

An investigation of kynurenine pathway metabolism, the hypothalamic-pituitary-adrenal axis, and inflammatory markers in Major Depressive Disorder

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I. Declaration

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II. Summary

Major Depressive Disorder (MDD) is a debilitating mental health problem with a lifetime prevalence rate of more than 16%. Despite its prevalence, a biological mechanism accounting for all aspects of the disorder has yet to be established. While the monoamine hypothesis of depression was the most widely supported biological theory of the disorder's causation for several decades, there is a lack of evidence that this is the primary dysfunction in MDD. Moreover, the most commonly prescribed type of antidepressant medication, the selective serotonin reuptake inhibitor, enhances serotonergic transmission yet about half of MDD patients who use this treatment do not experience remission. For this reason, it is essential to investigate other biological systems that have been hypothesised to play a role in depression. Shunting of tryptophan metabolism toward kynurenine pathway (KP) and away from 5-HT synthesis has been suggested as a possible aetiological factor in depression, though this theory has yet to be confirmed. Furthermore, the two systems that may promote activation of the KP, the hypothalamic-pituitary adrenal (HPA) axis and activation of the inflammatory response system are known to play a role in depression pathophysiology. This thesis aims to establish the dysfunction of each of these pathways and their relationship to one other in a cohort of depressed patients.

The HPA axis is the endocrine system responsible for stress responses which has frequently been shown to be hyperactive in depressed patients. Significant elevation of cortisol, a glucocorticoid produced by the HPA axis in response to stress, was detected at waking in the depressed cohort. The depressed group also exhibited a higher cortisol/cortisone ratio at waking. Cortisone is an inert glucocorticoid, thus increased cortisol/cortisone ratio indicates greater HPA activation. This ratio at waking was positively correlated to transcriptional expression of the gene encoding for the 11β -HSD1 enzyme which converts cortisone to cortisol, providing further supportive evidence for hypercortisolemia. These findings provide evidence in support of HPA axis dysregulation in depression.

Activation of the inflammatory response system was assessed in depressed patients by measurement of circulating concentrations and transcriptional expression of inflammatory cytokines. In addition, a volumetric analysis of brain structures of the limbic system was undertaken to ascertain if there were any associations with inflammatory markers. Gene expression of IL-1 β in whole blood was found to be elevated in patients compared to healthy controls however this was the only indicator of potential inflammation in the depression cohort. The hippocampal CA3 and CA4 subfields were found to have reduced volume in

depressed patients. Furthermore, significant associations between both inflammatory markers and hippocampal subfield volumes with markers of HPA axis activity were revealed by correlational analyses, suggesting the systems are potentially linked.

Activation of the HPA axis and the immune system are known to induce tryptophan metabolism into kynurenine via tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) respectively. Tryptophan, the precursor to 5-HT and kynurenine, was found to be depleted in depressed patients in this study, and relative ratios of downstream kynurenine metabolites were determined to be imbalanced in depressed patients. Specifically, circulating kynurenic acid concentration was decreased while the ratio of circulating quinolinic acid to kynurenine concentrations was increased. These findings assert that the imbalance of kynurenine metabolic products, which result in oxidative stress and potential excitotoxicity in the brain, are apparent in depressed patients. Moreover, several measures of KP activity were found to be correlated to measures of HPA axis and immune activation, suggesting that KP dysregulation exists in conjunction with alterations of these systems. Ultimately, these findings indicate that underlying biological causes and consequences of depression may be accounted for by a network that encompasses all of these pathways.

Due to the heterogeneous nature of MDD, an additional goal was to evaluate dysfunction of the HPA axis, immune system, and KP between depression subtypes and varying symptom profiles. The effect of early life adversity, atypical depressive subtype, recurrence of depressive episodes, and medication use on expression of biological measures were assessed to gain greater insight into the variation of dysfunction that exists within the disorder. Increased inflammation was witnessed in patients who had experienced early life adversity and in those with greater anxiety symptoms. Meanwhile, depressed patients with increased suicidality expressed greater KP induction which was associated with HPA axis indicators rather than markers of the inflammation response system. While these findings are preliminary, they emphasise the importance of designing future studies with the intention of dissecting biological differences between depressive symptom profiles. Taken together, this study demonstrates a dysfunctional biological network in depression involving HPA axis, immune system, and KP alterations, and emphasises the importance of assessing the interplay of these systems between depressive subtypes.

