> mRNA (FC)	0.82 (0.06)	1.00 (0.05)	t=2.45, p=0.019*
<i>CD4</i> mRNA (FC)	0.95 (0.10)	1.00 (0.07)	t=0.41, p=0.683
<i>CD8A</i> mRNA (FC)	0.78 (0.08)	1.00 (0.07)	t=2.23, p=0.032*
<i>CD8B</i> mRNA (FC)	0.85 (0.09)	1.00 (0.07)	t=1.30, p=0.204

Data are expressed as mean fold change (FC) with SEM in parentheses. Statistical analysis was performed using a Student's t-test. *p≤0.05 vs. ELA-.

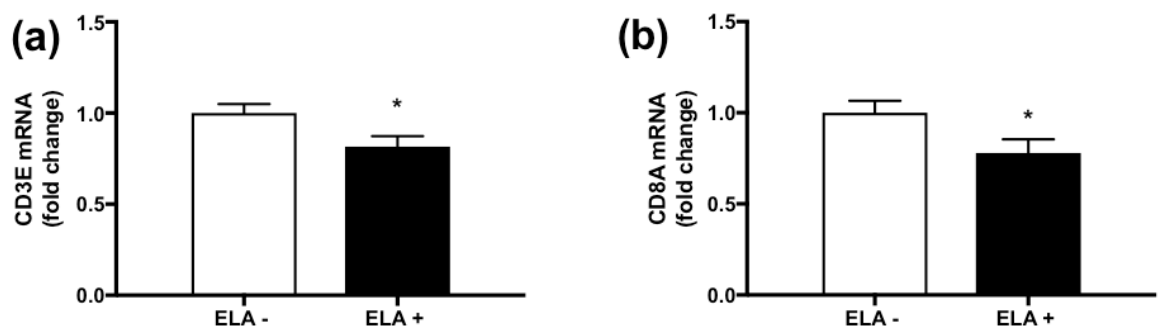


Figure 5.8 T cell surface glycoprotein gene expression data for depressed patients with and without ELA. Whole blood mRNA expression of (a) *CD3E* and (b) *CD8A* in depressed patients with and without ELA. Data expressed as mean fold change with SEM. *p≤0.05 vs. ELA- (Student's t-test).

5.3.14 Whole blood gene expression of T cell associated genes in depressed patients with and without ELA

Gene expression of *CD69*, *IL-4*, *IL-10* and *FOXP3* were measured in whole blood from 50 depressed patients: 26 ELA+ depressed patients and 24 ELA- depressed patients. The demographic and clinical profile of these 50 participants can be found in Section 3.3.30 and 3.3.31 respectively. A Student's t-test performed on log transformed mRNA data revealed no significant differences in the expression of *CD69*, *IL-4*, *IL-10* or *FOXP3* between the two groups [Table 5.13].

Table 5.13 Whole blood gene expression of T cell associated genes in depressed patients with and without ELA

	ELA+ (n=26)	ELA- (n=24)	Statistics (p-value)
<i>CD69</i> mRNA (FC)	1.76 (0.67)	1.00 (0.29)	t=0.23, p=0.818
<i>IL-4</i> mRNA (FC)	0.56 (0.13)	1.00 (0.29)	t=1.25, p=0.219
<i>IL-10</i> mRNA (FC)	0.92 (0.12)	1.00 (0.15)	t=0.11, p=0.911
<i>FOXP3</i> mRNA (FC)	0.95 (0.08)	1.00 (0.07)	t=0.69, p=0.494

Data are expressed as mean fold change (FC) with SEM in parentheses. Statistical analysis was performed using a Student's t-test. *p≤0.05 vs. ELA-.

5.3.15 *CD3E* mRNA expression profile following adaptive immune cell stimulation in depressed patients with and without ELA

CD3E gene expression was measured in PBMC mRNA of 33 depressed patients: 16 ELA+ depressed patients and 17 ELA- depressed patients. The demographic and clinical profile of these 33 participants can be found in Section 3.3.36 and 3.3.37 respectively. A Kruskal-Wallis One-Way ANOVA revealed a differential expression of *CD3E* in PBMCs isolated from depressed patients exposed to early life adversity (ELA+) and depressed patients not exposed to ELA (ELA-) following stimulation from the T-cell stimulus anti-CD3 ($\chi^2=13.335$, $df=1$, $p<0.001$). Subsequent Mann-Whitney U tests revealed that in the ELA- samples anti-CD3 ($Z=-2.372$, $p=0.017$) induced a significant decrease in *CD3E* mRNA expression relative to unstimulated control levels. In the ELA+ group, anti-CD3 ($Z=-2.750$, $p=0.005$) induced a significant decrease in *CD3E* mRNA expression relative to unstimulated control levels. However, analysis between the two groups revealed no significant differences in the unstimulated expression ($Z=-0.062$, $p=0.967$) or anti-CD3 ($Z=-0.624$, $p=0.552$) stimulated *CD3E* expression.

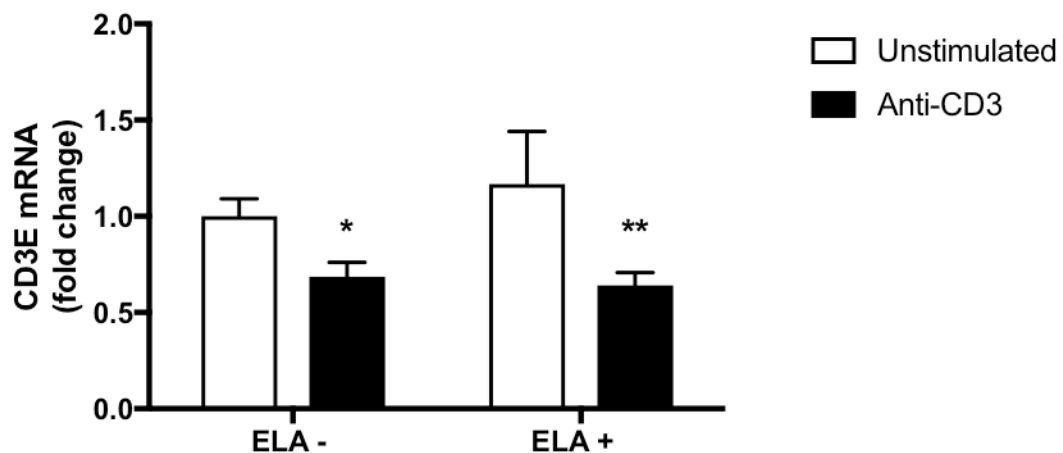


Figure 5.9 Anti-CD3 stimulated *CD3E* expression in PBMCs from depressed patients with and without ELA. PBMCs isolated from depressed patients with and without were incubated for 48 hours with anti-CD3 at a concentration of 0.1 $\mu\text{g/ml}$. Relative mRNA expression for *CD3E* was quantified. Data are expressed as mean fold change with SEM. * $p\leq 0.05$ vs. unstimulated *CD3E* expression; ** $p\leq 0.001$ vs. unstimulated *CD3E* expression (Kruskal-Wallis One-Way ANOVA followed by Mann-Whitney U tests).

5.3.16 IFN- γ mRNA expression profile following adaptive immune cell stimulation in depressed patients with and without ELA

IFNG gene expression was measured in PBMC mRNA of 33 depressed patients: 16 ELA+ depressed patients and 17 ELA- depressed patients. The demographic and clinical profile of these 33 participants can be found in Section 3.3.36 and 3.3.37 respectively. A Kruskal-Wallis One-Way ANOVA revealed a differential expression of *IFNG* in PBMCs isolated from ELA+ and ELA- depressed patients following stimulation with the innate immune stimulus LPS or the T-cell stimulus anti-CD3 ($\chi^2=5.976$, $df=2$, $p=0.05$). Subsequent Mann-Whitney U tests revealed that in the ELA- samples, LPS ($Z=-1.857$, $p=0.073$) and anti-CD3 ($Z=-1.403$, $p=0.179$) did not induce a significant increase in *IFNG* expression relative to unstimulated control levels. There was not a significant difference between LPS stimulated and anti-CD3 stimulated induction of *IFNG* mRNA expression ($Z=-0.911$, $p=0.393$). In the ELA+ group, LPS ($Z=-0.289$, $p=0.815$) and anti-CD3 ($Z=-1.557$, $p=0.131$) did not induce a significant increase in *IFNG* expression relative to unstimulated control levels. A trend towards a significant increase in anti-CD3 stimulated *IFNG* mRNA relative to LPS stimulated *IFNG* mRNA expression was observed in the ELA+ group ($Z=-1.982$, $p=0.051$). Analysis between the two groups revealed no significant differences in the unstimulated expression ($Z=-1.414$, $p=0.181$), LPS ($Z=-0.810$, $p=0.463$) or anti-CD3 ($Z=-0.492$, $p=0.649$) stimulated *IFNG* expression.

5.3.17 Evaluation of relationships between *CD3E* DNA methylation and depression and sleep disturbances in depressed patients

Spearman's rho analysis revealed that no association existed between *CD3E* DNA methylation at cg06164961 and with HAM-D, CES-D or PSQI scores in depressed patients [Table 5.14].

5.3.18 Evaluation of relationships between T cell surface glycoprotein and T cell associated gene mRNA expression and depression and sleep disturbances in depressed patients

CD8B mRNA expression was negatively associated with CES-D scores in depressed patients. No correlations existed between depression and sleep rating scales and gene expression of *CD3E*, *CD4*, *CD8A*, *CD69*, *IL-4*, *IL-10* or *FOXP3* in depressed patients [Table 5.14].

5.3.19 Evaluation of relationships between *CD3E* DNA methylation and childhood trauma severity in all participants

Spearman's rho analysis revealed that *CD3E* DNA methylation at cg06164961 was almost positively correlated ($p=0.051$) with global CTQ scores, specifically physical neglect in depressed patients [Table 5.15].

5.3.20 Evaluation of relationships between T cell surface glycoprotein and T cell associated gene mRNA expression and childhood trauma severity in all participants

CD3E mRNA expression was negatively associated with physical neglect severity. No significant correlations existed between trauma severity and gene expression of *CD4*, *CD8A* or *CD8B* mRNA. *CD69* mRNA expression was negatively associated with total CTQ scores, specifically sexual abuse. *IL-10* expression was negatively associated with physical abuse in all depressed patients. No correlations existed between trauma severity and gene expression of *IL-4* or *FOXP3* [Table 5.15].

Table 5.14 Correlational analysis between specific immune markers and measures of depression severity and sleep disturbances in depressed patients

	Total HAM-D	Total CES-D	Total PSQI
<i>CD3E</i> methylation	$r=0.284$ ($p=0.093$)	$r=0.235$ ($p=0.181$)	$r=0.100$ ($p=0.575$)
<i>CD3E</i> mRNA	$r=0.082$ ($p=0.609$)	$r=-0.078$ ($p=0.637$)	$r=-0.117$ ($p=0.478$)
<i>CD4</i> mRNA	$r=-0.119$ ($p=0.459$)	$r=0.161$ ($p=0.329$)	$r=0.074$ ($p=0.654$)
<i>CD8A</i> mRNA	$r=-0.164$ ($p=0.325$)	$r=-0.213$ ($p=0.206$)	$r=-0.098$ ($p=0.564$)
<i>CD8B</i> mRNA	$r=-0.112$ ($p=0.497$)	$r=-0.320$ ($p=0.050$)*	$r=-0.193$ ($p=0.246$)
<i>CD69</i> mRNA	$r=0.093$ ($p=0.520$)	$r=-0.051$ ($p=0.729$)	$r=0.053$ ($p=0.721$)
<i>IL-4</i> mRNA	$r=-0.004$ ($p=0.978$)	$r=-0.048$ ($p=0.758$)	$r=0.061$ ($p=0.692$)
<i>IL-10</i> mRNA	$r=0.025$ ($p=0.864$)	$r=0.095$ ($p=0.522$)	$r=-0.268$ ($p=0.066$)
<i>FOXP3</i> mRNA	$r=0.045$ ($p=0.758$)	$r=-0.140$ ($p=0.347$)	$r=0.106$ ($p=0.477$)

Statistical analysis was performed using Spearman rho correlation, $p \leq 0.05$ was considered significant. Bold type face represents significant findings.

Table 5.15 Correlational analysis between specific immune markers and measures early life adversity

	Total CTQ	Emotional Abuse	Physical Abuse	Sexual Abuse	Emotional Neglect	Physical Neglect
<i>CD3E</i> meth	$r=0.328$ ($p=0.051$)	$r=0.238$ ($p=0.169$)	$r=0.324$ ($p=0.058$)	$r=0.142$ ($p=0.416$)	$r=0.161$ ($p=0.356$)	$r=0.378$ ($p=0.025$)*
<i>CD3E</i> mRNA	$r=-0.302$ ($p=0.055$)	$r=-0.108$ ($p=0.507$)	$r=-0.199$ ($p=0.218$)	$r=-0.304$ ($p=0.056$)	$r=-0.252$ ($p=0.116$)	$r=-0.421$ ($p=0.007$)*
<i>CD4</i> mRNA	$r=-0.012$ ($p=0.939$)	$r=-0.134$ ($p=0.409$)	$r=0.050$ ($p=0.761$)	$r=0.143$ ($p=0.377$)	$r=-0.179$ ($p=0.268$)	$r=-0.026$ ($p=0.873$)
<i>CD8A</i> mRNA	$r=-0.293$ ($p=0.074$)	$r=-0.174$ ($p=0.303$)	$r=-0.267$ ($p=0.110$)	$r=-0.167$ ($p=0.323$)	$r=-0.202$ ($p=0.230$)	$r=-0.280$ ($p=0.094$)
<i>CD8B</i> mRNA	$r=-0.221$ ($p=0.175$)	$r=-0.002$ ($p=0.991$)	$r=-0.258$ ($p=0.118$)	$r=-0.224$ ($p=0.176$)	$r=-0.121$ ($p=0.469$)	$r=-0.265$ ($p=0.108$)
<i>CD69</i> mRNA	$r=-0.273$ ($p=0.055$)	$r=-0.049$ ($p=0.739$)	$r=-0.210$ ($p=0.148$)	$r=-0.467$ ($p=0.001$)*	$r=-0.268$ ($p=0.062$)	$r=-0.133$ ($p=0.362$)
<i>IL-10</i> mRNA	$r=-0.142$ ($p=0.324$)	$r=-0.084$ ($p=0.654$)	$r=-0.334$ ($p=0.019$)*	$r=-0.158$ ($p=0.279$)	$r=-0.078$ ($p=0.596$)	$r=0.128$ ($p=0.381$)
<i>IL-4</i> mRNA	$r=-0.044$ ($p=0.771$)	$r=-0.141$ ($p=0.356$)	$r=-0.056$ ($p=0.713$)	$r=0.094$ ($p=0.539$)	$r=-0.023$ ($p=0.878$)	$r=-0.230$ ($p=0.128$)
<i>FOXP3</i> mRNA	$r=-0.123$ ($p=0.400$)	$r=-0.118$ ($p=0.423$)	$r=0.005$ ($p=0.974$)	$r=-0.051$ ($p=0.730$)	$r=-0.143$ ($p=0.332$)	$r=-0.162$ ($p=0.272$)

Statistical analysis was performed using Spearman rho correlation, $p \leq 0.05$ was considered significant. Bold type face represents significant findings.

5.4 Discussion

In the present study, I sought to investigate the potential role that T cell alterations play in the pathophysiology of depression. Similar to Chapter 3 and 4, epigenetics were a primary focus of the investigation. DNA methylation analysis was performed to assess if epigenetic modifications could be contributing to any observed differences between groups. As it is not possible to study DNA methylation patterns in the brains of living subjects, the possibility that epigenetic modifications at T cell genes are associated with depression is of massive potential clinical significance.

5.4.1 The impact of early life adversity on *CD3E* alterations in depression

The Illumina® CpG site cg06164961, located in the body of the *CD3E* gene, has previously been used in studies as a CD3+ T cell marker in clinical samples (Cao-Lei et al., 2015), with increased methylation at this site associated with decreased *CD3E* mRNA expression (Guénard et al., 2013). However, to my knowledge, assessment of DNA methylation status at this site has never been performed in a cohort of depressed patients.

A significant increase in DNA methylation at cg06164961 of 10% was observed in depressed patients compared to healthy controls. These methylation levels were strongly correlated with HAM-D and CES-D scores, suggesting that severity of depression is associated with the degree of methylation. Furthermore, it was observed that DNA methylation levels at cg06164961 were highly correlated with total CTQ scores, emotional and physical abuse and neglect severity in all participants. As such a significant proportion of the depressed individuals in our cohort were exposed to early life adversity (48% vs. 12% prevalence in controls), resulting in a strong correlation between depression and trauma severity scores ($r=0.508$, $p<0.001$), the depressed group were divided on whether or not they were exposed to ELA to assess if these methylation changes were driven by trauma, rather than depression per se. It was revealed that currently depressed individuals who were exposed to childhood trauma had significantly higher methylation levels at cg06164961 compared to currently depressed patients who did not have a history of significant adversity. In fact, upon further inspection, control subjects with a history of adversity had higher mean methylation levels than currently depressed patients without a history of adversity, suggesting that increased methylation at cg06164961 may be a useful ELA trait marker. Additionally when assessing the relative expression of *CD3E* mRNA, a significant decrease in expression was observed in

depressed ELA+ patients when compared to depressed ELA- patients. When examining the expression of *CD3E* mRNA in depressed patients versus controls, although the depressed group had lower mean values, no significant differences were revealed. This result strengthens the role of *CD3E* changes in depression in individuals with a history of adversity.

5.4.2 CD3E DNA methylation and tryptophan depletion in depression

As presented in Chapter 4, a significant reduction (21%) in circulating tryptophan concentration was observed in depressed patients relative to control subjects. The essential amino acid tryptophan is crucial in the regulation of T cell activation. In vitro experiments have demonstrated that T cell activation is suppressed in a tryptophan-depleted environment, with tryptophan catabolism associated with decreased T cell activity, allowing for the control of T cell responses (Munn et al., 1999). To date, no direct link between tryptophan concentrations and the activity of DNA methyltransferase (DNMT) enzymes, which establish and maintain DNA methylation in humans, have been established. However, a recent study has shown a strong negative association between tryptophan concentrations and global DNA methylation in certain brain regions (Lee et al., 2014), suggesting that tryptophan could be involved in the regulation of DNA methylation.

When examining the entire cohort, a reduction in plasma tryptophan concentration was significantly correlated with an increase in DNA methylation at cg06164961. Furthermore a trend towards a significant association ($p=0.064$) was revealed between decreased plasma tryptophan concentrations and *CD3E* mRNA expression, suggesting a relationship between decreased tryptophan, increased DNA methylation and decreased T cell activity.

This study presents evidence for the first time that decreased tryptophan concentrations, commonly observed in depressed individuals, may mediate DNA methylation patterns which ultimately contribute to T cell activity alterations associated with depression.

5.4.3 Anti-CD3 stimulated T cell gene expression in depression

Anti-CD3 was the mitogen used in this study to stimulate T cells. Many studies use anti-CD3 in combination with its co-stimulatory molecule CD28 (Thompson et al., 1989), however, carrying out in vitro stimulations with anti-CD3 only is believed to more closely represent the

in vivo environment as the co-stimulatory molecules are provided by activated monocytes (Hughes, 2014b).

Relative expression of *CD3E* was measured from peripheral blood mononuclear cells (PBMCs) at baseline and after anti-CD3 stimulation to assess potential differences in T cell activity between groups. Stimulation with anti-CD3 did not significantly alter expression of *CD3E* mRNA in control subjects, however, it did significantly decrease expression of *CD3E* mRNA in the cells of the depressed cohort. Further probing the impact of anti-CD3 stimulation on the expression of *CD3E* mRNA in depressed patients, it was discovered that ELA+ depressed patients had the most significant decrease in mRNA gene expression. As anti-CD3 stimulation is believed to have proliferative effects on T cells, it is possible that depressed individuals, particularly those exposed to adversity early in life, have dysregulated T cell function. However, no increase in *CD3E* gene expression was observed in control samples, suggesting that perhaps anti-CD3 concentrations used in this setting were not at a sufficient concentration to stimulate further *CD3E* mRNA expression; these findings are potentially more representative of baseline *CD3E* expression levels and further investigation is necessary before making any conclusions.

Relative expression of the T helper 1 (T_H1) cell effector molecule interferon gamma (*IFN- γ*) was measured from peripheral blood mononuclear cells (PBMCs) at (1) baseline, (2) after LPS stimulation and (3) after anti-CD3 stimulation to assess potential differences in T cell activity. Interestingly, while stimulation with anti-CD3 induced a significant increase in *IFN- γ* at the message level in the control participants, mitogen stimulation with anti-CD3 did not have a significant impact on transcriptional *IFN- γ* expression in the depressed cohort relative to unstimulated expression levels. However, no significant difference was observed when comparing stimulated *IFN- γ* mRNA expression between depressed patients and control subjects. This could be the result of *IFN- γ* in the unstimulated sample set from the depressed cohort already being at a saturated expression level, preventing further expression upon secondary stimulation with anti-CD3.

Although it appears that differences in T cell responses or activity are present in depressed patients, further exploration of T cell effector molecules and T cell associated gene expression is required to fully understand the complex physiological consequences of a dysregulated adaptive immune system in depression.

5.4.4 Associations with T cell associated genes and clinical measures

The relative expression of a selection of T cell associated genes were measured in whole blood. While the expression of the genes measured did not significantly differ between groups, a number of interesting associations were revealed. Of particular interest were associations observed between mRNA expression and trauma severity in depressed patients. *CD69* expression is induced upon activation of T cells and may also play a role in their proliferation (NCBI, 2017). In depressed patients, a strong association was observed between decreased *CD69* mRNA expression and childhood sexual abuse (CSA) severity, indicating a reduction in T cell activity in individuals exposed to CSA.

IL-10 is anti-inflammatory cytokine that inhibits the activity of T_h1 cells, natural killer (NK) cells and macrophages (Couper et al., 2008). It is one of the main effector molecules of T_h2 and T_{reg} cells (Dong and Martinez, 2010). In depressed patients, a strong association was observed between decreased *IL-10* mRNA expression and the degree of physical abuse severity, indicating a reduction in anti-inflammatory T cell activity in individuals exposed to trauma early in life.

5.4.5 Conclusions

In conclusion, this study supports the presence of DNA methylation-mediated alterations in T cell activity in depression. It may be suggested that exposure to early life adversity negatively primes the adaptive immune system to a point which then may become exacerbated at some stage later in life, contributing to a depressive phenotype. While further study is required to fully elucidate the mechanistic basis to the findings in this chapter, this study highlights the need for a focus on adaptive immune system alterations in depressed patients, especially those who have been victims of trauma early in life.

Chapter 6

**Maternal depression during pregnancy:
implications for baby epigenetic
signature and stress response system**

6.1 Introduction

The ‘fetal programming hypothesis’ proposes that “alterations in fetal nutrition and endocrine status result in developmental adaptations that permanently change structure, physiology and metabolism, thereby predisposing to cardiovascular, metabolic and endocrine disease in adult life” (Barker, 1998). This mechanism is believed to act as a pre-emptive measure so that the fetus is prepared for potential external challenges once they are born. It is proposed that the body’s stress response system, the hypothalamic-pituitary-adrenal (HPA) axis, is programmed by circulating glucocorticoids at critical time points during gestation (Kapoor et al., 2006b).

Depression has consistently been associated with alterations in HPA axis activity (Calfa et al., 2003). The measurement of cortisol in saliva provides a reliable tool for investigations of HPA axis activity (Kirschbaum and Hellhammer, 1989). Several studies have demonstrated increased morning cortisol concentrations in current and remitted depression (Bhagwagar et al., 2005; Vreeburg et al., 2009) as well as blunted cortisol responses to stressors (Burke et al., 2005b).

Maternal depression during pregnancy has been found to be associated with impaired cognitive and emotional development of the offspring during childhood, and increased rates of psychiatric disorder during adolescence and adulthood (Davis et al., 2007; Pawlby et al., 2009). Associations between maternal anxiety during pregnancy and salivary cortisol levels in their children have been found (O’Connor et al., 2005b), highlighting the possibility that the developing fetal stress system is skewed in response to high levels of cortisol in the pregnant woman.

It is not fully understood how exposure to maternal depression and/or high cortisol during pregnancy “programmes” the baby’s HPA axis. However, epigenetic modifications provide a promising possible mechanism to explain this programming. These modifications are believed to mediate the gene-environment dialogue in early life and give rise to persistent programming of adult physiology (Murgatroyd and Spengler, 2011). The epigenetic modification DNA methylation refers to the addition of a methyl group to a CG site. Addition of a methyl group to a CG site typically results in gene silencing as the chromatin becomes tightly coiled which prevents transcriptional machinery accessing DNA promoter regions (Farrell and O’Keane, 2016).

In relation to major depressive disorder and alterations in HPA axis activity, one of the most extensively investigated epigenetic modifications is methylation at a nerve growth factor-inducible protein A (NGFI-A) binding site located in the hippocampal glucocorticoid receptor gene (*NR3C1*) exon 1F promoter. In human studies, increased methylation at this site has been associated with depression severity (Radtke et al., 2015) and decreased GR mRNA expression (McGowan et al., 2009).

The GR co-chaperone FK506 binding protein 5 (FKBP5) has recently attracted attention as a protein possibly implicated in the area of epigenetic mediated gene-environment interaction, leading to adult mental health disturbances. FKBP5 is involved in maintaining HPA axis homeostasis (Binder et al., 2004b) and its dysregulation has been reported in depression (O'Leary et al., 2013). It is important to investigate the mechanisms by which this dysregulation may occur. Demethylation at the *FKBP5* gene intron 7 observed in individuals with post-traumatic stress disorder (PTSD). Early life abuse (ELA) has been found to be associated with an increase in FKBP5 induction by GR activation, subsequent GR resistance and dysregulation of the stress response system (Klengel et al., 2013a).

The main aims of this study were to:

1. Assess whether babies born to women suffering with depression during pregnancy have altered DNA methylation levels at the *NR3C1* and/or *FKBP5* gene at six months postpartum;
2. Examine the possible impact that DNA methylation has on baby cortisol concentrations and cortisol reactivity;
3. Examine possible effects that depression during pregnancy may have on maternal DNA methylation patterns at 6 months postpartum.

6.2 Methods

6.2.1 Participant recruitment

Pregnant women were recruited from the National Maternity Hospital, Holles Street and the Coombe Women & Infants University Hospital by Dr. Chaitra Jairaj and Ms. Niamh O'Leary, Trinity College Dublin. The women were recruited at between 20 and 30 weeks gestation. Screening for the study involved assessing whether each woman fulfilled criteria for a Major Depressive Episode from the DSM-IV, scored >17 on the Hamilton Depression Rating Scale (HAM-D), was not suffering from a psychotic or substance abuse disorder and was not suffering from any chronic medical illnesses. Only singleton pregnancies were included. Healthy pregnant women were recruited in to the study for comparison. Once the woman met the criteria, they were invited to participate in the study. If they agreed to participate, the study was thoroughly explained and they signed a consent form. Women were then administered the same clinical instruments as in the depression study (see Section 2.2.1.2-2.2.1.7) and the Perceived Stress Scale (Cohen et al., 1983). Extensive demographic and other clinical data were collected.

6.2.2 Saliva sample collection

Mother and baby dyads had saliva samples collected on the same day, at six months postpartum, to coincide with baby's planned vaccination day.

6.2.2 (a)

For the women, saliva samples were collected in Salivette® tubes (Sarstedt) by the study participant at five time points throughout the course of the day. These time points were 0, 30 and 60 minutes, and 12 and 12.5 hours, after waking. This collection procedure was performed upon recruitment (between 20-30 weeks gestation) and at six month postpartum, to correspond with the babies' samples. The participants were given detailed collection instructions in person and also via an information sheet (Appendix 3a). The tubes were either collected by the researcher or returned by post. Once returned to the lab, the tubes were centrifuged at 1,000 x g for 2 minutes at room temperature. Saliva was aliquoted and stored in microtubes at -80°C until all samples had been collected.

6.2.2 (b)

For the babies, saliva samples were collected into Salimetrics® Infant Saliva Storage Tubes at 6 months postpartum. The 6-month vaccination that is routinely given to infants as part of the Public Health system in Ireland was used as a stress test. A sample was collected prior to the baby's vaccination for a baseline measure, and again 30 minutes after the vaccination as a stress response measurement. The tubes were brought back to the lab and were subsequently centrifuged at 1,000 x g for 2 minutes and room temperature and stored in microtubes at -80°C until all samples had been collected. This work was performed by Ms. Niamh O'Leary.

6.2.3 Saliva collection for epigenetic analysis

At 6 months post-partum, both mothers and babies also had saliva collected in Orogene DNA Saliva tubes (Genotek). These tubes contain a proprietary solution that protects and preserves DNA collected from saliva. The samples were stored at room temperature until all samples were collected to allow for batch analysis. This work was carried out by Ms. Niamh O'Leary.

6.2.4 Liquid chromatography-mass spectrometry (LC-MS) for salivary cortisol

LC-MS was performed on the saliva samples for calculation of cortisol concentrations at the University of Manchester in the Department of Clinical Biochemistry. The work was performed by members of Prof. Brian Keevil's laboratory. Calculation of all cortisol measurements (mother and baby) were analysed by Dr. Chaitra Jairaj.

6.2.5 Isolation of DNA from saliva samples

DNA was isolated from saliva stored in Orogene DNA Saliva Tubes by manual ethanol precipitation using the prepIT®L2P reagent (Genotek) as per the manufacturer's recommended protocol. DNA sample quantity and quality were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Subsequently the DNA samples were equalised to contain 1000 ng gDNA in 40 µl RNase-free water for bisulfite-conversion and PCR.

6.2.6 DNA methylation analysis of HPA axis markers in saliva

Methylation levels of the glucocorticoid receptor gene (*NR3C1*) exon 1F and FK506 binding protein 5 (*FKBP5*) intron 7 was measured in DNA isolated from saliva by pyrosequencing, performed on bisulfite-treated DNA.

For *NR3C1* exon 1F, NR3C1F_LR_F1-biotin (5Biosg/GTTTTTAATTTTTTAGGAAAAAGGGT) and NR3C1F_LR_R1 (ACCCCTTTCCAAATAACACACTTC) were the forward and reverse primers, respectively, for the PCR reaction. NR3C1F_LR_S3 (AACTCCCAATAAATCTAAAC) and NR3C1F_LR_S4 (AAAAAACTCCATAACCCTCT) were the sequencing primers used in the pyrosequencing reaction.

```
GTTTTTAATTTTTTAGGAAAAAGGGTGGCGGCGGTAGCGGCGGGGGTCGATTTGGTTTTTTGGGG  
CGGCG41TTAAGAGGGTTATCG40AGTTTTTTTAGTTTTTTTTT39CG38TTTT37T  
TTAGTTT36CG35GTTTATGATTTATTCGGGAGTTCGTTTTGTTTTTGGCGGTAAGCGTCGTTAGTG  
TTTTGCGGGTGATAGCGGGCGGGTTATAAGAGTCGGGGCGTTTTTCACGGTTGAGTTGCGTGAGT  
GGTCGCGTCGACGTCGTCGGGTCGAGTTGCGTGAAGTGTGTTATTTCGAAAGGGGT
```

Figure 6.1. 319 bp PCR amplicon of *NR3C1* exon 1F, lower strand, converted sequence. Measured CG sites are highlighted. NGFI-A binding site is underlined.

For *FKBP5* intron 7, FKBP5_in7_F2 (TGGGATAATAATTTGGAGTTATAGTGTAGG) and FKBP5_in7_R2-biotin (5Biosg/AAATTTATCTCTTACCTCCAACACTAC) were the forward and reverse primers respectively, for the PCR reaction. FKBP5_S4 (GTTGATATATAGGAATAAAATAAGA) was the sequencing primer used in the pyrosequencing reaction.

```
TGGGATAATAATTTGGAGTTATAGTGTAGGTTTTTCGTGATTTTTGTGAAGGGTATAATTCGTTTAG  
TTTTGAAAAGTTGATTTTTATTTTTTAAGGAGTTATTTGGTAGAACGTGAATTTTTGTTTTAATTT  
AGGAAAAAAGTATAAAAGAATAAGTTTAGGAATAAATAAGGGAATAAGTTTTGGATTTATTT  
AAAAAAGTTAAAAAAGTTGATATATAGGAATAAATAAGAATAC6GAGTTTTTT7CG  
TT
```

Figure 6.2. 341 bp PCR amplicon of *FKBP5* intron 7, upper strand, converted sequence. Measured CpG sites are highlighted.

In total, percentage methylation was measured at eight CG sites: six in *NR3C1* exon 1F and two in *FKBP5* intron 7. Pyrosequencing was performed using the Q96 platform (Qiagen). Percentage methylation levels and quality control were analysed using PyroMark CpG Software (Version 1.0.11, Qiagen). Detailed methodology of the pyrosequencing procedure is outlined in Chapter 2 – Section 2.2.3.

6.2.7 Statistical Analysis

Data are presented as mean with standard deviation (SD) and standard error of the mean (SEM) where appropriate. Data normality was assessed using the Shapiro-Wilk test of normality. Differences in demographic variables were tested using Mann-Whitney U test, Chi-square (χ^2) test and/or Fisher's exact test. Depending on data distribution, differences in biological variables were assessed using Student's t-test, Mann-Whitney U test or univariate analysis of variance. Spearman's rho correlation was used to assess relationships between biological variables. All statistical analyses were considered significant when $p \leq 0.05$. Statistics were performed using SPSS (Version 22, IBM) and all graphs were generated using GraphPad Prism Software (Version 6, GraphPad Inc.).

6.3 Results

6.3.1 Demographic data for pregnant women recruited into the study

A proportion of the total women in the study were involved in the epigenetic study and for the purposes of my study, I have analysed this sub-sample. 29 mother-baby dyads were included in my study: 12 women who experienced depression during pregnancy and 17 healthy controls. There were no significant differences in BMI or smoking prevalence between the groups but the women in the depressed group in this sub-sample were significantly younger than those in the control group [Table 6.1].

Table 6.1 Demographic data for all recruited pregnant women

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
Age (years)	31.25 (3.75)	35.18 (5.51)	Z=-2.01, p=0.048*
BMI	22.65 (1.03)	22.61 (2.80)	Z=-0.33, p=0.857
Smoking (Y/N)	0/12	0/17	n/a

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann-Whitney U test (Age and BMI) and Chi squared (χ^2) test (Smoking). BMI=Body Mass Index. *p<0.05 vs controls.

6.3.2 Clinical data for pregnant women recruited into the study

Consistent with a diagnosis of depression, pregnant women with Major Depressive Disorder (MDD) had significantly higher clinical scores on measures of depression compared to women with no history of psychiatric disorders. Examination of ELA scores in the groups demonstrated that the depressed women had significantly higher CTQ scores compared to controls. 50% of depressed women were receiving pharmacological treatment for the management of depression at the time of recruitment [Table 6.2].

Table 6.2 Clinical data for all recruited pregnant women

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
HAM-D	21.50 (7.08)	1.06 (1.25)	Z=-4.57, p<0.001***
CES-D	37.40 (14.91)	6.59 (5.82)	Z=-3.69, p<0.001***
PSQI	10.80 (3.55)	4.24 (1.99)	Z=-4.02, p<0.001***
PSS	35.70 (8.10)	18.12 (7.57)	Z=-3.66, p<0.001***
CTQ Score	42.80 (15.55)	30.12 (5.84)	Z=-3.13, p=0.001***
ELA (Y/N)	5/7	3/14	n/a, p=0.402
AD Use (Y/N)	6/6	0/17	n/a, p=0.011*

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann-Whitney U test (HAM-D, CES-D, PSQI, PSS, CTQ Score) and Fisher's exact test (ELA, Antidepressant use). HAM-D=Hamilton Rating Scale for Depression; CES-D=Center for Epidemiologic Studies Depression Scale; PSQI=Pittsburgh Sleep Quality Index; CTQ=Childhood Trauma Questionnaire; AD=antidepressant.

6.3.3 Cortisol Awakening Response (CAR) measurements in pregnant women with depression and controls

A univariate analysis of variance performed on log transformed cortisol data, with age as a covariate, revealed that the depressed group had significantly increased cortisol concentrations 30 minutes from wakening as well as significantly increased morning average cortisol output. [Table 6.3].

Table 6.3 Mother cortisol measurements during pregnancy

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
Cortisol T0	17.39 (6.80)	11.68 (1.30)	F=1.09, p=0.310
Cortisol T30	17.69 (3.34)	11.11 (0.84)	F=12.5, p=0.001**
Cortisol T60	11.89 (3.25)	10.69 (0.82)	F=0.02, p=0.894
Cortisol AM average	16.80 (4.55)	11.41 (0.88)	F=7.03, p=0.017*
Cortisol T720	2.92 (0.82)	2.49 (0.49)	F=0.02, p=0.888
Cortisol T750	2.79 (0.72)	2.43 (0.46)	F=0.07, p=0.798
Cortisol PM average	2.86 (0.77)	2.46 (0.47)	F=0.05, p=0.833
Cortisol Reactivity	-5.51 (3.91)	0.66 (1.82)	F=1.13, p=0.323

Data are expressed as mean cortisol concentrations with SEM in parentheses. A univariate analysis of variance was performed on log transformed cortisol data. *p≤0.05 vs controls. **p≤0.001 vs. controls.

6.3.4 Cortisol Awakening Response (CAR) measurements in women with depression during pregnancy and controls at six months postpartum

At six months postpartum, all 12 women who had suffered from depression during their pregnancy were euthymic. A univariate analysis of variance performed on log transformed cortisol data, with age as a covariate, revealed a significant increase at T750 (evening) cortisol concentrations between the depressed group compared to the controls [Table 6.4].