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VII. List of abbreviations

3-HAA	3-Hydroxyanthranilic acid
3-HK	3-Hydroxy kynurenine
5-HT	Serotonin
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of Variance
AUC	Area Under Curve
CA	cornus ammonis
CAR	Cortisol Awakening Response
CES-D	Center for Epidemiological Studies – Depression
CHAID	CHi-squared Automatic Interaction Detection
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CTQ	Childhood Trauma Questionnaire
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTI	Diffusion Tensor Imaging
EDTA	Ethylenediaminetetraacetic acid
ELA	Early life adversity
ELISA	Enzyme Linked Immunosorbent Assay
GLM	General linear model
GM	Grey matter
HAM-D	Hamilton Rating Scale of Depression
HPA	Hypothalamic-Pituitary-Adrenal
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
HSE	Health and Safety Executive
IDO	Indoleamine 2,3-dioxygenase
IFN- γ	Interferon gamma
IL1- β	Interleukin 1-beta

IL-6	Interleukin-6
ISCED	International standard classification of education
KATs	Kynurenine aminotransferases
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
KynA	Kynurenic acid
LC-MS	Liquid chromatography - mass Spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MAOI	Monoamine oxidase inhibitor
MDD	Major depressive disorder
MINI	Mini-International Neuropsychiatric Interview
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic acid
PSQI	Pittsburgh Sleep Quality Index
qPCR	quantitative polymerase chain reaction
RT	Reactivity
SD	Standard deviation
SEM	Standard error of the mean
SLE	Stressful life event
SPM	Statistical Parametric Mapping
SPSS	Statistical Package for the Social Sciences
SSRI	Selective serotonin reuptake inhibitor
SNRI	Serotonin norepinephrine reuptake inhibitor
TCA	Tricyclic antidepressant
TDO	Tryptophan 2,3-dioxygenase
TIV	Total intracranial volume
TNF- α	Tumour necrosis factor alpha
WHO	World Health Organisation
WM	White matter

Chapter 1

Introduction

1.1 Major Depressive Disorder

1.1.1 Epidemiology

Major depressive disorder (MDD) is one of the most common psychiatric conditions in adolescents and adults, and is a significant public health issue. MDD is predicted to be the leading cause of disability by 2030 according to the World Health Organisation (2008). Large epidemiological studies of MDD have been conducted and have determined a lifetime prevalence of 19.2%, and a yearly prevalence of 8.3% (Kessler et al., 2010), though the prospective lifetime prevalence is predicted to increase to approximately 41% (Moffitt et al., 2010). Occurrence of depression increases dramatically between childhood and adolescence, though the mean age of onset falls between 24.8 and 34.8 years of age according to a survey of 10 countries (Costello et al., 2006; Weissman et al., 1996). However, an episode of depression in adolescence significantly increases the risk of depression in adulthood (Costello et al., 2003). Women experience depression at a higher rate than men across all age groups. For example, women of childbearing age have a lifetime prevalence of MDD of 25.2% compared to 16.8% in men of the same age (Kessler et al., 2010).

Several sociodemographic factors are associated with depression diagnosis. Poorer academic achievement, unemployment, and poverty are each associated with higher rates of depression (Kessler et al., 2003; Inoue et al., 2010; Riglin et al., 2014), though prevalence of depression is higher in high-income countries (Weissman et al., 1996).

There are many personal and public health consequences associated with MDD. Depression is known to be a recurrent disorder over the course of a patient's lifetime, with a rate of relapse of at least 50% (American Psychiatric Association, 2000). On average, patients who have experienced depression will have between five and nine episodes in their lifetime (Kessler et al., 1997). Sadly, depression is associated with increased mortality and is strongly associated with increased suicide attempts, which is the most serious outcome of the disorder (Harris and Barraclough, 1997; Kessler, 2012). Finally, there is a massive economic burden associated with MDD, as it is estimated to cost \$26.1 billion in health services and care in the United States each year, and contributes to economic losses as a result of unemployment caused by depression (Greenberg et al., 2003).

1.1.2 Diagnosis

Major depressive disorder is characterised by the core symptoms of low mood and anhedonia, along with a combination of other biological and cognitive symptoms that persist for at least 2 weeks. Major depressive disorder, or a depressive episode, is diagnosed by a psychiatrist following a clinical interview. There are two systems of disease classification that give criteria for MDD. The first is the Diagnostic and Statistical Manual (DSM-5) (American Psychiatric Association, 2013), and the other is the International Classification of Disease (ICD-10) (World Health Organisation, 1992), though each contain nearly identical requirements for diagnosis. The DSM requires that patients meet 5 of the following 9 criteria to warrant a diagnosis of MDD:

- Depressed mood or irritable most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful).
- Decreased interest or pleasure in most activities, most of each day
- Significant weight change (5%) or change in appetite
- Change in sleep: Insomnia or hypersomnia
- Change in activity: Psychomotor agitation or retardation
- Fatigue or loss of energy
- Guilt/worthlessness: Feelings of worthlessness or excessive or inappropriate guilt
- Concentration: diminished ability to think or concentrate, or more indecisiveness
- Suicidality: Thoughts of death or suicide, or has suicide plan

If a person is experiencing at least 5 of the above symptoms nearly every day, has had a change from their normal baseline mood, and is experiencing impaired function regarding social, education, or occupation, they would qualify for a diagnosis of MDD according to the DSM-5.