Table 6.4 Mother cortisol measurements at six months postpartum

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
Cortisol T0	9.93 (1.00)	8.13 (1.26)	F=1.02, p=0.326
Cortisol T30	9.75 (1.32)	9.23 (0.71)	F=0.18, p=0.677
Cortisol T60	6.50 (0.72)	6.26 (0.74)	F=0.39, p=0.540
Cortisol AM average	8.52 (0.80)	8.20 (0.61)	F=0.08, p=0.781
Cortisol T720	1.90 (0.70)	1.06 (0.20)	F=0.99, p=0.336
Cortisol T750	1.90 (0.50)	0.81 (0.01)	F=10.3, p=0.006**
Cortisol PM average	1.56 (0.33)	0.93 (0.10)	F=6.11, p=0.027
Cortisol Reactivity	-2.85 (0.85)	-1.86 (1.12)	F=0.01, p=0.969

Data are expressed as mean cortisol concentrations with SEM in parentheses. Statistical analysis was performed using a univariate analysis of variance on log transformed cortisol data, with age as a covariate. **p≤0.01 vs controls.

6.3.5 Cortisol stress response measurements in babies at six months postpartum

A Student's t-test performed on log transformed cortisol data revealed no significant differences in cortisol stress response measurements between the groups [Table 6.5].

Table 6.5 Baby cortisol measurements at six months

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
Cortisol T0	8.36 (4.25)	2.11 (0.22)	t=-1.44, p=0.170
Cortisol T30	11.14 (3.77)	5.89 (1.21)	t=-1.07, p=0.297
Cortisol AM average	9.76 (2.78)	3.50 (0.46)	t=-1.78, p=0.096
Cortisol Reactivity	2.79 (5.80)	2.82 (0.83)	t=-0.16, p=0.875

Data are expressed as means SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed cortisol data

6.3.6 Overall DNA methylation levels at *NR3C1* exon 1F promoter in women with depression and controls

At six months postpartum, all 12 women who had suffered from depression during their pregnancy were euthymic. Overall *NR3C1* exon 1F methylation levels were calculated at 6 CG sites. A univariate analysis of variance performed on log transformed *NR3C1* methylation data, with age as a covariate, found no significant differences in overall DNA methylation status between the two groups [Table 6.6, Figure 6.3].

Table 6.6 Overall *NR3C1* methylation status for women with depression during pregnancy and controls

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
mean <i>NR3C1F</i>	4.16 (0.30)	4.51 (0.24)	F=0.56, p=0.466

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate analysis of variance, with age as a covariate, on log transformed methylation data.

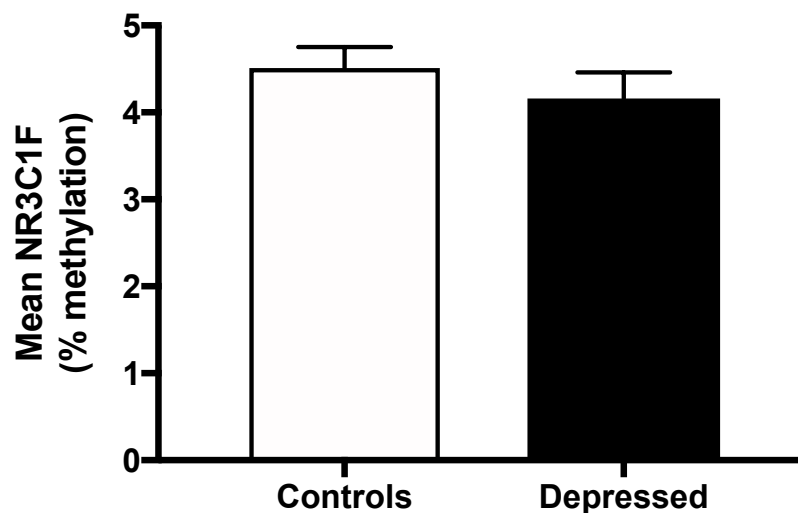


Figure 6.3 *NR3C1* exon 1F methylation in women who suffered with depression during pregnancy and controls. Mean *NR3C1* exon 1F DNA methylation in women who suffered with depression during pregnancy and healthy controls. Data expressed as mean with SEM, Univariate Analysis of Variance, controlling for age.

6.3.7 DNA methylation levels of specific CG sites in the *NR3C1* exon 1F promoter in women with perinatal depression and controls

A univariate analysis of variance, with age as a covariate, was performed on log transformed *NR3C1* methylation data to assess whether there were differences at any individual CG site measured.

Analysis revealed that the depressed group had significantly decreased levels of DNA methylation at both CG38 and CG39 [Figure 6.4 (d) and (e)]. There were no significant differences in methylation at CG35, CG36, CG37 and CG41 between the two groups [Table 6.7].

Table 6.7 Methylation levels at specific CG sites in *NR3C1* exon 1F promoter for women with depression during pregnancy and controls

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
<i>NR3C1F</i> CG35	6.51 (0.94)	4.90 (0.80)	F=1.37, p=0.255
<i>NR3C1F</i> CG36	3.02 (0.22)	3.35 (0.31)	F=0.01, p=0.981
<i>NR3C1F</i> CG37	2.74 (0.20)	3.36 (0.19)	F=2.50, p=0.129
<i>NR3C1F</i> CG38	4.36 (0.28)	6.28 (0.65)	F=6.65, p=0.018*
<i>NR3C1F</i> CG39	4.38 (0.33)	5.44 (0.37)	F=5.75, p=0.026*
<i>NR3C1F</i> CG41	2.73 (1.11)	2.42 (0.59)	F=1.10, p=0.342

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate analysis of variance on log transformed methylation data, with age as a covariate. *p<0.05 vs controls.

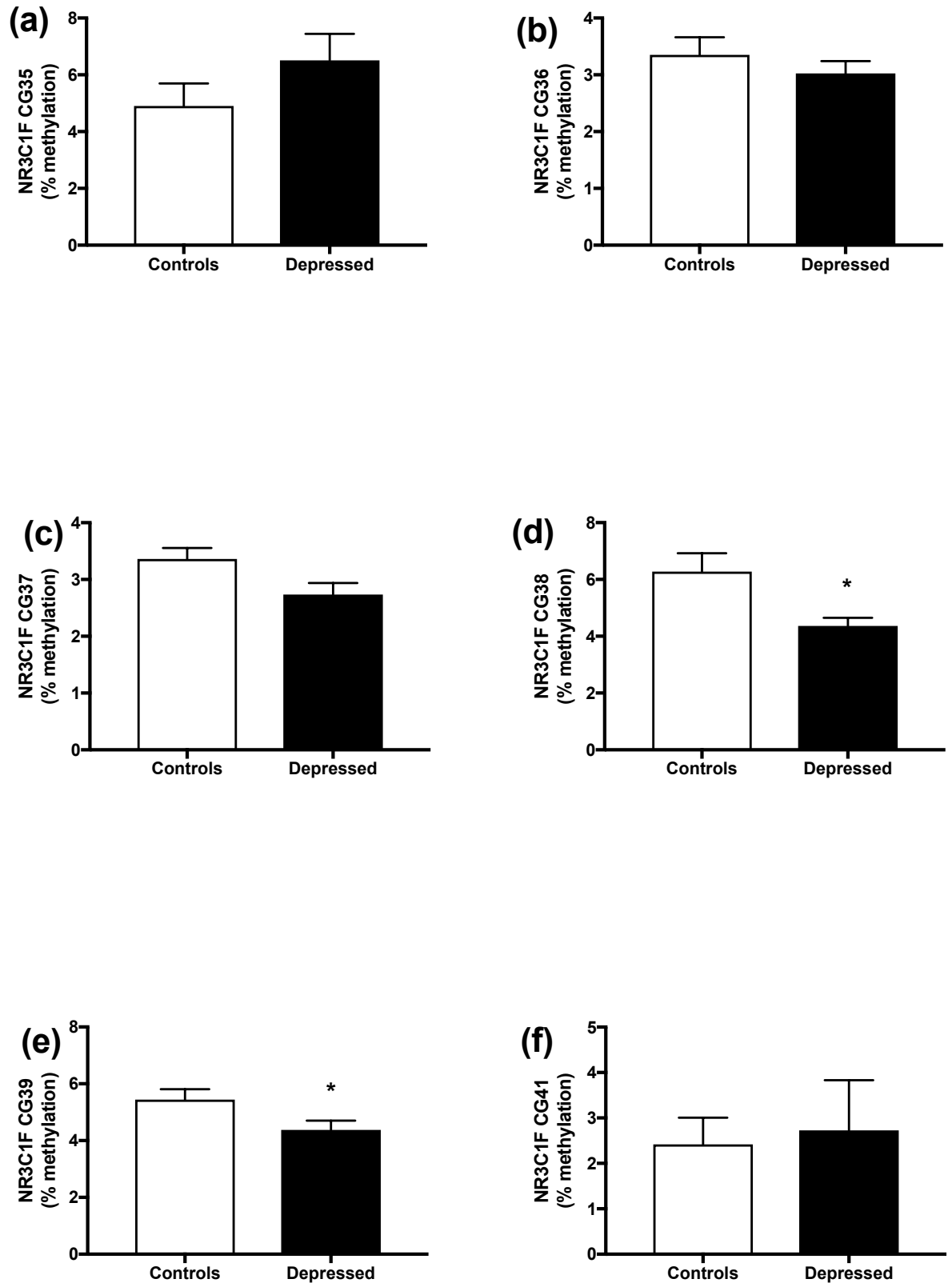


Figure 6.4 Site-specific *NR3C1* exon 1F methylation in women who suffered with depression during pregnancy and controls. *NR3C1* exon 1F DNA methylation at (a) CG35, (b) CG36, (c) CG37, (d) CG38, (e) CG39 and (f) CG41 in women who suffered with depression during pregnancy and healthy controls. Data expressed as mean with SEM, Univariate Analysis of Variance, controlling for age. * $p \leq 0.05$ vs controls.

6.3.8 Overall DNA methylation levels at *FKBP5* intron 7 promoter in women with perinatal depression and controls

Overall *FKBP5* methylation levels were calculated at 2 CG sites. A univariate analysis of variance performed on log transformed *FKBP5* methylation data, with age as a covariate, found no significant differences in overall DNA methylation status between the two groups [Table 6.8].

Table 6.8 Overall *FKBP5* methylation status for women with depression during pregnancy and controls

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
mean<i>FKBP5</i>	60.80 (1.91)	60.16 (1.21)	F=0.20, p=0.659

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using a univariate analysis of variance on log transformed methylation data, with age as a covariate.

6.3.9 DNA methylation levels of specific CG sites in the *FKBP5* intron 7 promoter in women with depression and controls

A univariate analysis of variance was performed on log transformed *FKBP5* methylation data, with age as a covariate, to assess whether there were differences at any individual CG site measured. There was no significant differences in methylation at CG6 or CG7 between the two groups [Table 6.9].

Table 6.9 Methylation levels at specific CG sites in *FKBP5* intron 7 promoter for women with depression during pregnancy and controls

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
<i>FKBP5</i> CG6	54.90 (1.83)	54.16 (1.10)	F=0.37, p=0.549
<i>FKBP5</i> CG7	66.71 (2.72)	69.48 (3.04)	F=0.44, p=0.515

Data are expressed as mean methylation percentage with SEM in parentheses. Statistical analysis was performed using univariate analysis of variance on log transformed methylation data, with age as a covariate.

6.3.10 Overall DNA methylation levels at *NR3C1* exon 1F promoter in babies born to women with perinatal depression and controls

Overall *NR3C1* exon 1F methylation levels were calculated at the same 6 CG sites as in their mothers'. A Student's t-test performed on log transformed *NR3C1* methylation data revealed no differences in overall DNA methylation status between the two groups [Table 6.10].

Table 6.10 Overall *NR3C1* methylation status for babies born to women with depression during pregnancy and controls

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
mean <i>NR3C1F</i>	4.33 (0.28)	4.05 (0.24)	t=-0.81, p=0.431

Data are expressed as means SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed methylation data.

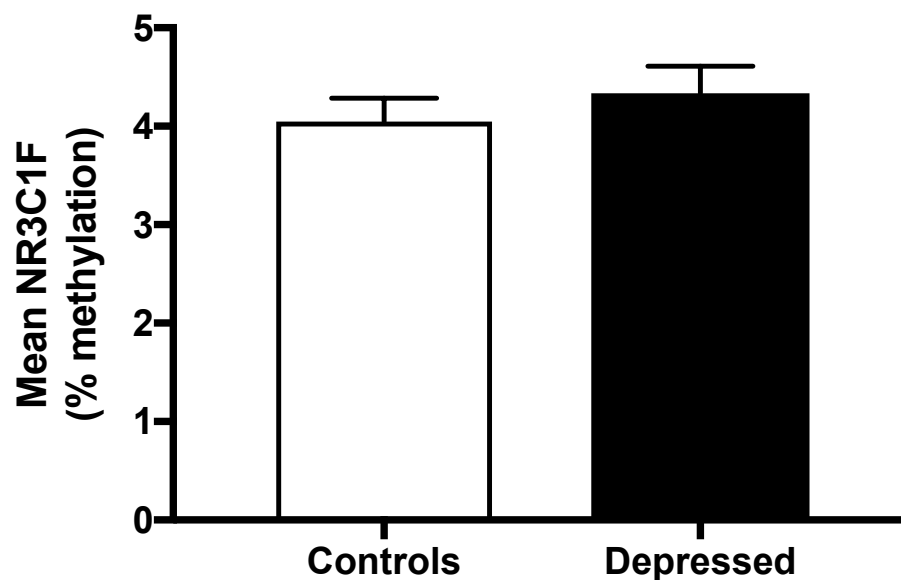


Figure 6.5 *NR3C1* exon 1F methylation in babies born to women who suffered with depression during pregnancy and controls. Mean *NR3C1* exon 1F DNA methylation in babies born to women who suffered with depression during pregnancy and healthy controls. Data expressed as mean with SEM, Student's t-test.

6.3.11 DNA methylation levels of specific CG sites in the NR3C1 exon 1F promoter in babies born to women with perinatal depression and controls

A Student's t-test was performed on log transformed NR3C1 methylation data to assess whether there were differences at any individual CG site measured.

Babies born to women who suffered with perinatal depression had increased DNA methylation levels at CG35 compared to the control group [Figure 6.6 (a)]. There were no significant differences in methylation at CG36, CG37, CG38, CG39 and CG41 between the two groups [Table 6.11].

Table 6.11 Methylation levels at specific CG sites in NR3C1 exon 1F promoter for babies born to women with depression during pregnancy and controls

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
NR3C1F CG35	6.10 (0.91)	3.98 (0.55)	t=-2.77, p=0.013*
NR3C1F CG36	3.24 (0.42)	3.91 (0.37)	t=-0.01, p=0.994
NR3C1F CG37	3.23 (0.30)	3.29 (0.24)	t=0.25, p=0.809
NR3C1F CG38	4.74 (0.38)	5.21 (0.38)	t=0.84, p=0.413
NR3C1F CG39	4.63 (0.44)	4.84 (0.26)	t=0.64, p=0.529
NR3C1F CG41	2.88 (1.30)	3.53 (0.95)	t=-0.54, p=0.607

Data are expressed as means SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed methylation data. *p≤0.05 vs controls.

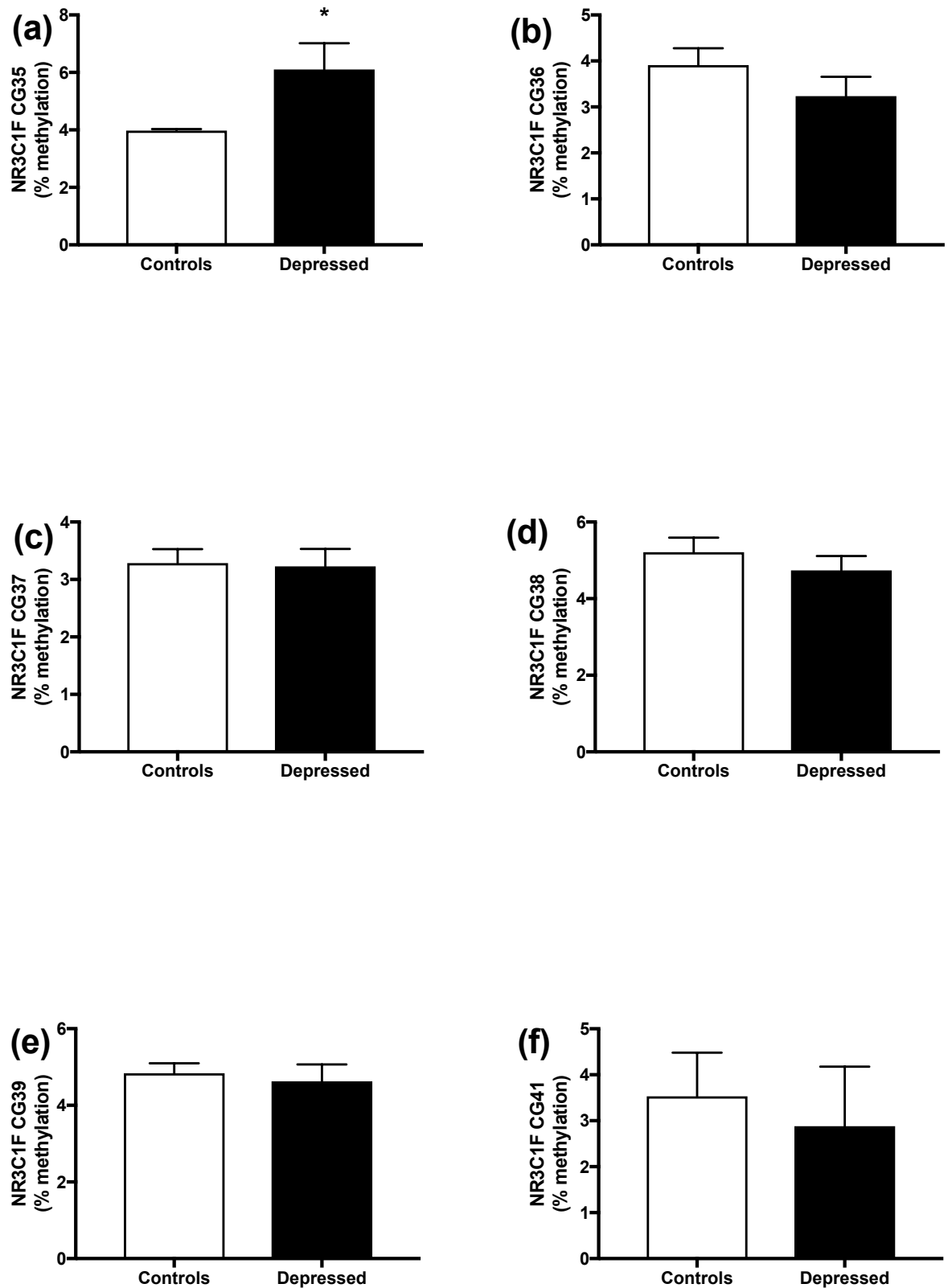


Figure 6.6 Site-specific *NR3C1* exon 1F methylation in babies born to women who suffered with depression during pregnancy and controls. *NR3C1* exon 1F DNA methylation at (a) CG35, (b) CG36, (c) CG37, (d) CG38, (e) CG39 and (f) CG41 in babies born to women who suffered with depression during pregnancy and healthy controls. Data expressed as mean with SEM, Univariate Analysis of Variance, controlling for age. *p<0.05 vs controls.

6.3.12 Mean DNA methylation levels of *FKBP5* intron 7 promoter in babies born to women with perinatal depression and controls

A Student's t-test performed on log transformed *FKBP5* methylation data revealed no differences in overall DNA methylation status between the two groups [Table 6.12].