1.1.3 Depressive subtypes

The DSM-5 contains diagnostic criteria for several depression subtypes. Atypical and melancholic depression are two subtypes that will be detailed in the following section and explored in this thesis. The DSM-5 describes other affective disorders including dysthymic depression, bipolar depression, psychotic depression, post-traumatic stress disorder, and post-partum depression which are not investigated in this thesis.

1.1.3.1 Melancholic subtype

Depression with melancholic features, sometimes called “melancholia”, is the most widely recognised subtype of depression, and its prevalence is between 20-30% (Rush et al., 2009; Bobo et al., 2011). Melancholic depression is described in the DSM-5 (American Psychiatric Association, 2013). The melancholic subtype requires a patient to have either anhedonia (the inability to find pleasure in positive things) or lack of mood reactivity. Additionally, the patient is required to exhibit at least three of the following symptoms:

- Depression that is subjectively different from grief or loss
- Severe weight loss or loss of appetite
- Psychomotor agitation or retardation
- Early morning waking
- Excessive guilt
- Worse mood in the morning

Melancholia is a more homogeneous classification than a general diagnosis of MDD, describing a more specific range of symptoms and it has been argued that it should be identified in the DSM as a distinct affective illness with its own diagnosis (Parker et al., 2010).

1.1.3.2 Atypical subtype

The atypical subtype of depression is characterized in the DSM by mood reactivity, or brightening of mood in response to actual or potential positive events, plus at least two other criteria. The other criteria include

- Significant weight gains or increased appetite
- Hypersomnia
- Lead paralysis or the sensation of heavy limbs
- Long-standing pattern of interpersonal rejection sensitivity that results in significant social or occupational impairment

According to the DSM, a person who has met the criteria for melancholic features of MDD cannot be classified as the atypical subtype. The prevalence of the atypical subtype among depressed patients is between 15-50% (Thase, 2007), and is up to four times more prevalent in women than in men (Parker et al., 2007). Because this classification involves aspects of a

personality disorder, its identification as a subtype of depression has been disputed (Horwath et al., 2002; Angst et al., 2002).

While diagnosis of melancholic and atypical subtypes cannot exist simultaneously according to the DSM, it has been noted that patients may fluctuate between episodes of atypical and melancholic depression and that the more chronic the illness, the more likely a patient is to have experienced both subtypes (O'Keane et al., 2012).

Research findings have suggested that patients with atypical depression and melancholic depression differ in their response to treatment with medication. Atypical depressives are more responsive to treatment with monoamine oxidase inhibitors (MAOIs), while selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (SNRIs) are more effective in melancholic depression (Brown, 2007; Pae et al., 2009).

1.1.4 Comorbidities

Major depressive disorder often occurs concurrently with other physical and psychiatric illnesses. Depression occurs more frequently in people with physical illnesses such as diabetes, cardiovascular disease, cancer and irritable bowel syndrome than in the general population, at a rate as high as 40% (Goodnick and Hernandez, 2000; Clarke and Curry, 2009). Depression also frequently co-occurs with anxiety and sleep disturbances (Costello et al., 2003; Murphy and Peterson, 2015).

The most commonly co-occurring psychiatric conditions are depression and anxiety. In fact, comorbidity of anxiety disorders with major depression is the norm rather than the exception (Costello et al., 2003; Kessler et al., 2005). Both anxiety and depression are highly recurrent (Carballo et al., 2011; Lahey et al., 2014), and assessment for depression diagnosis contains items related to anxiety (American Psychiatric Association, 2013). Moreover, antidepressants are approved for treating specific anxiety disorders (Stahl, 2010), lending evidence that there are overlapping biological mechanisms associated with the two conditions.

Sleep disturbance is one of the most common symptoms of depression. Insomnia is reported by up to 88% of patients with depression, with terminal insomnia (early morning wakening) being particularly common (Yates et al., 2004; Peterson et al., 2008). Meanwhile, hypersomnia is experienced by up to 27% of patients (Yates et al., 2004). Depressed patients often report non-restorative sleep and fatigue, though it has been suggested that people

with MDD might have poor insight into their sleep quality (Yates et al., 2004; Matousek et al., 2004). It appears that there is a bidirectional relationship between sleep disturbance and severity of depression symptoms as increased mood disturbance is typically accompanied by more severe insomnia and vice versa (Peterson et al., 2008). Likewise, improving sleep quality has been shown to improve mood in depressed patients (McCall et al., 2010). The high rate of comorbidity associated with depression contributes to the heterogeneity of the disorder among patients, and hinders the ability to assess biological mechanisms of depression in isolation.

1.1.5 Risk factors for depression

It has been determined that many factors contribute to the development of depression. Most significantly, women experience depression to a much greater extent with lifetime occurrence rates possibly twice as high as in men (Kuehner, 2016). Beyond gender differences, family history of depression plays a major role. Children of MDD patients are at least three times more likely to experience depression than those without a family history, especially if the affected family member is the mother (Sullivan et al., 2000; Weissman et al., 2006). However, genome-wide association studies (GWAS) have not yet consistently identified specific genes associated with MDD (Lewis et al., 2010; Muglia et al., 2010). Rather, it has been suggested that individual gene contributions to depression are likely to have only minor effects and it is more likely that the presence of multiple single nucleotide polymorphisms (SNPs) with small effect sizes make cumulative contribution to MDD occurrence (Lewis et al., 2010; Zuk et al., 2012).

Environmental factors such as life stressors and childhood trauma have also been identified as risk factors of MDD. For example, stressful life events (SLEs) such as divorce, conflicts in the workplace, or a death in the family increase the likelihood of MDD occurrence (Paykel, 2001; Farmer and McGuffin, 2003; Hosang et al., 2012; Pemberton and Fuller Tyszkiewicz; 2016). Depression occurs in up to 20% of individuals that experience severe SLEs, and the number of SLEs experienced by the individual increases the likelihood of onset and recurrence of MDD (Bifulco, 1994; Kessler, 1997; Kendler et al., 1998). Additionally, depression is more common among people with lower income or greater financial strain (Zimmerman and Katon, 2005). Physical abuse and neglect, emotional abuse and neglect, and sexual abuse that occurs in childhood is known to be associated with a higher incidence of depression in adulthood, increased suicidal ideation, and altered stress response systems

(Kaufman and Charney, 2001; Meaney, 2001; Fisher et al., 2013). It is believed that early life adversity such as childhood abuse and neglect could potentially sensitize individuals to being more susceptible to future stresses or depressive episodes (Monroe and Harkness, 2001). Social support also plays a large role in the onset of depression. A lack of social support has been shown to be associated with a higher rate of onset and relapse of depression (Paykel, 1994).

Since both inherited and environmental factors have been shown to play a role in the onset of depression, gene-environment interactions may be essential to understanding the pathophysiology of depression (Lesch, 2004; Farrell and O'Keane, 2016). Since GWAS have failed to identify specific genes associated with MDD yet the disorder has been shown to be highly heritable, the explanation could be the existence of alterations in epigenetics as a result of environmental stress (Keers and Uher, 2011; Klengel and Binder, 2013). Epigenetic modification of the genome can occur via environmental changes that induce methylation and alter gene expression without changing the actual sequence of DNA. Environmental and genetic risk factors for depression may interact through this mechanism and play a role in onset of depression.

1.2 Approved pharmacological treatments for Major Depression

A wide range of pharmacological treatments for MDD exist, yet each has pitfalls including lack of efficacy, adverse side effects, and dangerous drug interactions. Only about 40% of patients with MDD achieve remission within two adequate trials of antidepressant treatment (Sinyor et al., 2010). The available antidepressant drugs do not address all aspects of depression symptoms, and none work by mechanisms that could explain the entire pathophysiology of the disorder. The discovery of these drugs and their ability to alleviate depression symptoms lead to the development of the initial hypotheses surrounding the biological dysfunction of depression, though a biological theory of depression based on these drugs is inadequate. This section will detail the mechanisms of existing antidepressant drugs and their use in depressed populations.

1.2.1 Selective serotonin reuptake inhibitors

Serotonin is a monoamine neurotransmitter primarily found in the gastrointestinal (GI) tract and the central nervous system (CNS) of humans. In the CNS of humans, serotonin is believed to be responsible for mood, appetite, and sleep (Mohammad-Zadeh et al., 2008). Selective serotonin re-uptake inhibitors (SSRIs) are currently the first line treatment for clinical depression and they are the most widely prescribed antidepressant (National Health Service, UK, 2016). The exact mechanism of action of SSRIs is unknown, but they are believed to increase the availability of extracellular serotonin by preventing its reabsorption into presynaptic neurons [Figure 1.1]. The SSRI binds to the serotonin transporter (SERT) preventing reuptake of serotonin. This is believed to increase concentrations of serotonin in the synaptic cleft, ultimately promoting binding to the postsynaptic neuron and enhancing serotonergic neurotransmission. While the onset of action of SSRIs typically occurs after 2 weeks, full response usually takes 6 weeks, during which time patients may be left vulnerable to suicidal ideation. The delay in peak efficacy of SSRIs is believed to be because of adaptive effects of sustained use of SSRIs. With continuous use of SSRIs, it is believed that the increased concentration of serotonin in the synaptic cleft results in downregulation of post-synaptic serotonin receptors (5HT_{1A} receptors). After the number of 5HT_{1A} receptors has been reduced, the neuron is disinhibited to release more serotonin into the synaptic cleft (Elbe, 2010).

While SSRIs are widely prescribed in treatment of MDD, as well as for treatment of some comorbidities of depression including anxiety or obsessive compulsive disorder, only 40% of depressed patients achieve remission with two adequate trials (Thase et al., 2001). The most commonly prescribed SSRIs are citalopram, escitalopram, paroxetine, fluoxetine, fluvoxamine, and sertraline. Each has a risk of inducing adverse side effects including nausea, dry mouth, sexual dysfunction, insomnia, diarrhoea, dizziness, and restlessness (Ferguson, 2001). Despite the SSRI being the most prominently used treatment for MDD, its low efficacy and numerous side effects are problematic.