Table 6.12 Overall *FKBP5* methylation status for babies born to women with depression during pregnancy and controls

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
mean<i>FKBP5</i>	40.91 (0.94)	43.19 (1.27)	t=1.33, p=0.206

Data are expressed as means SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed methylation data.

6.3.13 DNA methylation levels of specific CG sites in the *FKBP5* intron 7 promoter in babies born to women with perinatal depression and controls

A Student's t-test performed on log transformed *FKBP5* methylation data revealed no differences in DNA methylation status at either CG6 or CG7 site between the two groups [Table 6.13].

Table 6.13 Methylation levels at specific CG sites in *FKBP5* intron 7 promoter for babies born to women with depression during pregnancy and controls

	Patients (n=12)	Controls (n=17)	Statistic (p-value)
<i>FKBP5</i> CG6	37.00 (0.76)	40.09 (1.33)	t=1.81, p=0.093
<i>FKBP5</i> CG7	44.82 (1.25)	46.29 (3.04)	t=0.81, p=0.432

Data are expressed as means SEM in parentheses. Statistical analysis was performed using a Student's t-test on log transformed methylation data.

6.3.14 Evaluation of the relationship between mother DNA methylation and baby DNA methylation

(a) *NR3C1* exon 1F promoter

Spearman's rho correlation analysis revealed that no associations existed between mother *NR3C1* exon 1F promoter methylation status and baby *NR3C1* exon 1F promoter methylation status. This was the case when examining mean methylation status and methylation at specific CG sites [Figure 6.7 and Figure 6.8 respectively].

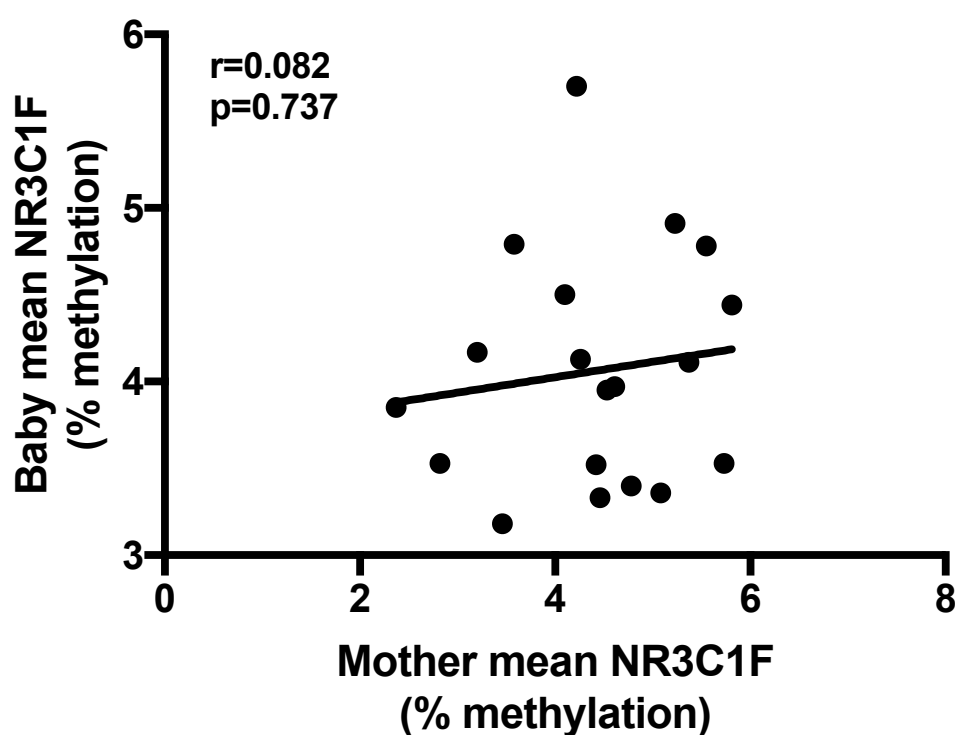


Figure 6.7 Relationship between overall mother and baby *NR3C1* methylation. No correlation exists between overall levels of DNA methylation at the mother *NR3C1* exon 1F and overall levels of DNA methylation at the baby *NR3C1* exon 1F.

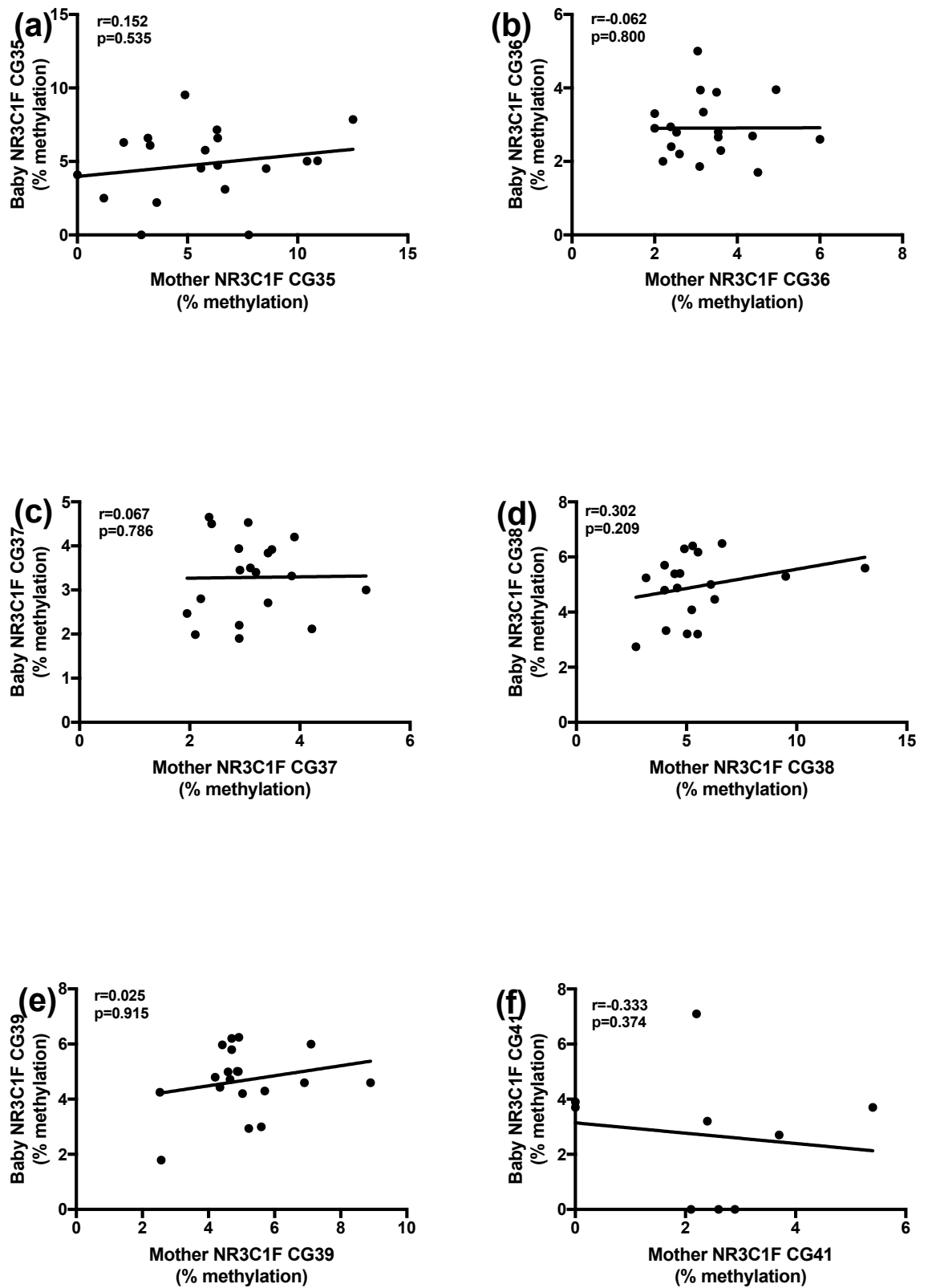


Figure 6.8 Relationships between individual mother and baby *NR3C1F* CG site methylation. No correlation exists between DNA methylation at any of the specific CG sites measured at the *NR3C1* exon 1F between mothers and their babies.

(b) *FKBP5* intron 7 promoter

There were no associations between mother *FKBP5* intron 7 methylation status and baby *FKBP5* intron 7 methylation status. This was the case when examining mean methylation status and methylation at specific CG sites [Figure 6.9].

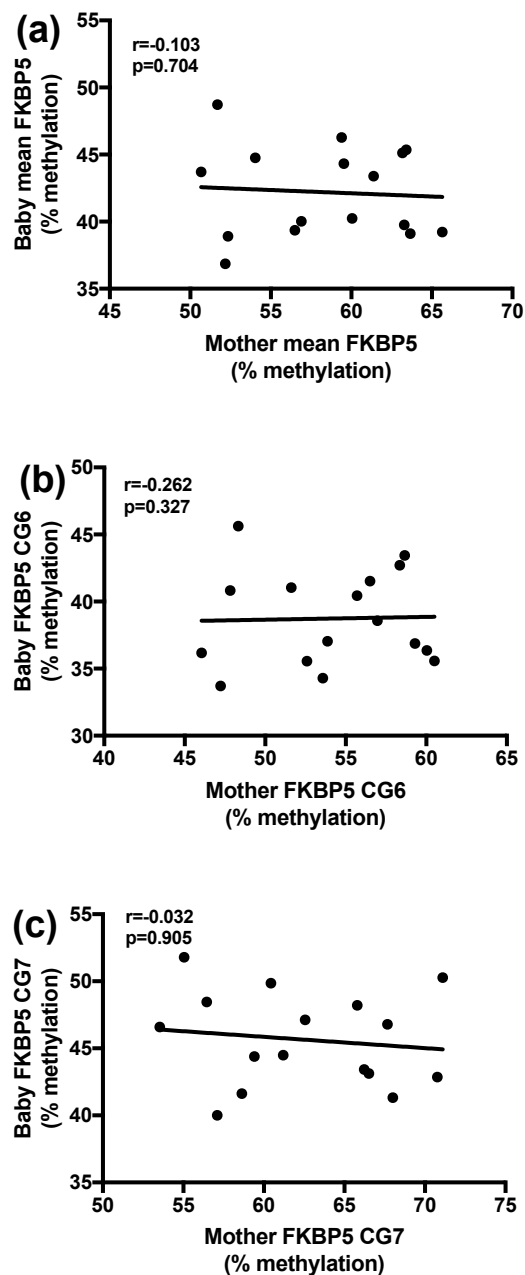


Figure 6.9 Relationships between mother and baby *NFKBP5* methylation. No correlation exists in overall or site-specific levels of DNA methylation at the *FKBP5* intron 7 in mothers or their babies.

6.3.15 Evaluation of the relationship between maternal depression, sleep disturbances, stress and trauma and baby DNA methylation

Spearman's rho correlational analysis was performed to assess potential relationships between maternal stressors, measured during pregnancy, and baby DNA methylation at the *NR3C1* exon 1F promoter. Analysis revealed that baby *NR3C1F* CG35 DNA methylation was positively correlated with maternal CES-D, PSQI and PSS scores during pregnancy [Figure 6.10 a-c]. Baby *NR3C1F* CG35 DNA methylation was also positively correlated maternal CTQ scores [Figure 6.10 d]. A significant negative relationship was identified between baby *NR3C1F* CG38 DNA methylation and maternal CTQ scores [Figure 6.10 e] [Table 6.14].

FKBP5 intron 7 CG6 and mean *FKBP5* DNA methylation was positively correlated with maternal PSS scores during pregnancy [Figure 6.11 a,b] [Table 6.15].

6.3.16 Evaluation of the relationship between mother cortisol concentrations and baby DNA methylation.

There were no associations between mother cortisol concentrations during pregnancy and baby *NR3C1* exon 1F DNA methylation [Table 6.16 and Table 6.17 respectively].

There were no associations between mother cortisol concentrations during pregnancy and baby *FKBP5* intron 7 DNA methylation [Table 6.18 and Table 6.19 respectively].

Table 6.14 Correlational analysis of baby *NR3C1* DNA methylation at 6 months and maternal rating scales during pregnancy.

	HAM-D	CES-D	PSQI	PSS	CTQ
<i>NR3C1</i> CG35	r=0.384 (p=0.086)	r=0.563 (p=0.012)*	r=0.565 (p=0.012)*	r=0.544 (p=0.016)*	r=0.568 (p=0.011)
<i>NR3C1</i> CG36	r=-0.166 (p=0.472)	r=-0.039 (p=0.875)	r=0.049 (p=0.841)	r=0.090 (p=0.713)	r=-0.021 (p=0.931)
<i>NR3C1</i> CG37	r=-0.099 (p=0.668)	r=0.189 (p=0.439)	r=0.069 (p=0.780)	r=0.232 (p=0.339)	r=0.081 (p=0.741)
<i>NR3C1</i> CG38	r=-0.242 (p=0.290)	r=-0.437 (p=0.061)	r=-0.333 (p=0.164)	r=-0.316 (p=0.187)	r=-0.600 (p=0.007)**
<i>NR3C1</i> CG39	r=-0.188 (p=0.414)	r=-0.051 (p=0.837)	r=-0.105 (p=0.669)	r=0.159 (p=0.515)	r=-0.382 (p=0.106)
<i>NR3C1</i> CG41	r=-0.021 (p=0.951)	r=-0.216 (p=0.549)	r=-0.567 (p=0.088)	r=-0.210 (p=0.561)	r=-0.231 (p=0.520)
mean<i>NR3C1F</i>	r=-0.012 (p=0.958)	r=0.075 (p=0.759)	r=-0.018 (p=0.943)	r=0.156 (p=0.524)	r=-0.060 (p=0.035)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. Bold type face represents significant findings. HAM-D: Hamilton Depression Rating Scale, CES-D: Center for Epidemiological Studies Depression Scale, PSQI: Pittsburgh Sleep Quality Index, PSS: Perceived Stress Scale, CTQ: Childhood Trauma Questionnaire.

Table 6.15 Correlational analysis of baby *FKBP5* DNA methylation at 6 months and maternal rating scales during pregnancy.

	HAM-D	CES-D	PSQI	PSS	CTQ
<i>FKBP5</i> CG6	r=-0.327 (p=0.217)	r=-0.301 (p=0.276)	r=-0.280 (p=0.312)	r=-0.612 (p=0.015)*	r=-0.258 (p=0.353)
<i>FKBP5</i> CG7	r=-0.084 (p=0.757)	r=-0.218 (p=0.435)	r=-0.103 (p=0.715)	r=-0.461 (p=0.084)	r=-0.096 (p=0.734)
mean<i>FKBP5</i>	r=-0.208 (p=0.439)	r=-0.265 (p=0.340)	r=-0.193 (p=0.490)	r=-0.529 (p=0.043)*	r=-0.166 (p=0.544)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. Bold type face represents significant findings. HAM-D: Hamilton Depression Rating Scale, CES-D: Center for Epidemiological Studies Depression Scale, PSQI: Pittsburgh Sleep Quality Index, PSS: Perceived Stress Scale, CTQ: Childhood Trauma Questionnaire

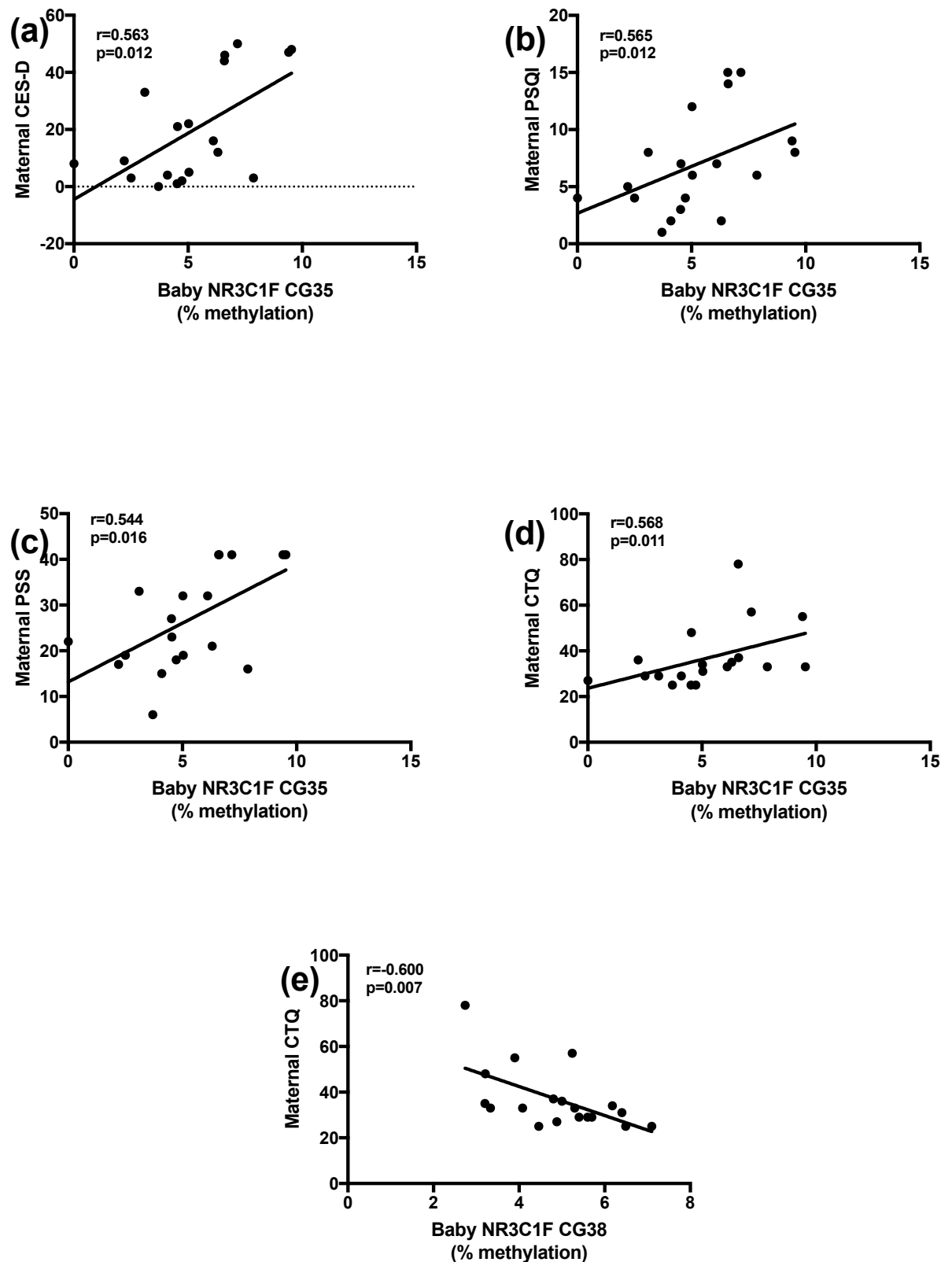


Figure 6.10 Relationships between baby *NR3C1* DNA methylation and maternal rating scales during pregnancy. A significant positive correlation exists between baby CG35 methylation and maternal (a) CES-D scores, (b) PSQI scores, (c) PSS scores and (d) CTQ scores. (e) A significant negative correlation exists between baby CG38 methylation and maternal CTQ scores.

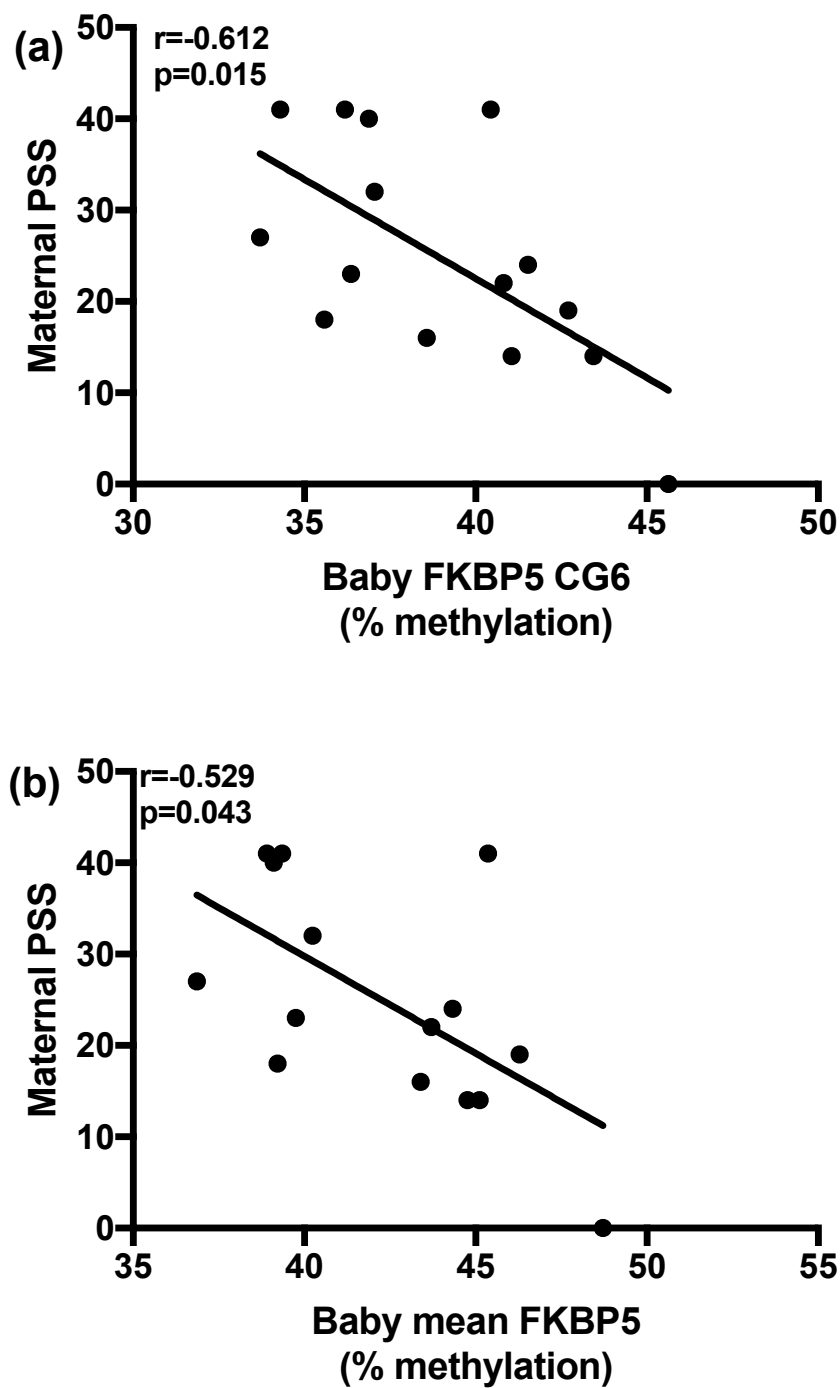


Figure 6.11 Relationships between baby *FKBP5* DNA methylation and maternal stress severity during pregnancy. A significant negative correlation exists between maternal PSS scores during pregnancy and (a) baby *FKBP5* intron 7 CG6 methylation and (b) baby mean *FKBP5* intron 7 methylation.

Table 6.16 Correlational analysis of baby *NR3C1* DNA methylation at 6 months and mother salivary cortisol levels during pregnancy.

	Cortisol T0	Cortisol T30	Cortisol T60	Cortisol T720	Cortisol T750
<i>NR3C1</i> CG35	r=0.425 (p=0.114)	r=0.171 (p=0.577)	r=0.152 (p=0.605)	r=-0.443 (p=0.098)	r=-0.399 (p=0.141)
<i>NR3C1</i> CG36	r=0.400 (p=0.140)	r=0.000 (p=1.000)	r=0.226 (p=0.436)	r=-0.134 (p=0.634)	r=-0.085 (p=0.764)
<i>NR3C1</i> CG37	r=-0.325 (p=0.237)	r=-0.264 (p=0.383)	r=-0.349 (p=0.221)	r=-0.279 (p=0.314)	r=-0.386 (p=0.155)
<i>NR3C1</i> CG38	r=-0.104 (p=0.713)	r=-0.077 (p=0.802)	r=-0.213 (p=0.464)	r=-0.079 (p=0.781)	r=-0.173 (p=0.537)
<i>NR3C1</i> CG39	r=-0.054 (p=0.849)	r=-0.142 (p=0.643)	r=-0.209 (p=0.473)	r=-0.363 (p=0.184)	r=-0.264 (p=0.342)
<i>NR3C1</i> CG41	r=0.092 (p=0.814)	r=-0.036 (p=0.939)	r=-0.060 (p=0.888)	r=-0.312 (p=0.380)	r=-0.139 (p=0.701)
mean<i>NR3C1F</i>	r=0.216 (p=0.439)	r=-0.041 (p=0.894)	r=-0.194 (p=0.507)	r=-0.539 (p=0.038)	r=-0.547 (p=0.035)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. No significant correlations were observed between mother cortisol levels during pregnancy and baby DNA methylation levels of *NR3C1* exon 1F promoter.

Table 6.17 Correlational analysis of baby *NR3C1* DNA methylation at 6 months and mother salivary cortisol levels at 6 months.

	Cortisol T0	Cortisol T30	Cortisol T60	Cortisol T720	Cortisol T750
<i>NR3C1</i> CG35	r=0.287 (p=0.281)	r=0.211 (p=0.451)	r=-0.123 (p=0.661)	r=0.254 (p=0.361)	r=0.422 (p=0.133)
<i>NR3C1</i> CG36	r=0.088 (p=0.745)	r=0.232 (p=0.405)	r=-0.086 (p=0.761)	r=0.041 (p=0.885)	r=0.462 (p=0.097)
<i>NR3C1</i> CG37	r=0.106 (p=0.696)	r=0.179 (p=0.524)	r=0.465 (p=0.081)	r=0.287 (p=0.300)	r=0.191 (p=0.513)
<i>NR3C1</i> CG38	r=-0.225 (p=0.402)	r=0.032 (p=0.909)	r=0.000 (p=1.000)	r=-0.045 (p=0.875)	r=0.022 (p=0.941)
<i>NR3C1</i> CG39	r=0.055 (p=0.841)	r=0.315 (p=0.253)	r=0.313 (p=0.256)	r=-0.212 (p=0.449)	r=0.038 (p=0.898)
<i>NR3C1</i> CG41	r=-0.396 (p=0.257)	r=0.101 (p=0.796)	r=-0.118 (p=0.763)	r=-0.198 (p=0.402)	r=0.061 (p=0.866)
mean<i>NR3C1F</i>	r=-0.081 (p=0.766)	r=0.400 (p=0.139)	r=0.204 (p=0.466)	r=0.147 (p=0.600)	r=0.516 (p=0.059)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. No significant correlations were observed between mother cortisol levels at 6 months and baby DNA methylation levels of *NR3C1* exon 1F promoter.

Table 6.18 Correlational analysis of baby *FKBP5* DNA methylation at 6 months and mother salivary cortisol levels during pregnancy.

	Cortisol T0	Cortisol T30	Cortisol T60	Cortisol T720	Cortisol T750
<i>FKBP5</i> CG6	r=-0.509 (p=0.110)	r=-0.345 (p=0.298)	r=0.218 (p=0.519)	r=-0.328 (p=0.325)	r=-0.046 (p=0.894)
<i>FKBP5</i> CG6	r=-0.018 (p=0.958)	r=0.064 (p=0.853)	r=0.618 (p=0.043)	r=0.187 (p=0.582)	r=0.361 (p=0.276)
mean<i>FKBP5</i>	r=-0.155 (p=0.650)	r=-0.027 (p=0.937)	r=0.555 (p=0.077)	r=0.109 (p=0.749)	r=0.301 (p=0.368)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. No significant correlations were observed between mother cortisol levels during pregnancy and baby DNA methylation levels of *FKBP5* intron 7.

Table 6.19 Correlational analysis of baby *FKBP5* DNA methylation at 6 months and mother salivary cortisol levels at 6 months.

	Cortisol T0	Cortisol T30	Cortisol T60	Cortisol T720	Cortisol T750
<i>FKBP5</i> CG6	r=0.382 (p=0.276)	r=-0.097 (p=0.789)	r=-0.261 (p=0.467)	r=0.452 (p=0.261)	r=0.401 (p=0.373)
<i>FKBP5</i> CG7	r=0.309 (p=0.285)	r=-0.365 (p=0.300)	r=-0.406 (p=0.244)	r=0.483 (p=0.225)	r=-0.089 (p=0.849)
mean<i>FKBP5</i>	r=0.394 (p=0.260)	r=-0.316 (p=0.374)	r=-0.333 (p=0.347)	r=0.452 (p=0.261)	r=0.045 (p=0.924)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. No significant correlations were observed between mother cortisol levels at 6 months and baby DNA methylation levels of *FKBP5* intron 7.

6.3.17 Evaluation of the relationship between baby DNA methylation and cortisol concentrations

There was a significant negative correlation between baby methylation at both *NR3C1* CG35 and CG36 with baby cortisol reactivity at six months postpartum [Table 6.20] [Figure 6.12], but not with individual cortisol concentrations pre and post-vaccination [Table 6.20].

Spearman's rho analysis revealed no significant associations between baby *FKBP5* DNA methylation and any baby cortisol measurements at six months [Table 6.21].

Table 6.20 Correlational analysis of baby *NR3C1* DNA methylation at 6 months and salivary cortisol pre- and post-vaccination.

	Cortisol T0	Cortisol T30	Mean Cortisol	Cortisol Reactivity
<i>NR3C1</i> CG35	r=0.501 (p=0.097)	r=0.132 (p=0.668)	r=0.309 (p=0.329)	r=-0.692 (p=0.013)*
<i>NR3C1</i> CG36	r=0.270 (p=0.397)	r=-0.374 (p=0.209)	r=-0.119 (p=0.712)	r=-0.622 (p=0.031)*
<i>NR3C1</i> CG37	r=-0.007 (p=0.983)	r=-0.071 (p=0.817)	r=0.018 (p=0.957)	r=0.140 (p=0.665)
<i>NR3C1</i> CG38	r=-0.336 (p=0.285)	r=-0.484 (p=0.094)	r=-0.547 (p=0.065)	r=0.294 (p=0.354)
<i>NR3C1</i> CG39	r=-0.206 (p=0.521)	r=-0.204 (p=0.504)	r=-0.248 (p=0.437)	r=0.084 (p=0.795)
<i>NR3C1</i> CG41	r=0.017 (p=0.965)	r=-0.339 (p=0.372)	r=-0.264 (p=0.493)	r=-0.559 (p=0.117)
<i>meanNR3C1F</i>	r=0.202 (p=0.529)	r=-0.360 (p=0.226)	r=-0.167 (p=0.604)	r=-0.459 (p=0.134)

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

Table 6.21 Correlational analysis of baby *FKBP5* DNA methylation at 6 months and salivary cortisol pre- and post-vaccination.

	Cortisol T0	Cortisol T30	Mean Cortisol	Cortisol Reactivity
<i>FKBP5</i> CG6	r=0.600 (p=0.208)	r=0.367 (p=0.332)	r=0.261 (p=0.618)	r=0.143 (p=0.787)
<i>FKBP5</i> CG7	r=0.771 (p=0.072)	r=0.317 (p=0.406)	r=0.551 (p=0.257)	r=-0.257 (p=0.623)
<i>meanFKBP5</i>	r=0.657 (p=0.156)	r=0.317 (p=0.406)	r=0.377 (p=0.461)	r=-0.086 (p=0.872)

Statistical analysis was performed using the Spearman's rho correlation, with *p≤0.05 considered significant. No significant correlations were observed between baby *FKBP5* DNA methylation levels and baby cortisol measurements at six months.

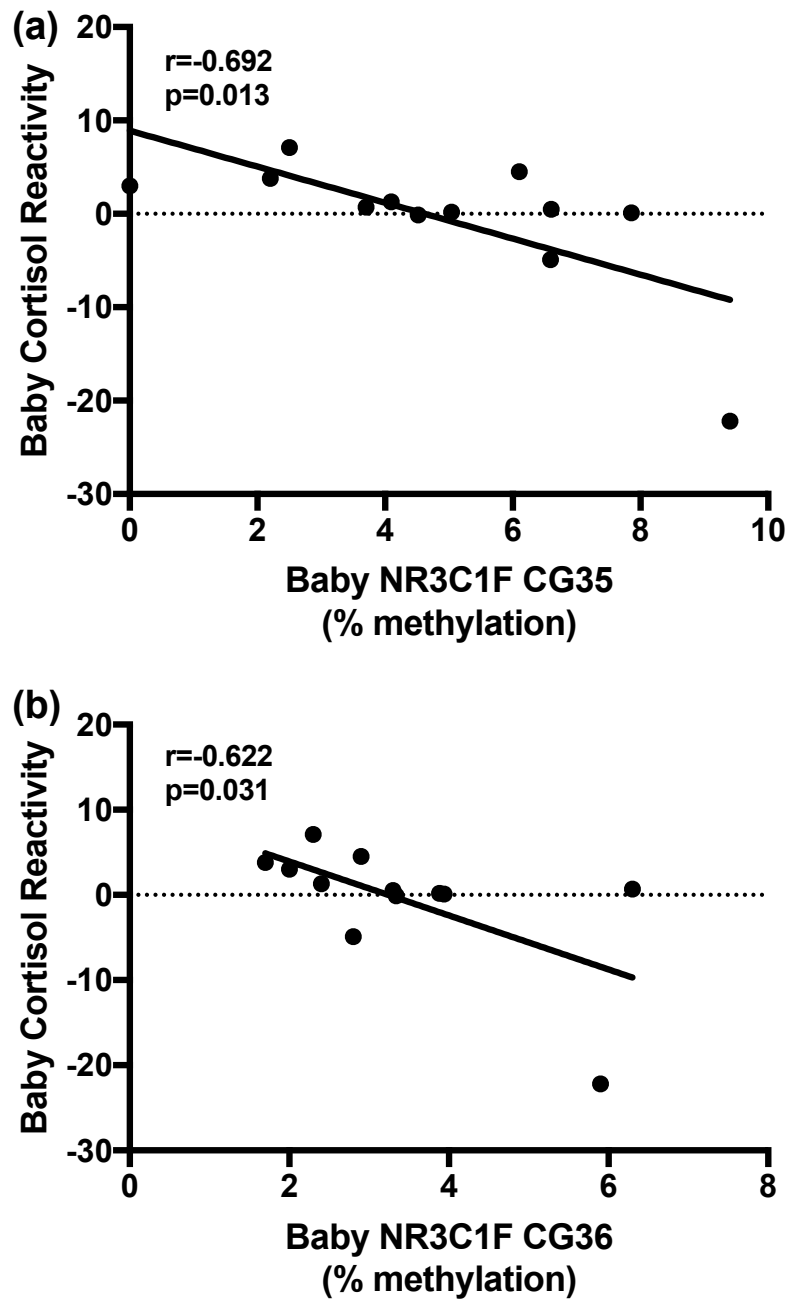


Figure 6.12 Relationships between baby *NR3C1* exon 1F DNA methylation and baby cortisol reactivity at six months. A significant negative correlation exists between baby cortisol reactivity and (a) baby *NR3C1* exon 1F CG35, and (b) baby *NR3C1* exon 1F CG36 methylation.

6.4 Discussion

In the present study, I assessed the levels of DNA methylation at two separate genes that have been implicated in altered stress systems and depression in a cohort of depressed women during pregnancy and at six months postpartum. DNA methylation was also assessed in their babies at six months postpartum. These samples were taken from the larger REDEEM (Research in Depression, Endocrinology, Epigenetics and Neuroimaging) project, that explores the relationships of the possible effects of depression during pregnancy on baby neuroendocrine systems and neurodevelopment. The major findings from this study were that babies born to women who suffered with depression during pregnancy had significantly increased DNA methylation at the *NR3C1* exon 1F promoter at six months postpartum. This DNA methylation was associated with decreased cortisol stress reactivity after vaccination. To my knowledge, this is the first study to assess both *NR3C1* and *FKBP5* methylation in women who suffered with depression during pregnancy and in the mother-baby dyads; with HPA axis measures at both time points

6.4.1 *NR3C1* exon 1F DNA methylation in babies born to women with depression and controls

DNA methylation of the *NR3C1* gene and its association to stress-related disorders has been of interest since the pivotal work of Ian Weaver and colleagues, which suggested a causal relationship among epigenomic state, GR expression and stress responses in the offspring (Weaver et al., 2004a). While a small number of studies have examined maternal mood and baby *NR3C1* DNA methylation, the majority of the research to date has reported findings using self-rated stress and/or self-rated depression scales (Oberlander et al., 2008; Braithwaite et al., 2015) and not among a clinically diagnosed population. In this study, babies born to women who were diagnosed with clinical depression during pregnancy had significantly increased DNA methylation at the *NR3C1* exon 1F promoter CG35 at six months postpartum. Methylation at this site in babies was found to be significantly correlated with maternal self-rated depression and stress scores during pregnancy, as well as with sleep disturbances. Furthermore, methylation at this site was positively associated with maternal exposure to ELA. These findings suggest that the methylation status of *NR3C1* exon 1F CG35 in babies at six months postpartum is highly sensitive to prenatal maternal mood and, interestingly, to mother's history of ELA.

6.4.2 Association between *NR3C1* exon 1F promoter and cortisol measurements in babies at six months postpartum

Cortisol reactivity refers to a phase in which cortisol concentrations increase from baseline levels following the onset of a stressor (Burke et al., 2005a). Both blunted and elevated cortisol reactivity in response to stressors have been shown in adults with depression (Suzuki et al., 2013). Much less, however, is known about baby stress response systems following exposure to prenatal depression. For assessment of cortisol reactivity in the babies, cortisol samples were collected pre- and post-vaccination. While no differences existed in cortisol reactivity between babies born to depressed women and babies born to control women, a strong negative correlation was revealed between baby DNA methylation at *NR3C1* exon 1F CG35 and CG36 and baby cortisol reactivity at six month postpartum. Increased methylation at the *NR3C1* indicates a reduced functional capacity of the GR. As DNA methylation at CG35 in babies born to women suffering with perinatal depression was significantly increased, this finding suggests that reduced GR function in baby is associated with maternal depression during pregnancy. This provides an epigenetic link between antenatal depression in women and an altered stress system in their baby offspring.