1.2.2 Serotonin-norepinephrine reuptake inhibitors

Another class of antidepressant is the serotonin-norepinephrine reuptake inhibitor (SNRI). They prevent reabsorption of both serotonin and norepinephrine into the presynaptic neuron, causing dual inhibition and promoting the availability of both neurotransmitters in the synaptic cleft. Because of its action on multiple neurotransmitters, use of SNRIs may relieve a wider range of symptoms than an SSRI, hence its use in the treatment of not only MDD but anxiety disorders, obsessive-compulsive disorder, attention deficit disorder, and neuropathic pain (Weilburg, 2004). They have been shown to be effective for treating severe forms of MDD (Thase, 2008). However, SNRIs are associated with the same and more adverse side effects than SSRIs, such as increase in blood pressure. SNRIs commonly prescribed for depression treatment include venlafaxine and duloxetine.

1.2.3 Monoamine oxidase inhibitors

Monoamine oxidase inhibitors (MAOIs) have been used since the 1950s to treat unipolar and bipolar depression. The mechanism of action of this category of drug is to inhibit the activity of the enzyme monoamine oxidase that normally breaks down monoamine neurotransmitters including serotonin and norepinephrine. Preventing the decomposition of monoamines is hypothesised to increase their availability to bind to receptors on the postsynaptic neuron. MAOIs are particularly effective in the treatment of atypical depression (Singh et al., 2006), suggesting that the biological basis of atypical depressives rather than non-atypical or melancholic depressives, is more related to the dysfunction counteracted by this class of drugs. MAOIs are not used as a first line treatment as they have many adverse side effects and dangerous drug or diet interactions. For example, consuming

high quantities of alcohol or foods rich in dietary amines such as cheese could lead to a hypertensive crisis in patients being treated with MAOIs due to the tryamine contained in these foods which stimulates the release of norepinephrine, and causes blood vessels to constrict. MAO in the gut typically metabolises the excess norepinephrine, but since treatment with an MAOI inhibits MAO, the detrimental effects of excess norepinephrine are not prevented and could lead to serious health consequences for patients (Thase, 2012). Additionally, combination of an MAOI with an SSRI or SNRI is extremely dangerous and may induce serotonin toxicity, a condition that is characterised by fever, tremor, diarrhoea, and seizures (Fiedorowicz and Swartz, 2004). This adverse drug interaction is problematic for depressed patients switching between treatment with an SSRI to an MAOI and vice versa. Since the patient's system should be completely cleared of the original treatment before starting the other, the patient risks leaving depressive symptoms untreated for several weeks to avoid serotonin toxicity (Gitlin, 1997). The efficacy of MAOIs in MDD subtypes provides evidence that availability of monoamines is reduced in depression, yet the risks associated with use of this treatment are high and not all depressed patients find relief from their symptoms with this class of drug.

1.2.4 Tricyclic antidepressants

Tricyclic antidepressants (TCAs) were developed in the 1950s and are considered “first generation” antidepressants because they were the first widely used psychotropic drug in the treatment of depression (Gillman, 2007). They function in a similar manner to SNRIs, by blocking serotonin and norepinephrine transporters which results in sustained availability of monoamines in the synaptic cleft and ultimately improving neurotransmission. However, in addition to reuptake inhibition TCAs act as antagonists of 5-HT (serotonin) receptors and agonists of sigma receptors. Sigma receptors are not well understood but are known to play a role in pain relief and believed to have similar effects on opioid receptors, hence the use of TCAs for management of neuropathic pain (Gris et al., 2015). Furthermore, TCAs carry out other actions such as receptor antagonism that result in antihistamine and anticholinergic effects as well as blocking sodium channels (Richelson, 1979). The inhibition of sodium channels by TCAs makes them an efficient analgesic. However, it is also the reason they have the potential to cause cardiotoxicity in overdose that may result in delirium, coma, and death. A long list of adverse side effects may be experienced by patients using TCAs including constipation, appetite changes, weight gain, sexual dysfunction, memory

impairment, confusion, drowsiness, anxiety, anhedonia, restlessness, blurry vision, dizziness, weakness, and nausea (Gillman, 2007). The numerous and serious side effects associated with use of TCAs is problematic and thus they have largely been replaced by SSRIs in the clinical setting. Finally, about 50% of patients taking TCAs experience a clinical response which is substandard and indicates a need for alternative mechanisms of action for improved efficacy and tolerability (Arroll et al., 2005).

1.2.5 Other pharmacological treatments for MDD

There are several other pharmacological treatments often implemented for relief of depression symptoms.