6.4.3 Association between prenatal maternal stress and *FKBP5* intron 7 DNA methylation in babies at six months postpartum

FKBP5 is a glucocorticoid receptor (GR) co-chaperone protein that is an important modulator of stress responses. It typically exerts an inhibitory role on GR signalling by delaying nuclear translocation of the GR and decreasing GR-dependent transcriptional activity (Zannas et al., 2016). Demethylation at *FKBP5* intron 7 has been linked to long-term dysregulation of the stress hormone system (Klengel et al., 2013a). It has been hypothesised that a decrease in DNA methylation at the *FKBP5* gene could result in a subsequent increase of *FKBP5* mRNA and protein, thus leading to alterations in stress responses. This study has shown, for the first time, a strong and significant association between increased maternal stress experienced during pregnancy and demethylation at *FKBP5* intron 7 in babies at six months postpartum. This correlation was present between prenatal maternal stress scores and baby DNA methylation at *FKBP5* intron 7 CG6 as well as with overall methylation measured at *FKBP5* intron 7. Although not significant but probably worth exploring further, babies in the depressed group had lower mean *FKBP5* intron 7 methylation levels at all sites at six months postpartum.

6.4.4 Absence of relationship between mother and baby epigenetic signatures

DNA methylation may be heritable (Bird, 2002). The heritability of epigenetic markers between generations is often used to explain the aetiology of traits that do not follow Mendelian patterns of inheritance but the patterns and prevalence of this possible inheritance are poorly understood (Kile et al., 2010). On examination of relationships in DNA methylation levels at the *NR3C1* and *FKBP5* gene, no associations were revealed between mother and baby methylation levels at any CG site examined in either gene. This finding is in accordance with the literature (Oberlander et al., 2008a). This indicates that methylation patterns at regulatory regions in the genes examined in this study are not subject to epigenetic inheritance but could be more sensitive to environmental cues, such as stress in the intrauterine environment. This is strongly supported by the finding of multiple and strong relationships between baby methylation and mother clinical scores during pregnancy.

6.4.5 NR3C1 exon 1F DNA methylation in women at six months postpartum who experienced depression during pregnancy and controls

Women who were clinically depressed during pregnancy, but who were euthymic at six months postpartum, had significantly decreased DNA methylation levels at *NR3C1* exon 1F promoter CG38 and CG39 at six months postpartum. To my knowledge, this is the first and only study to examine *NR3C1* exon 1F promoter methylation in women who experienced clinical depression during pregnancy and a comparable control group. This finding is extremely interesting as CG38 is the location of a nerve growth factor-inducible protein A (NGFI-A) transcription factor binding site (Palma-Gudiel et al., 2015) and methylation at this site has been associated with a reduction in GR mRNA expression (McGowan et al., 2009). Cortisol levels steadily increase throughout gestation (Sandman et al., 2006) and pregnancy, compared to the non-pregnant state, is associated with relative and incremental hypercortisolism. Moreover, increases in cortisol secretion were observed in the sample of women who were depressed in our study, compared to controls; a finding that has been observed in another study by O'Keane et al, (2011). This observed decrease in methylation at *NR3C1* exon 1F sites CG38 and CG39 in women at six months postpartum, who were depressed during pregnancy, seems at odds with what one would expect as it predicts enhanced feedback cortisol mechanisms. My findings of higher evening cortisol levels, commonly found in depression and indicating increased diurnal cortisol secretion, is indicative of reduced feedback. Theoretically, one would think that that this ought to be associated with increased methylation. An

assessment of GR mRNA expression in these women would be useful in further understanding the implications of the finding of DNA methylation decrease in this population for postpartum women. It is possible that epigenetic mechanisms are more plastic than previously hypothesised and the altered methylation at six months postpartum could be regulatory mechanism countering the hypercortisolaemic state of pregnancy.

6.5.6 Limitations and further directions

As is often the case with preliminary studies examining the study would have benefited from a larger sample size. The relatively small sample size is partly the result of the difficulty in recruiting women who are clinically depressed during pregnancy and who are willing to participate in clinical research. This is understandable as pregnancy, under normal conditions, is already a time of high stress. Women are less likely to be asked or to volunteer that they are depressed while pregnant probably for fear of societal stigma, as pregnancy is a time conventionally associated with joy (Jairaj et al., 2017). Another factor that limited the sample size was the difficulty in obtaining sufficient saliva from babies for both epigenetic and cortisol analysis at six months. For the purposes of pyrosequencing, at least 1000ng is required for bisulfite-conversion of DNA. Babies frequently did not provide adequate saliva to isolate DNA for analysis. A larger sample would improve the power of statistical analysis and should be more representative of the population.

Further studies should endeavour to assess the association between mother and infant methylation levels in the sites tested at different longitudinal points to develop a greater understanding of the relationship between inherited and de novo methylation patterns at genes involved in the regulation of stress responses in the developing infant.

The REDEEM study is following-up on the mother-baby dyads included in this study. The follow-up studies include assessment of maternal sensitivity, and baby neurodevelopment and behaviour. I hope to assess mother and baby DNA methylation status at follow-up to assess the following:

- Permanence or transience of methylation at regions measured in both mothers and babies;
- Impact of maternal sensitivity on baby methylation;
- Relationship, if any, between baby DNA methylation and neurodevelopment and behaviour.

In future work it would be worthwhile including DNA methylation analysis from fathers as a recent animal study has demonstrated that hippocampal *NR3C1* DNA methylation can mediate part of preconception paternal stress effects in rat offspring (Niknazar et al., 2017).

While methylation of *NR3C1* exon 1F CG35 was significantly correlated with cortisol reactivity measurements in babies, suggestive of a relationship between the two, more direct evidence, such as GR mRNA expression should be performed to further link methylation status at this site with possible functional consequences.

6.5.7 Conclusions

This study gives strong evidence to support that, at six months postpartum, infant methylation at both the *NR3C1* exon 1F and *FKBP5* intron 7 is related to prenatal maternal mood. Furthermore, the findings suggest that the degree of methylation at the *NR3C1* exon 1F promoter in infants is related to cortisol stress responses at six months postpartum.

Results from this study also suggest that depression experienced during pregnancy alters DNA methylation patterns at the *NR3C1* exon 1F promoter at six months postpartum.

Chapter 7

Discussion and Future Directions

Major depressive disorder (MDD) is a debilitating psychiatric illness that affects at least 350 million individuals worldwide (Kessler et al., 1994). At least 50% of individuals who recover from a first episode of depression will go on to develop one or more additional episodes in their lifetime and it is estimated that 80% of individuals with a history of two or more episodes will experience a further recurrence (Burcusa and Lacono, 2007). Currently, the diagnosis of depression is based purely on symptoms/experiences described by the patient along with the clinical judgement of a trained professional. There are no biological diagnostic tools available to distinguish subtypes of depression despite the heterogeneity in presentations of the disorder, and hence the potential complexity in making a diagnosis. Additionally, despite a variety of pharmacological agents available for the treatment of depression, response rates are relatively poor. Approximately 50% of individuals requiring treatment for depression will need at least two trials of antidepressants to alleviate symptoms and 30% will ultimately be deemed treatment resistant (Rush et al., 2006b). This information highlights the importance of research into the biological mechanisms underlying depression. The aims of this thesis were to examine relationships among systems previously implicated in the pathophysiology of depression including the hypothalamic-pituitary-adrenal (HPA) axis, the serotonin system and the adaptive immune system, with a particular focus on epigenetic mechanisms and exposure to early life adversity.

7.1 Early life adversity and HPA axis alterations in depression

A consistent finding in the literature to date is the association of depression with glucocorticoid resistance and subsequent hyperactivity of the HPA axis (Calfa et al., 2003). An increasing body of research suggests that this hyperactivity may be mediated, in part, via epigenetic mechanisms at the glucocorticoid receptor gene (*NR3C1*) in response to trauma or chronic stress (Perroud et al., 2011b). The main objectives of both Chapter 3 and Chapter 6 were to assess HPA axis alterations in depression and, if present, the contribution of early life adversity (ELA) and epigenetics to these differences.

The primary findings in Chapter 3 were (1) an increase in overall DNA methylation at the *NR3C1* exon 1F promoter region and (2) higher morning cortisol concentrations upon wakening accompanied by decreased cortisol reactivity in depressed patients relative to healthy controls. The findings of high baseline and flattened cortisol reactivity to stress have been reported consistently in the literature (Cleare et al, 1996; Suzuki et al., 2013) and support a state of HPA axis dysregulation in depression. Site-specific increases in DNA methylation at

CG38 were associated with decreased expression of glucocorticoid receptor (GR) mRNA in peripheral blood cells. Furthermore, there was a strong and positive relationship between DNA methylation at the NGFI-A binding site CG38 and morning cortisol concentrations. Taken together, these findings indicate a significant association between increases in DNA methylation at the *NR3C1* exon 1F, decreases in GR mRNA expression and high baseline cortisol with flattened stress responses. The data suggest that in depression alterations in HPA axis function are associated with DNA methylation changes at the *NR3C1* exon 1F promoter. A significant positive relationship was revealed between childhood emotional abuse severity and DNA methylation at CG37 and CG38 when examining the depressed group only, suggests that the relationship between depression and GR methylation may be mediated through ELA. These studies provide further evidence of the HPA axis hyperactivity known to be associated with depression; and that the altered dynamics in the HPA axis are probably, at least partly, mediated by epigenetic alterations at the GR (*NR3C1*). A specific site within the gene, CG38, was associated with decreased expression of GR mRNA; and increased morning cortisol secretion.

The mineralocorticoid receptor (MR or *NR3C2*) is another steroid hormone receptor that, in comparison to the GR, has been relatively overlooked in the literature examining HPA axis function and depression. It has been suggested that there may be an imbalance in the MR/GR ratio in MDD patients that contribute to the depressive state (Young et al., 2003). Brain MR expression has been shown to be reduced in the hippocampus, inferior frontal gyrus and cingulate gyrus of individuals who had suffered with depression (Klok et al., 2011), further supporting the hypothesis that MR differences may contribute to the depressive phenotype. Negative feedback regulation of the HPA axis is not only dependent on functioning GRs but also on intact MR function. While no differences in the expression of MR mRNA were found between groups, an almost significant decrease in MR expression and a significant decrease in MR:GR ratio was observed in depressed individuals with a history of ELA exposure. This finding suggests again a greater effect of ELA, rather than depression per se, to changes in HPA axis in MDD. The MR findings suggest a potential role of the MR system in the HPA axis alterations in depression, especially in patients with a history of adversity.

The studies contained in Chapter 6 reported on the HPA axis alterations and *NR3C1* exon 1F DNA methylation levels in a group of mother-baby dyads. The mother-baby dyads were from a cohort of women who suffered with depression during pregnancy and a group of women with no history of psychiatric illness. The study findings were that, at six months postpartum, babies born to women who were clinically diagnosed with depression during their pregnancy

had significantly increased DNA methylation at *NR3C1* exon 1F promoter CG35. Furthermore, in the group of babies examined, methylation levels at CG35, and its neighbour CG36, were negatively associated with cortisol reactivity. Exposure to maternal clinical depression during pregnancy can be considered as one of the earliest possible forms of environmental adversity. These findings indicate that depression during pregnancy may modify DNA methylation at the *NR3C1* exon 1F promoter CG35 in baby.

Overall, the findings from this group of studies provide strong evidence for adversity-driven DNA methylation changes at the glucocorticoid receptor gene in depression that are associated with altered HPA axis function, as measured by the CAR/diurnal test.

7.1.2 ELA and epigenetics: is ELA+ depression a discrete MDD subtype?

Child abuse and neglect are associated with increased risk for depression, anxiety, posttraumatic stress disorder, and substance use disorders (Teicher & Samson 2013: psychopathology), and a range of physical health problems including obesity, diabetes, cardiovascular disease, and cancer. Epigenetic regulation of gene function can be viewed as the adaptation of the genome to environmental influences, a process that has been referred to as genomic plasticity. Thus, it is possible to conceptualize the epigenome as an interface through which the environment can influence genetic processes and, as a result, regulate behavior at least partially in response to environmental needs (Turecki et al, 2014). Regulation of the HPA axis, via epigenetic modifications at the GR which is responsible for regulation of HPA axis negative feedback, has been implicated in the psychopathology in adulthood that follows on from ELA. In relation to major depressive disorder and alterations in HPA axis activity, the most extensively investigated epigenetic modification to date has been hypermethylation of a nerve growth factor-inducible protein A (NGFI-A) binding site located in the hippocampal *NR3C1* gene exon 1F promoter (Swirloff and Milbrandt, 1995b). The findings to date indicate a probable role of exon 1F promoter hypermethylation in the pathogenesis of depression (Smart et al., 2015). This hypermethylation essentially blocks the binding of NGFI-A to its binding site at the promoter and subsequently affects the level of GR protein in the cytoplasm of the cell. If this were happening in brain limbic tissues, this would result in dysregulation of HPA axis negative feedback, uncontrolled cortisol release and ultimately depression. Pivotal animal studies identified that exposure to an adverse environment early in life negatively impacted the HPA axis activity via DNA methylation at the GR gene (Weaver et al., 2004b). Follow on studies examining this phenomenon found similar

alterations in DNA methylation in depressed patients as well as individuals diagnosed with borderline personality disorder and noted that exposure to ELA further altered DNA methylation (Perroud et al., 2011). The above studies in this thesis demonstrate possible biological consequences of exposure to ELA that are consistent with the literature. My study adds to this literature by (1) demonstrating that CG38 of the *NR3C1* exon 1F promoter is related to the severity of the abuse; (2) CG35 of the *NR3C1* exon 1F is altered in response to maternal depression during pregnancy; (3) demonstrating clear functional consequences of this methylation on GR gene expression; and (4) demonstrating a pattern of associations with HPA axis function that supports the GR epigenetic hypothesis.

The significant decrease in MR/GR ratio observed only in depressed patients with a history of ELA is a novel finding. As HPA axis regulation, specifically the synthesis and release of corticotrophin-releasing hormone (CRH), is controlled through a glucocorticoid negative feedback system mediated by both MR and GR (Szyf et al., 2005) further investigations should be performed, particularly at the epigenetic level, to assess the clinical importance and implications of this preliminary finding.

There is growing consensus that patients with depression, anxiety, and substance use disorders with a history of maltreatment represent a clinically and biologically distinct subtype with earlier age at onset, greater symptom severity, more comorbidity, a greater risk for suicide, and poorer treatment response than non-maltreated individuals with the same diagnoses (Teicher and Samson, 2013). Findings from this thesis suggest that ELA+ depression do indeed have additional biological changes, that probably contribute to the neuroendocrine pathology seen in MDD generally, and that this is very likely to lead to a poorer outcome. This is of central importance as it has been reported that depression occurring in those with a history of childhood maltreatment is associated with a poorer response to treatment for depression, particularly to antidepressant medication (Nanni et al., 2011). It is believed that adversity goes largely unreported when an individual presents with a depressive episode (Petersen et al., 2014) as the diagnosis of depression relies purely on information given by the patient. Thus, while ELA+ and ELA- depression may present in a similar fashion, the evidence from my studies adds to the existing literature demonstrating that those with ELA+ depression have different aetiopathologies and clinical needs that are currently not being met. The clinical phenotypes have largely been established. Differentiating the neurobiology of depressed individuals who have experienced ELA will help to target the underlying pathophysiology consequent upon developmental adversity. Epigenetic changes, specifically at key CG sites in the GR, may be one such target.

7.2 Serotonin neurotransmission in depression

Serotonin (5-hydroxytryptamine/5-HT), a monoamine involved in mood, sleep, appetite, motor activity and cognitive function (Meltzer and Nash, 1988), is the neurotransmitter most frequently implicated and investigated in the depression literature. It is believed that a decrease in 5-HT neurotransmission may be aetiologically relevant in the depressed state (Young and Leyton, 2002). 5-HT activity is regulated in the brain by the serotonin transporter/solute carrier family 6 member 4 (5-HTT/SLC6A4) that effectively terminates the action of 5-HT by transporting it back into the presynaptic neuron. Over 20 years ago, a functional polymorphism in the promoter region of the *SLC6A4* gene was discovered (Heils et al., 1996), referred to as 5-HTTLPR: a 44bp deletion/insertion that results in two different alleles; the short (s) variant having less transcriptional activity than the long (l) variant. Given the hypothesised role of 5-HT/5-HTT in depression, and an increasing body of literature suggesting epigenetic changes contributing to various psychiatric disorders (Nestler, 2009), investigations into *SLC6A4* promoter DNA methylation changes in depression are of particular interest.

Much research has examined the potential role of the precursor tryptophan in the development of MDD. The synthesis of 5-HT in humans is dependent on the bio-availability of the essential amino acid tryptophan (Fernstrom, 1983). A suggested mechanism to explain altered 5-HT neurotransmission is that individuals with depression have decreased tryptophan concentrations resulting in reduced availability for 5-HT synthesis. It has been hypothesised that tryptophan is depleted in depression as a result of an increased inflammatory profile and increased stress hormones that activate the kynurenine pathway (KP). The kynurenine pathway uses available tryptophan thus theoretically reducing the amount of tryptophan available for serotonin production (Muller and Schwarz, 2007).

Although associations between HPA axis output, with high basal secretion of cortisol and blunted cortisol stress responses, and the altered 5-HT function are both seen in depression, a direct link between the two has not been unequivocally established. The aims of Chapter 4 were to investigate *SLC6A4* promoter DNA methylation status in depression and assess this methylation in relation to both HPA axis activity and circulating tryptophan/kynurenine levels. *SLC6A4* promoter DNA methylation was significantly decreased in depressed patients. Tryptophan concentrations were also significantly lower in the depressed, compared to the control, group. Interestingly, a significant negative relationship between *SLC6A4* DNA methylation and tryptophan concentrations was revealed. This indicates that depressed

patients had two separate and related elements of the 5-HT system altered i.e. reduced tryptophan availability for 5-HT synthesis and decreased 5-HTT expression. Both may modify 5-HT neurotransmission and might work in synergy to reduce 5-HT concentrations to critical levels at which the depressed state becomes apparent. Increases in clinical rating scales, the most robust marker of the depressed state, were also associated with methylation at CG8. Neither the methylation findings at the 5-HTT or the circulating tryptophan concentrations were related to a history of ELA.

Methylation at the CG8 site of the *SLC6A4* was significantly associated with multiple cortisol measures in the whole group and in the depressed group only. An inverse relationship between increases in HPA axis activity and reduced 5-HT function, as found in my study, supports the idea of a functional relationship between increased HPA axis output and reduced 5-HT neurotransmission (Heisler, 1998).

My finding in this series of epigenetic studies of 5-HT function are supportive of the “Serotonin Hypothesis” and add to the existing literature by (1) identifying reduced methylation at specific sites in the *SLC6A4* gene in depression, which supports current literature (Devlin et al., 2010) (2) identifying a linear relationship between *SLC6A4* methylation and clinical scores of depression; (3) demonstrating a relationship between this and the frequently replicated finding of reduced circulating tryptophan levels; and (4) finding inverse relationships between methylation of the *SLC6A4* gene and HPA axis output. Thus, decreased levels of DNA methylation at specific sites within the *SLC6A4* promoter are present in depression, are related to both the clinical severity of the episode and to HPA axis function, and may represent a marker for the depressive state.

7.3 Acquired immunity in depression: relationship to ELA

A significant body of research has been conducted in the last 20 years linking increased innate immune system activity to the development of depression. However, significantly less attention has focused on the potential role of adaptive immune system activity, including those of T cells, in the pathophysiology of depression (Miller, 2010). Various mechanisms suggesting how T cell function is altered in depression have been proposed, including tryptophan depletion (Fallarino et al., 2002b) and increases in circulating glucocorticoids (Bauer et al., 2003b). All circulating T cells are characterised by the presence of the T cell receptor (TCR)-CD3 complex (Wegener et al., 1992). The CD3 ϵ polypeptide, a component of

the CD3 complex, has been previously used as a marker for T cell activity. DNA methylation levels at cg06164961, located in the body of the *CD3E* gene, have been used as a CD3+ T cell marker in earlier studies (Cao-Lei et al., 2015). Despite this, to our knowledge, methylation at this site has never been assessed in the context of the depressed state. In light of this, we sought to evaluate differences in DNA methylation at the *CD3E* gene along with the mRNA expression of T cell activation markers, surface glycoproteins and effector molecules in depressed patients to see whether an altered T cell profile was present.

A significant increase in DNA methylation at the CG site in the *CD3E* gene was revealed in depressed patients relative to control subjects. This is a novel finding. These increased methylation levels were strongly correlated with decreases in *CD3E* mRNA expression, indicating that DNA methylation at the site measured was related to subsequent gene expression. Demonstration of the direct functional consequences of altered methylation reinforces the potential importance of this novel and potentially useful epigenetic marker. In an attempt to separate the effects of depression from ELA, analysis was conducted on the depressed group only who were divided on the basis of their exposure to adversity. This analysis revealed that *CD3E* methylation levels were highest in the ELA+ group, suggesting that DNA methylation levels at cg06164961 represent a potential marker for developmental adversity as a risk factor for adult psychopathology. Altered acquired immunity would be consistent with the neurobiological findings in chronic stress, given the profound effects of stress on immunity. Interestingly, DNA methylation of the *CD3E* gene was significantly associated with circulating tryptophan concentrations. To our knowledge, this is also a novel finding that suggests a mechanistic link between altered T cell activity and 5-HT neurotransmission in depression.

Clearly, this has to remain a preliminary finding until replicated. It is worthy of further exploration given the relationship between stress, immunity, inflammation, HPA axis and 5-HT.

7.4 Epigenetic mechanisms and the pathophysiology of depression

While the heritability of depression is estimated at 37% (Sullivan et al., 2000), genome wide association studies (GWAS) have not been able to consistently reproduce gene loci that significantly contribute to the disorder. Epigenetic mechanisms may provide an aetiological link between environmental stressors and the syndrome of depression, that genetics or

environment alone cannot explain (Dalton et al., 2014). Overall, this thesis supports a role for DNA methylation alterations in depression. Differences in DNA methylation were observed at genes that regulate systems hypothesised to be dysregulated in depression. These methylation alterations may be able to link dysregulation reported in discrete physiological systems such as the HPA axis and the serotonin system.

7.5 Limitations

A limitation of the studies presented in this thesis is their cross-sectional design. Findings from cross-sectional studies (i.e. observational studies that analyse data collected from a specific point in time) need to be interpreted with caution as they assume that model parameters are stable over time (Bowen and Wiersema, 1999). This is not the case in depression where there is overwhelming evidence that the depressive phenotype changes over the course of an illness (O’Keane et al., 2012b). All diseases modify body systems over time.

As is often the case with clinical research the studies described throughout the thesis would have benefited from a larger sample size. It is important to note the relatively small sample size as a limitation in these studies as it could result in the statistics being underpowered. Underpowered statistical analyses run the risk of Type II errors (i.e. false negative results) and this should be considered when interpreting these results. Furthermore, as mentioned in Section 1.8 (Aims), given the novelty of DNA methylation analysis in psychiatric research, much of the work presented throughout the thesis 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 throughout the thesis. 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.

7.6 Future directions

A preliminary finding in this thesis was the decreased mineralocorticoid receptor/glucocorticoid receptor (MR/GR) ratio, exclusive to depressed patients with a history of childhood adversity. This is certainly worth a replication/confirmatory study.

In chapter 3, stimulation of PBMCs with dexamethasone at different concentrations did not reveal any differences in cell response. It would be worthwhile to assess the gene expression profile after stimulation with LPS and dexamethasone. The cells ability to suppress a robust LPS-induced inflammatory challenge may uncover differences in GR or FKBP5. In the same vein; while the focus of HPA axis markers in this thesis was on GR and FKBP5, it would be of interest to examine the expression of other GR co-chaperone proteins, including glucocorticoid-induced leucine zipper (*GILZ*) and serum and glucocorticoid-regulated kinase 1 (*SGK1*) to assess the potential role that they may play in altered GR activity in depression.

A suggested future direction would be reassessment of the participants included in these studies at 12 and 24 months after their initial presentation. Examination of participant mood state, biological profile and treatment response would be extremely useful in determining whether differences recorded were state or trait markers for depression. Furthermore, the collection of information pertaining to treatment response and length of episode would be useful in examining the persistence/permanence of biological differences observed.

The reassessment of DNA methylation status would be of particular interest, especially in the mother-baby dyads, as the literature examining longitudinal DNA methylation patterns is extremely limited (if not non-existent), especially in infants. The information collected from follow ups could more accurately reveal new potential drug targets, especially in the new field of so-called epigenetic-based medicine (Egger et al., 2004).

While this thesis sought to examine epigenetic alterations in genes involved in the function of physiological systems implicated in depression i.e. the HPA axis, the 5-HT system and the adaptive immune system, there are other systems implicated in depression that were not assessed at the epigenetic level that would be of interest in future studies. Examples of markers that have been examined at the epigenetic level that could be of interest in this work include brain-derived neurotrophic factor (*BDNF*) (Fuchikami et al., 2011), *IL-11* (Powell et al., 2013) and monoamine oxidase-A (*MAO-A*) (Domschke et al., 2015).

Finally, while the associations between *CD3E* DNA methylation and mRNA expression of T cell surface glycoproteins, it would be of great interest to perform flow cytometry analysis to get a comprehensive T cell profile on participants. This could have the potential to validate our novel finding of *CD3E* DNA methylation alterations in depression and in those exposed to ELA.

7.7 Conclusions

Putting together all of the studies in this thesis, particular trends emerge. The findings suggest that stress and immune systems may change over time in those who have experienced past adversity. These changes may be caused by epigenetic mechanisms. Changes in methylation in the promoter regions of the GR and the CD3E T cell receptor appear to be related to a history of childhood adversity. This is compatible with the functions of these physiological systems in homeostatic processes. On the other hand, epigenetic changes in the serotonin transporter would appear to be related to state depression. The three systems investigated, the immune, HPA and 5-HT systems, are interrelated in complex ways. One striking relationship, with the epigenetic markers used in my studies, was that between increased activity of the HPA axis and reduced 5-HT function.

Chapter 8

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Appendices

Appendix 1: List of Publications

Published papers

Tozzi L., Doolin K., **Farrell C.**, Joseph S., O'Keane V., Frodl T. (2017). Functional magnetic resonance imaging correlates of emotion recognition and voluntary attentional regulation in depression: a generalized psycho-physiological interaction study. *J Affect Disord*, 208: 535-544.

Farrell C. and O'Keane V. (2016). Epigenetics and the glucocorticoid receptor: a review of the implications in depression. *Psychiatry Res*, 242: 349-356.

Manuscripts submitted for publication

Jairaj C., Fitzsimons C.M, McAuliffe F., O'Leary N., Joyce N., McCarthy A., Cassidy E., Healy E., Donnelly J., Tully E., Sheehan J., Burke J., Imcha M., Ni Bhainnean M., Austin J., Doolin K., **Farrell C.**, O'Keane V. (2017). A population survey of prevalence rates of antenatal depression in the Irish obstetric services using the Edinburgh Postnatal Depression Scale (EPDS). Manuscript submitted for publication.

Tozzi, L., **Farrell C.**, Doolin K., Booij L, Pomares F., Chiarella J., O'Keane V., Frodl, T. (2017). Role of demethylation of the FKBP5 gene in major depressive disorder: related imaging and stress hormone changes. Manuscript submitted for publication.

Appendix 2: List of conference presentations

IAWMH World Congress 2017, RDS, Dublin: Symposium presentation – Depression during pregnancy and childhood trauma in mothers: effects on the epigenetic signature of their babies

ECNP Congress 2016, Vienna, Austria: Poster presentation – Association of maternal emotional abuse with decreased serotonin transporter gene (SLC6A4) methylation

Young Neuroscientists Symposium 2016, Trinity College Dublin: Poster presentation – DNA methylation changes at the CD3E gene promoter in depression

CINP Thematic Meeting 2015, Trinity College Dublin: Poster presentation – Investigating glucocorticoid receptor dysregulation in a cohort of first presentation depressives: a

Young Neuroscientists Symposium 2014, Trinity College Dublin: Poster presentation – First onset depression and the hypothalamic-pituitary-adrenal axis: an investigation of state and trait biomarkers

NCRC Clinical PhD Open Day 2014, OLCHC, Dublin: Oral presentation - An investigation on the effects of maternal depression during pregnancy on the baby's hypothalamic-pituitary-adrenal (HPA) axis and development during the first year of life

Appendix 3: REDEEM study information and consent pack

a.

		THE UNIVERSITY OF DUBLIN TRINITY COLLEGE SCHOOL OF MEDICINE Tel.: 01-896-1000	
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INFORMATION SHEET

Title of the Project:

The REDEEM Study
Research in Depression: Endocrinology, Epigenetics and neuroIMaging

LEADERS OF STUDY
The Principal Investigators in The REDEEM Study are Professor Veronica O'Keane, Trinity College Dublin & Tallaght Psychiatry Services
& Professor Thomas Frodl, Trinity College Dublin & University of Regensburg, Germany;

This project is funded by the Health Research Board (HRB), the EU (Marie Curie Fellowship) and the Meath Foundation.

SUMMARY OF STUDY
Depression is a complicated disorder. Your genes, personality type and life stress all contribute to causing depression. Some people are more likely to become depressed because they may have a strong family history of depression: for example, if both parents have suffered from depression. Most people, however, become depressed because of a combination of things. For example, they may be stressed because of things that are happening, and/or they may be a sensitive personality type, and/or they may have had a difficult childhood. Often people who become depressed do not have a person to confide in that they can trust emotionally.

In the REDEEM Study we are interested in finding out how the brain and body changes when someone becomes depressed. We want to see what happens in the **body's** stress and immune systems and in brain structure and function when someone is depressed. We want to then look at any changes in these systems over a period of time (approximately one and a half years).

WHY HAVE I BEEN CHOSEN?
You have been chosen because you have either major depression or are a healthy person who can be a "**control**" for (someone to compare with) a depressed person. We are looking at specific groups of depressed people: those who are experiencing their first episode of depression; those who have had previous episodes of depression and those who were tested by our group five years ago. For those who are experiencing their first episode of depression, we would like you to repeat some of the tests in six months, and some at one year following this.



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DO I HAVE TO TAKE PART?

No. It is completely your decision whether you take part or not. If you want to get involved with the study, this Information Sheet will be explained in detail to you. You will be asked to sign the Consent Form if you decide to participate. You may withdraw from the study at any time you wish and without giving a reason. This will not affect the care you receive from your doctor.

WHAT WILL HAPPEN IF I AGREE TO TAKE PART IN THIS STUDY?

If you decide to participate, we will ask you to do an interview and fill out some questionnaires to look at your symptoms and experiences. This will take about 40 minutes.

During this appointment a doctor or Research Worker will arrange the following tests

- (1) collection of blood, saliva and urine samples
- (2) a brain scan

(1) Collection of blood, saliva and urine samples

We wish to examine the levels of the stress hormone cortisol. The stress hormone cortisol is the main stress hormone in our bodies. Cortisol affects our brain and mind and is the reason why we have difficulty sleeping, concentrating and why we feel anxious when stressed or depressed. If we are very stressed, our levels of cortisol are high. People suffering from depression have high levels of cortisol throughout the day.

We want to measure how cortisol levels increase following waking up and in the evening. Depressed people tend to secrete high cortisol levels when they awake and to have high cortisol levels in the evening. We want to see if the secretion of cortisol goes down following treatment for depression.

What we will ask you to do:

Saliva: we will give you small tubes in which to collect samples of saliva. The instructions are written down and we will go through this with you.

Urine: we will ask you to collect your morning sample of urine in a plastic container that we will supply. This collection will allow us to measure the total amount of cortisol that you secrete over a night.

Our research worker will arrange collection of the samples.

Blood: we will take some blood samples from you: in total about 50ml in five blood tubes. The blood samples will be taken by one of the research workers (Kelly, Chloe, Leonardo) either when you fill out the questionnaires or when you go to Trinity College to have your scan. Your blood samples will be anonymous (will only have a number, not your name).

Most of the blood will be analysed in the Trinity College Institute of Neuroscience Dublin by our research team. We want to measure cortisol and immune substances in the blood.

Some of the samples will be sent to our research partners in McGill University Montreal, Canada as well as a company in Europe to see how your genes are working. At a later stage these results may form part of a collaborative study with researchers in Ireland and abroad.



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(2) Brain scan

A brain scan, or neuroimage, allows us to see the different structures in the brain, giving a picture of the different parts of the brain. The type of scan that we will use is an MRI scan. It is a safe procedure that usually takes about 60 minutes.

We will do the MRI scan in Trinity College, in the Lloyd Building. The MRI machine uses a magnetic field to take pictures of the brain. Before you enter the room where the machine is located, you will have to remove all the metallic things you may be wearing such as bracelets, earrings, watch, or keys. You will be asked to lie on a couch for about 60 minutes while the MRI machine gathers information.

While you are lying on the couch, you will be able to speak with the operator of the scanner at any time. In addition, you will have a button in your hand that you can use to stop the measurement at any time.

You will be asked to look at pictures on a screen while lying on the couch. While viewing these pictures you have to provide answers by pressing a button in your hand. What you need to do will be explained to you before you go to the scanner and you will have the opportunity to see a short example of the test.

WHAT WILL BE THE POSSIBLE BENEFITS OF TAKING PART?

It is unlikely that any of the information from the **"biological"** part of the study will tell you or us much about you or what treatments might be good for you. If there is some unexpected finding in your blood tests or in your scan, such as an abnormal blood vessel in the brain, we will provide whatever care you may need.

No individual genetic or non-genetic result will be available from the study, to you or to anyone else, and this study does not involve screening for genetic diseases. If you wish, we will keep you informed of the progress of the study in general.

WHAT ARE THE POSSIBLE DISADVANTAGES OF TAKING PART?

The risk for the taking of the blood sample is the same as for any standard blood-taking procedure and is very safe. There is the usual minor discomfort/pain with the procedure but it will be in your arm where the puncture is and will be present for a short while only.

The MRI machine uses a strong magnet to make images of the brain. You will be asked to lie on a long narrow couch for about 60 minutes while the machine gathers data. During this time you will be exposed to a magnetic field that you will not feel. You will, however, hear repetitive tapping noises that arise from the scanner around your body. We will provide earplugs and headphones that you will be required to wear so that it is not loud. The space within the large magnet in which you lie is somewhat narrow, although we have taken many steps to relieve the "claustrophobic" feeling.

There are no known significant risks with this procedure since the main magnetic field at the strengths used are felt to be without harm. MRI in pregnant women will be avoided in the



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present study. People who cannot be scanned using MRI include persons who have piercings that cannot be removed, cardiac pacemaker or a certain type of metallic clip in their body (i.e., an aneurysm clip in the brain); persons who have worked with metal or had a piece of metal removed from the eye(s); or persons who have shrapnel, bullets, or buckshot in their body.

There is a risk of heating from the imaging coils, and/or the cables from monitoring devices that record physiologic processes such as heart beats per minute. Please report any heating sensation immediately. You may have the scan stopped at any time if this occurs.

There is a possibility that you will experience a localized twitching sensation due to the magnetic field changes during the scan. This is not unexpected and **shouldn't** be painful. However, you may have the scan stopped at any time if this occurs.

While participating in this study, you should not take part in any other research project without approval from all of the investigators. This is to protect you from possible injury arising from such things as extra blood drawing, effects of research drugs, or similar hazards.

WILL MY TAKING PART BE CONFIDENTIAL?

Yes. All information, which is collected about you, during the course of the research, will be kept strictly confidential. Your name will not be attached to any information about you that leaves the hospital.

You will only be identified by a number in the REDEEM Study. The key to this number code and any personal information will be kept confidentially by Professor Veronica O'Keane in Trinity College Dublin.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results will be published in scientific journals and presented at conferences. We hope to present the results of the study in the general media and public venues as well.

LEGAL ISSUES:

The doctors involved in this study are covered by standard medical malpractice insurance.

PERMISSION:

This study has been approved by the St. James's and Tallaght Hospital Ethics Committee



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FURTHER INFORMATION:

If you would like to obtain further information about the nature of the study you can do so by contacting:

Prof. Veronica O'Keane
Department of Psychiatry
Tallaght Psychiatry Services
Sheaf House
Belgard Square North
Tallaght
Dublin 24
Ireland
Tel: 01 463 5200

Professor Thomas Frodl
Department of Psychiatry
Centre for General Psychiatry II, University of Regensburg
Clinical Director
Universitaetstr. 84
93051 Regensburg
Germany,
Tel.: 0049-941-9412017

Thank you for your help with this project.

b.



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Consent Form

Title of research study:

The REDEEM Study

Research in Depression: Endocrinology, Epigenetics and neuroiMaging

Principal Investigators: Professor Veronica O'Keane, Trinity College Dublin & Tallaght Psychiatry Services &
Professor Thomas Frodl, Trinity College Dublin & University of Regensburg, Germany,

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study. I have received a copy of this agreement

PLEASE TICK YOUR RESPONSE IN THE APPROPRIATE BOX

- I have read and understood the attached Participant Information Leaflet
Yes ☐ No ☐
- I have had the opportunity to ask questions and to discuss the study
Yes ☐ No ☐
- I have received satisfactory answers to all my questions
Yes ☐ No ☐
- I understand that I am free to withdraw from the study at any time without giving a reason and without this affecting my future medical care
Yes ☐ No ☐

PARTICIPANT'S NAME:

PARTICIPANT'S SIGNATURE:

Date:

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits and risks of this research study. I have offered to answer any questions and fully answered any questions asked. I believe that the participant understands my explanation and has freely given informed consent.

Physician's/Scientist's NAME:

Physician's/Scientist's SIGNATURE:

Date:



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The REDEEM Study

Research in Depression: Endocrinology, Epigenetics and neuroiMaging

For participant aged less than 18 years

If you are under 18 years old the signature of your parent or guardian must be obtained

NAME OF PARENT or GUARDIAN: _____

SIGNATURE: _____

DATE: _____

RELATION TO PARTICIPANT: _____

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to; this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Physician's/Scientist's signature: _____

Date: _____

Appendix 4: Questionnaires and rating scales

a.