For example, melatonergics such as agomelatine are often used in depressed patients in combination with SSRIs or on their own. Melatonergics function by agonising the melatonin receptor and antagonising the 5-HT_{2C} receptor, and are known to resynchronize circadian rhythms to restore healthy sleep patterns which are often altered during MDD (Guaiana et al., 2013). While melatonergics are known to have fewer side effects than other antidepressants and is tolerated at a higher rate than venlafaxine, however has not shown to be more effective at relieving depressive symptoms compared to SSRIs (Guaiana et al., 2013).

Benzodiazepines are often used to treat the anxiety symptoms experienced in conjunction with a depressive episode. These include the drugs alprazolam (Xanax) and diazepam (Valium). Benzodiazepines are a class of psychoactive drugs that act selectively on gamma-aminobutyric acid-A (GABA-A) receptors in the brain to enhance the effects of GABA. This results in a sedative and anxiolytic effect which is intended for short-term use, as long-term use may result in dependence and withdrawal.

Sleeping medication is often prescribed for depressed patients who are experiencing insomnia. Two such drugs are eszopiclone, which are categorised as non-benzodiazepine hypnotics (Wagner and Wagner, 2000). These drugs are intended for short term use, and are less likely to be associated with dependency or abuse potential than benzodiazepines (Hajak et al., 2003).

1.3 Monoamine hypothesis of Major Depressive Disorder

The monoamine hypothesis of depression is the theory that depletion of neurotransmitters such as serotonin, dopamine, and norepinephrine in the central nervous system is the physiological cause of depression (Delgado, 2000; Hindmarch, 2002) [Figure 1.1]. The theory was first suggested in the 1950s after patients being treated for hypertension with reserpine, a drug that depletes monoamines, exhibited depressive symptoms (Freis, 1954). Based on the efficacy of drugs such as TCAs, MAOIs and later SSRI and SNRIs to improve symptoms in MDD, research continued to focus on serotonin dysfunction as the key to understanding depression pathophysiology (Manji et al., 2001; Morilak and Frazer, 2004). Drugs that augment serotonergic and noradrenergic transmission in the brain were shown to modify mood, therefore the monoamine hypothesis continued to reign over depression research (Morilak and Frazer, 2004). However, the monoamine hypothesis alone does not fully account for the mechanism of action of antidepressants, nor does it provide a complete understanding of the underlying biology of depression. As described in the previous section, each of the pharmacological drugs available for antidepressant use are coupled with adverse side effects and are only effective in relieving symptoms in a fraction of patients who use them (Montgomery et al., 1994).

The mechanisms of antidepressants are poorly understood. While part of the function of TCAs and MAOIs is to improve transmission of serotonin and norepinephrine, there are other functions carried out by these drugs that might be facilitating antidepressant effects that are not acknowledged by the monoamine hypothesis. Evidence that contradicts the monoamine hypothesis exists, too. For example, the drug tianeptine, a TCA which acts by increasing serotonin reuptake rather than decreasing it, exhibits antidepressant effects (Loo et al., 2001). The monoamine hypothesis led to a strong focus on serotonergic dysfunction in depression research during the 21st century while much evidence of involvement of other pathways exists. Development of a newer model of depression should not neglect evidence of the involvement of other biological dysfunctions in conjunction with disturbed serotonergic transmission. Alterations of systems including the hypothalamic-adrenal-axis, the immune system, the limbic system, and the kynurenine pathway have been shown between depressed patients and the healthy population, yet a model that entails each of these paired with the existing monoamine hypothesis has yet to be substantiated (Maes, 1994; Holsboer, 2000; Maes et al., 2009; Young, 2013).

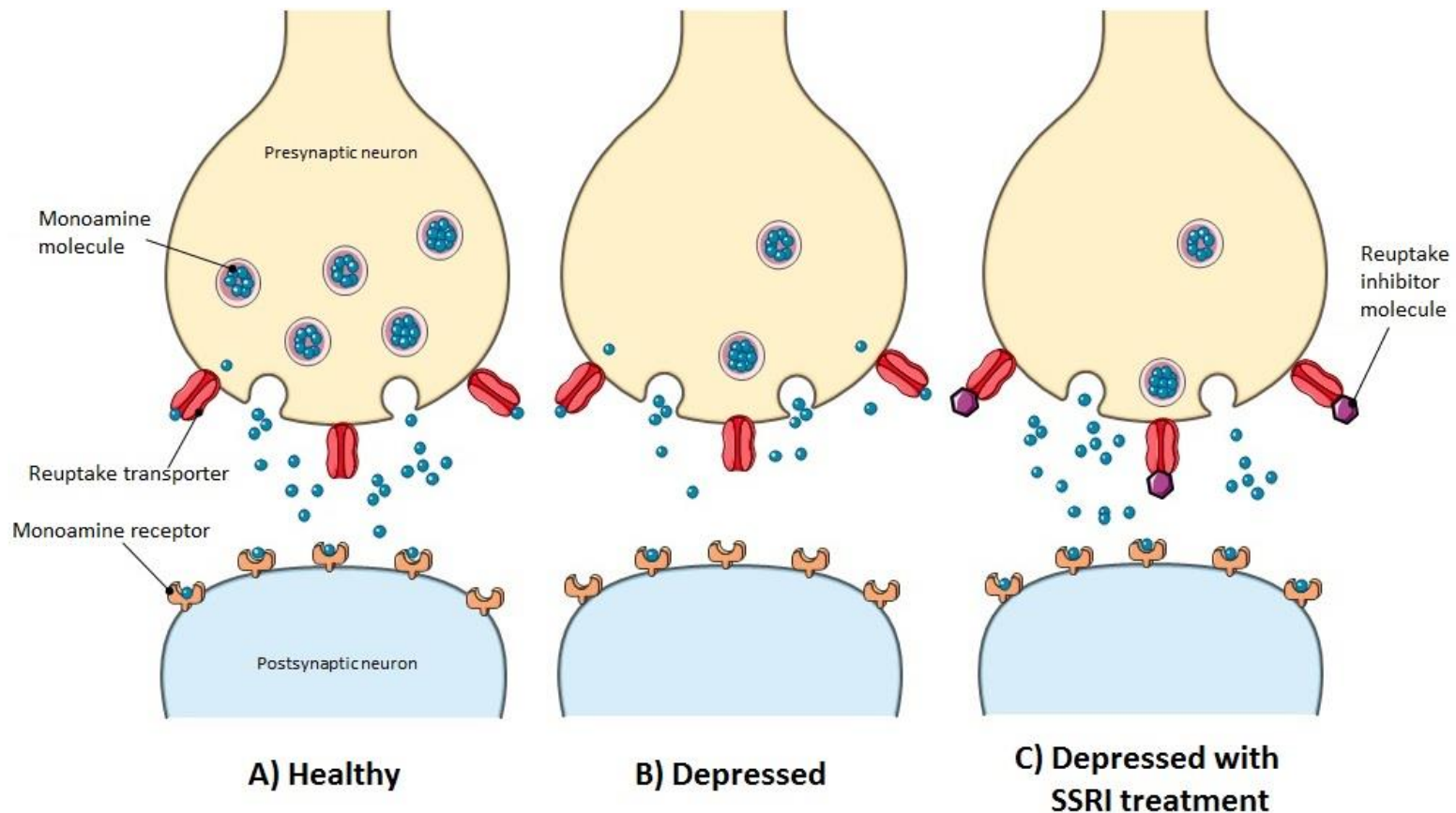


Figure 1.1 Monoamine hypothesis and function of SSRI treatment. (a) In the healthy brain, monoamine molecules are released by the presynaptic neuron and bind to monoamine receptors on the postsynaptic neuron; (b) In depression, it is hypothesised that fewer monoamine molecules are available for binding to receptors, contributing to the existence of a mood disorder; (c) Treatment with a reuptake inhibitor increases the number of monoamine molecules in the synaptic cleft by preventing reuptake into the presynaptic neuron. Use of SSRIs results in increased availability of monoamines to bind to receptors on the postsynaptic neuron which is hypothesised to improve the low mood experienced in MDD. With sustained use of SSRIs, it is believed that the increased concentration of serotonin in the synaptic cleft results in downregulation of post-synaptic serotonin receptors (5HT_{1A} receptors). After the number of 5HT_{1A} receptors is reduced, the neuron is disinhibited to release more serotonin into the synaptic cleft. This is the adaptive effect of antidepressant use which accounts for the delay in peak efficacy of SSRIs.

1.4 Hypothalamic-pituitary-adrenal axis and depression

1.4.1 Anatomy of the hypothalamic-pituitary-adrenal axis

The hypothalamic pituitary adrenal (HPA) axis is a major part of the neuroendocrine system that controls the body's response to stress [Figure 1.2]. In response to stress, the peptides corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) are released from the paraventricular nucleus of the hypothalamus. Once CRH and AVP enter the pituitary portal blood, it triggers the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH). Consequently, ACTH enters the peripheral bloodstream and stimulates the release of glucocorticoids including cortisol from the adrenal cortex (Dinan and Scott, 2005). A negative feedback system is exerted through the binding of cortisol to glucocorticoid and mineralocorticoid receptors at the hippocampus, hypothalamus, and the pituitary. Baseline HPA axis activity is regulated by the binding of glucocorticoids to mineralocorticoid receptors which they have a high affinity for and which are found primarily in the hippocampus. Meanwhile, glucocorticoid receptors are found predominantly in the hypothalamus and anterior pituitary gland and are involved only during stress and in phases of the circadian rhythm when concentrations of glucocorticoid are higher (Papadopoulos and Cleare, 2012).