Contact Information

Name: _____ Date: _____

Address: _____

Phone number: _____

Demographic Information

Date of Birth: _____ / _____ / _____ Age Today: _____

Gender: _____ Male _____ Female

Race/Ethnicity (check as many as apply):

_____ Caucasian/White _____ Asian/Pacific Islander _____ Other (Please specify: _____)
_____ African/Black/Caribbean _____ Hispanic/Latino

Marital Status:

_____ Single _____ Married _____ In partnership _____ Divorced
_____ Separated _____ Widowed

Employment Status:

_____ Employed Full Time _____ Employed Part Time _____ Student
_____ On leave _____ Unemployed

Highest Level of Education Achieved: _____

Health Information

Length of Current Depressive Episode: _____

Current Medications (List All) _____ Date of commencement of antidepressant _____

Height: _____ ft _____ in Weight: _____

Smoker: ____ Yes ____ No Alcohol: ____ Yes ____ No Other Drugs: ____ Yes ____ No
If Yes, units: _____ If Yes, specify: _____

For Recurrent Depression Study Only

Number of previous episodes: _____

Duration depression treated: _____

Duration depression not treated: _____

b.

Hamilton Depression Rating Scale (HAM-D)

Name: _____ Date of Assessment: _____
 Age: _____ Gender: _____ Male _____ Female _____

The HAM-D is designed to rate the severity of depression in patients. Although it contains 21 areas, calculate the patient's score on the first 17 answers.

☐ 1. DEPRESSED MOOD (Gloomy attitude, pessimism about the future, feeling of sadness, tendency to weep) 0=Absent 1=Sadness, etc. 2=Occasional weeping 3=Frequent weeping 4=Extreme symptoms	☐ 6. INSOMNIA – Delayed (Waking in early hours of the morning and unable to fall asleep again) 0=Absent 1=Occasional 2=Frequent
☐ 2. FEELINGS OF GUILT 0=Absent 1=Self-reproach, feels he/she has let people down 2=Ideas of guilt 3=Present illness is a punishment; delusions of guilt 4=Hallucinations of guilt	☐ 7. WORK AND INTERESTS 0=No difficulty 1=Feelings of incapacity, listlessness, indecision and vacillation 2=Loss of interest in hobbies, decreased social activities 3=Productivity decreased 4=Unable to work. Stopped working because of present illness only. (Absence from work after treatment or recovery may rate a lower score)
☐ 3. SUICIDE 0=Absent 1=Feels life is not worth living 2=Wishes they were dead or any thoughts of possible death to self 3=Suicidal ideas or gestures 4=Attempts at suicide	☐ 8. RETARDATION (Slowness of thought, speech, and activity; apathy; stupor) 0=Absent 1=Occasional 2=Frequent
☐ 4. INSOMNIA – Initial (Difficulty falling asleep) 0=Absent 1=Occasional 2=Frequent	☐ 9. AGITATION (Restlessness associated with anxiety.) 0=Absent 1=Occasional 2=Frequent
☐ 5. INSOMNIA – Middle (Complains of being restless and disturbed during the night. Waking during the night.) 0=Absent 1=Occasional 2=Frequent	☐ 10. ANXIETY – PSYCHIC 0=No difficulty 1=Tension and irritability 2=Worrying about minor matters 3=Apprehensive attitude 4=Fears

- ☐ 11. ANXIETY – SOMATIC
(Indigestion, cramps, palpitations, headaches, hyperventilation, sweating, tremor)
0=Absent
1=Mild
2=Moderate
3=Severe
4=Incapacitating

- ☐ 12. SOMATIC SYMPTOMS – GASTROINTESTINAL
0=Absent
1=Loss of appetite but eating without encouragement from others
2=Difficulty eating without urging from others, marked reduction of food intake

- ☐ 13. SOMATIC SYMPTOMS – GENERAL
0=Absent
1=Heaviness in limbs, back or head. Backaches, headache, muscle aches. Loss of energy and fatigability
2=Any clear-cut symptom rates 2

- ☐ 14. GENITAL SYMPTOMS (Loss of libido, impaired sexual performance, menstrual disturbances)
0=Absent
1=Mild
2=Severe

- ☐ 15. HYPOCHONDRIASIS
0=Not present
1=Self-absorption
2=Preoccupation with health
3=Querulous attitude
4=Hypochondriacal delusion

- ☐ 16. WEIGHT LOSS
0=No weight loss
1=Slight
2=Obvious

- ☐ 17. INSIGHT
(Insight must be interpreted in terms of patient's understanding and background.)
0=No loss
1=Partial or doubtful loss
2=Loss of insight

TOTAL ITEMS 1 TO 17: _____

0 – 7 = Normal
8 – 13 = Mild Depression
14 – 18 = Moderate Depression
19 – 22 = Severe Depression
≥ 23 = Very Severe Depression

- ☐ 18. DIURNAL VARIATION
(Symptoms worse in the morning or evening. Note which it is.)

0=No Variation
1=Mild variation; AM () PM ()
2=Severe variation; AM () PM ()

- ☐ 19. DEPERSONALIZATION AND DEREALIZATION
(Feelings of unreality, nihilistic ideas)
0=Absent
1=Mild
2=Moderate
3=Severe
4=Incapacitating

- ☐ 20. PARANOID SYMPTOMS
(Not with a depressive quality)
0=None
1=Suspicious
2=Ideas of reference
3=Delusions of reference and persecution
4=Hallucinations, persecutory

- ☐ 21. OBSESSIVE SYMPTOMS
(Obsessive thoughts and compulsions against which the patient struggles)
0=Absent
1=Mild
2=Severe

ATYPICAL FEATURES:

Check if any of the following are true:

- ☐ A. Mood Reactivity (mood brightens in response to actual or potential positive events)
- ☐ B. At least two of the following:
Significant weight gain or increase of appetite
Hypersomnia (Sleeping too much)
Leadens paralysis (Heavy feeling of arms and legs)
Longstanding pattern of interpersonal rejection sensitivity that results in significant social or occupational impairment.

If both A and B are true, depression is atypical.

C.

NAME: _____ DATE: _____

A. MAJOR DEPRESSIVE EPISODE

(→ MEANS : GO TO THE DIAGNOSTIC BOXES, CIRCLE NO IN ALL DIAGNOSTIC BOXES, AND MOVE TO THE NEXT MODULE)

A1	Have you been consistently depressed or down, most of the day, nearly every day, for the past two weeks?	NO	YES
A2	In the past two weeks, have you been much less interested in most things or much less able to enjoy the things you used to enjoy most of the time?	NO	YES
IS A1 OR A2 CODED YES?		→ NO	YES

A3 **Over the past two weeks, when you felt depressed or uninterested:**

a	Was your appetite decreased or increased nearly every day? Did your weight decrease or increase without trying intentionally (i.e., by $\pm 5\%$ of body weight or ± 8 lbs. or ± 3.5 kgs., for a 160 lb./70 kg. person in a month)? IF YES TO EITHER, CODE YES.	NO	YES *
b	Did you have trouble sleeping nearly every night (difficulty falling asleep, waking up in the middle of the night, early morning wakening or sleeping excessively)?	NO	YES
c	Did you talk or move more slowly than normal or were you fidgety, restless or having trouble sitting still almost every day?	NO	YES *
d	Did you feel tired or without energy almost every day?	NO	YES
e	Did you feel worthless or guilty almost every day?	NO	YES
f	Did you have difficulty concentrating or making decisions almost every day?	NO	YES
g	Did you repeatedly consider hurting yourself, feel suicidal, or wish that you were dead?	NO	YES

ARE 5 OR MORE ANSWERS (A1-A3) CODED YES?

IF PATIENT HAS CURRENT MAJOR DEPRESSIVE EPISODE CONTINUE TO A4,
OTHERWISE MOVE TO MODULE B:

A4 a During your lifetime, did you have other episodes of two weeks or more when you felt depressed or uninterested in most things, and had most of the problems we just talked about?

b In between 2 episodes of depression, did you ever have an interval of at least 2 months, without any depression and any loss of interest?

NO YES *

**MAJOR DEPRESSIVE
EPISODE, CURRENT**

A4 a During your lifetime, did you have other episodes of two weeks or more when you felt depressed or uninterested in most things, and had most of the problems we just talked about?

b In between 2 episodes of depression, did you ever have an interval of at least 2 months, without any depression and any loss of interest?

NO YES

**MAJOR DEPRESSIVE
EPISODE, RECURRENT**

* If patient has Major Depressive Episode, Current, use this information in coding the corresponding questions on page 5 (A6d, A6e).

MAJOR DEPRESSIVE EPISODE WITH MELANCHOLIC FEATURES (optional)

(→ MEANS : GO TO THE DIAGNOSTIC BOX, CIRCLE NO, AND MOVE TO THE NEXT MODULE)

IF THE PATIENT CODES POSITIVE FOR A CURRENT MAJOR DEPRESSIVE EPISODE (A3 = YES), EXPLORE THE FOLLOWING:

A5 a	During the most severe period of the current depressive episode, did you lose almost completely your ability to enjoy nearly everything?	NO	YES
b	During the most severe period of the current depressive episode, did you lose your ability to respond to things that previously gave you pleasure, or cheered you up? IF NO: When something good happens does it fail to make you feel better, even temporarily?	NO	YES
IS EITHER A5a OR A5b CODED YES?		→ NO	YES

A6 Over the past two week period, when you felt depressed and uninterested:

- | | | | |
|---|--|----|-----|
| a | Did you feel depressed in a way that is different from the kind of feeling you experience when someone close to you dies? | NO | YES |
| b | Did you feel regularly worse in the morning, almost every day? | NO | YES |
| c | Did you wake up at least 2 hours before the usual time of awakening and have difficulty getting back to sleep, almost every day? | NO | YES |
| d | IS A3c CODED YES (PSYCHOMOTOR RETARDATION OR AGITATION)? | NO | YES |
| e | IS A3a CODED YES FOR ANOREXIA OR WEIGHT LOSS? | NO | YES |
| f | Did you feel excessive guilt or guilt out of proportion to the reality of the situation? | NO | YES |

ARE 3 OR MORE A6 ANSWERS CODED YES?

NO	YES
Major Depressive Episode with Melancholic Features Current	

B. DYSTHYMIA

(→ MEANS : GO TO THE DIAGNOSTIC BOX, CIRCLE NO, AND MOVE TO THE NEXT MODULE)

IF PATIENT'S SYMPTOMS CURRENTLY MEET CRITERIA FOR MAJOR DEPRESSIVE EPISODE, DO NOT EXPLORE THIS MODULE.

B1	Have you felt sad, low or depressed most of the time for the last two years?	→ NO	YES
B2	Was this period interrupted by your feeling OK for two months or more?	NO	→ YES
B3	During this period of feeling depressed most of the time:		
a	Did your appetite change significantly?	NO	YES
b	Did you have trouble sleeping or sleep excessively?	NO	YES
c	Did you feel tired or without energy?	NO	YES
d	Did you lose your self-confidence?	NO	YES
e	Did you have trouble concentrating or making decisions?	NO	YES
f	Did you feel hopeless?	NO	YES
	ARE 2 OR MORE B3 ANSWERS CODED YES?	→ NO	YES

B4 Did the symptoms of depression cause you significant distress or impair your ability to function at work, socially, or in some other important way?

NO	YES
DYSTHYMIA CURRENT	

C. SUICIDALITY

In the past month did you:

		NO	YES	Points
C1	Suffer any accident? IF NO TO C1, SKIP TO C2; IF YES, ASK C1a,;			0
C1a	Plan or intend to hurt yourself in that accident either passively or actively? IF NO TO C1a, SKIP TO C2; IF YES, ASK C1b,;	NO	YES	0
C1b	Did you intend to die as a result of this accident?	NO	YES	0
C2	Think that you would be better off dead or wish you were dead?	NO	YES	1
C3	Want to harm yourself or to hurt or to injure yourself?	NO	YES	2
C4	Think about suicide?	NO	YES	6

IF YES, ASK ABOUT THE INTENSITY AND FREQUENCY OF THE SUICIDAL IDEATION:

Frequency

Intensity

Occasionally

Mild

Often

Moderate

Very often

Severe

Can you control these impulses
and state that you will not act
on them while in this program?

Only score 8 points if response is NO. NO YES 8

C5	Have a suicide plan?	NO	YES	8
C6	Take any active steps to prepare to injure yourself or to prepare for a suicide attempt in which you expected or intended to die?	NO	YES	9
C7	Deliberately injure yourself without intending to kill yourself?	NO	YES	4
C8	Attempt suicide? Hoped to be rescued / survive Expected / intended to die	NO	YES	10

In your lifetime:

C9	Did you ever make a suicide attempt?	NO	YES	4
----	--------------------------------------	----	-----	---

IS AT LEAST 1 OF THE ABOVE (EXCEPT C1) CODED YES?

IF YES, ADD THE TOTAL NUMBER OF POINTS FOR THE ANSWERS (C1-C9)
CHECKED 'YES' AND SPECIFY THE LEVEL OF SUICIDE RISK AS
INDICATED IN THE DIAGNOSTIC BOX:

MAKE ANY ADDITIONAL COMMENTS ABOUT YOUR ASSESSMENT
OF THIS PATIENT'S CURRENT AND NEAR FUTURE SUICIDE RISK IN
THE SPACE BELOW:

NO YES

SUICIDE RISK CURRENT

1-8 points Low
9-16 points Moderate
≥ 17 points High

d.

Center for Epidemiological Studies Depression Scale (CES-D), NIMH

Name: _____ Date: _____

Age: _____ Gender: _____ Male _____ Female

	During the Past Week			
	Rarely or none of the time (less than 1 day)	Some or a little of the time (1-2 days)	Occasionally or a moderate amount of time (3-4 days)	Most or all of the time (5-7 days)
1. I was bothered by things that usually don't bother me.				
2. I did not feel like eating; my appetite was poor.				
3. I felt that I could not shake off the blues even with help from my family or friends.				
4. I felt I was just as good as other people.				
5. I had trouble keeping my mind on what I was doing.				
6. I felt depressed.				
7. I felt that everything I did was an effort.				
8. I felt hopeful about the future.				
9. I thought my life had been a failure.				
10. I felt fearful.				
11. My sleep was restless.				
12. I was happy.				
13. I talked less than usual.				
14. I felt lonely.				
15. People were unfriendly.				
16. I enjoyed my life.				
17. I had crying spells.				
18. I felt sad.				
19. I felt that people dislike me.				
20. I could not get "going."				

SCORING: zero for answers in the first column, 1 for answers in the second column, 2 for answers in the third column, 3 for answers in the fourth column. The scoring of positive items is reversed. Possible range of scores is zero to 60, with the higher scores indicating the presence of more symptomology.

e.

Pittsburgh Sleep Quality Index (PSQI)

Name: _____

Age: _____ Gender: _____ Male _____ Female

Date: _____

The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all questions.

During the past month,

- When have you usually gone to bed? _____
- How long (in minutes) has it taken you to fall asleep each night? _____
- When have you usually gotten up in the morning? _____
- How many hours of actual sleep do you get at night? (This may be different than the number of hours you spend in bed) _____

5. During the past month, how often have you had trouble sleeping because you...	Not during the past month (0)	Less than once a week (1)	Once or twice a week (2)	Three or more times a week (3)
a. Cannot get to sleep within 30 minutes				
b. Wake up in the middle of the night or early morning				
c. Have to get up to use the bathroom				
d. Cannot breathe comfortably				
e. Cough or snore loudly				
f. Feel too cold				
g. Feel too hot				
h. Have bad dreams				
i. Have pain				
j. Other reason(s), please describe, including how often you have had trouble sleeping because of this reason:				
6. During the past month, how often have you taken medicine (prescribed or "over the counter" to help you sleep?				
7. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?				
8. During the past month, how much of a problem has it been for you to keep up enthusiasm to get things done?				
	Very good (0)	Fairly good (1)	Fairly bad (2)	Very bad (3)
9. During the past month, how would you rate your sleep quality overall?				

Scoring the Pittsburgh Sleep Quality Index

Component 1 #9 Score.....

Component 2 #2 Score (<15min=0; 16-30min=1; 31-60min=2; >60min=3) + #5a Score (if sum is equal to 0=0; 1-2=1; 3-4=2; 5-6=3).....

Component 3 #4 Score (>7=0; 6-7=1; 5-6=2; <5=3).....

Component 4 (total # of hours asleep)/(total # of hours in bed) x 100

>85%=0; 75%-84%=1; 65%-74%=2; <65%=3.....

Component 5 Sum of scores #5b to #5j (0=0; 1-9=1; 10-18=2; 19-27=3).....

Component 6 #6 Score.....

Component 7 #7 Score + #8 Score (0=0; 1-2=1; 3-4=2; 5-6=3)

Add the seven component scores together for the **Global PSQI Score**

f.

CTQ

Name: _____

Age: _____

Gender: _____ Male _____ Female

When I was growing up...	Never True	Rarely True	Sometimes True	Often True	Very Often True
1. I didn't have enough to eat.					
2. I knew that there was someone to take care of me and protect me					
3. People in my family called me things like "stupid," "lazy," or "ugly."					
4. My parents were too drunk or high to take care of the family.					
5. There was someone in my family who helped me feel that I was important or special.					
6. I had to wear dirty clothes.					
7. I felt loved.					
8. I thought that my parents wished I had never been born.					
9. I got hit so hard by someone in my family that I had to see a doctor or go to the hospital.					
10. There was nothing I wanted to change about my family.					
11. People in my family hit me so hard that it left me with bruises or marks.					
12. I was punished with a belt, a board, a cord, or some other hard object.					
13. People in my family looked out for each other.					
14. People in my family said hurtful or insulting things to me.					
15. I believe that I was physically abused.					
16. I had the perfect childhood.					
17. I got hit or beaten so badly that it was noticed by someone like a teacher, neighbor, or doctor.					
18. I felt that someone in my family hated me.					
19. People in my family felt close to each other.					
20. Someone tried to touch me in a sexual way, or tried to make me touch them.					
21. Someone threatened to hurt me or tell lies about me unless I did something sexual with them.					
22. I had the best family in the world.					
23. Someone tried to make me do sexual things or watch sexual things.					
24. Someone molested me.					
25. I believe that I was emotionally abused.					
26. There was someone to take me to the doctor if I needed it.					
27. I believe that I was sexually abused.					
28. My family was a source of strength and support.					

g.

PSS

Name: _____

Age: _____

The questions in this scale ask you about your feelings and thoughts during the last month. In each case, you will be asked to indicate *how often* you felt or thought a certain way. Although some of the questions are similar, there are differences between them and you should treat each one as a separate question. The best approach is to answer each question fairly quickly. That is, don't try to count up the number of times you felt a particular way, but rather indicate the alternative that seems like a reasonable estimate.

In the last month...	0 Never	1 Almost Never	2 Some- times	3 Fairly Often	4 Very Often
1. How often have you been upset because of something that happened unexpectedly?					
2. How often have you felt that you were unable to control the important things in your life?					
3. How often have you felt nervous and "stressed"?					
4. How often have you dealt successfully with irritating life hassles?					
5. How often have you felt that you were effectively coping with important changes that were occurring in your life?					
6. How often have you felt confident about your ability to handle your personal problems?					
7. How often have you felt that things were going your way?					
8. How often have you found that you could not cope with all the things that you had to do?					
9. How often have you been able to control irritations in your life?					
10. How often have you felt that you were on top of things?					
11. How often have you been angered because of things that happened that were outside of your control?					
12. How often have you found yourself thinking about things that you have to accomplish?					
13. How often have you been able to control the way you spend your time?					
14. How often have you felt difficulties were piling up so high that you could not overcome them?					