The HPA axis is affected by psychological, physical, and immunological stressors since the hypothalamus receives input from other brain regions including the hippocampus, amygdala, and brainstem which provoke excitatory and inhibitory responses. The presence of cortisol assists the body in preparing for stressors. For example, cortisol regulates gluconeogenesis, peripheral insulin resistance, and blood pressure. Moreover, glucocorticoids have an anti-inflammatory effect as glucocorticoids inhibit synthesis and secretion of inflammatory cytokines (Coelho et al., 1992). Cortisol affects the central nervous system in that it regulates neurogenesis, neuronal survival, neuronal excitability, memory acquisition and emotional processing of events (Pariante and Lightman, 2008). The enzyme 11-beta-steroid dehydroxygenase (11 β -HSD) catalyses the conversion of cortisol to inert cortisone and vice versa in peripheral tissue. This enzyme has two types: type 1 which converts cortisone to cortisol, and type 2 which performs the opposite function, thus regulating the amount of active corticosteroid present (Wyroll et al., 2011).

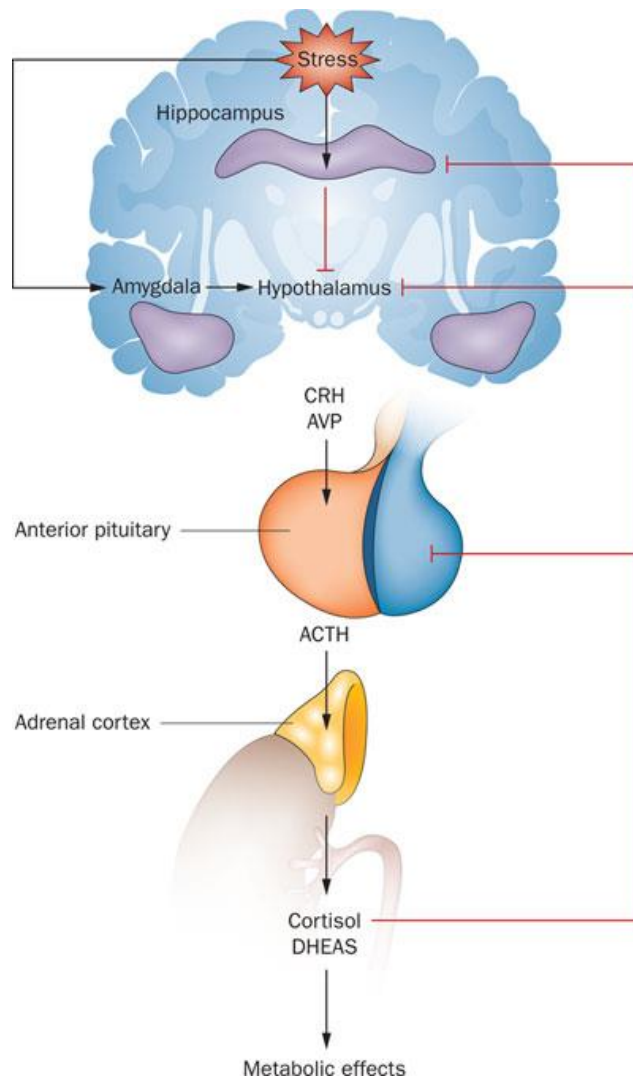


Figure 1.2 Representation of the hypothalamic-pituitary-adrenal axis. In response to stress, the hypothalamus secretes CRH and AVP, which stimulate the production of ACTH from the anterior pituitary. Circulating ACTH stimulates the adrenal cortex of the adrenal gland to produce glucocorticoids including cortisol. Corticosteroids regulate levels of their own secretion through a negative feedback control mechanism to maintain homeostasis of the HPA axis. This is exerted through binding of glucocorticoids to mineralocorticoid and glucocorticoid receptors at the level of the hippocampus, hypothalamus and pituitary gland. Black lines represent stimulation while red indicates inhibition. (Papadopoulos and Cleare, 2012).

1.4.2 Cortisol awakening response and diurnal variation

The HPA axis has an endogenous circadian rhythm. This is regulated by the suprachiasmatic nucleus, also known as the biological clock of the hypothalamus. This is evidenced by a 24-hour cycle of HPA activity, referred to as diurnal variation, in which cortisol levels are lowest around midnight and rise gradually from between 2:00am to 3:00am, peaking within 30

minutes of waking [Figure 1.3]. Following the peak cortisol concentration in the morning, levels decline throughout the day until midnight (Lightman and Conway-Campbell, 2010). The surge in cortisol concentration that occurs within the first hour of waking is referred to as the cortisol awakening response (CAR) (Pruessner et al., 1997). In healthy adults, salivary cortisol concentrations increase by between 50 and 160% in the first 30 min immediately after waking up (Clow et al., 2004). The cortisol awakening response is hypothesised to combat what is known as “sleep inertia,” the period after waking in which the body struggles to regain full alertness and cognitive function in anticipation of the upcoming day (Fries et al., 2009). The CAR is known to be part of healthy human circadian physiology, therefore deviations from a typical CAR pattern are believed to reflect maladaptive neuroendocrine functioning (Stalder et al., 2016). Stress is highly associated with altered CAR activity including elevated waking cortisol concentrations (Schlotz et al., 2004; Chida and Steptoe, 2009; Fries, 2009). Physical illness such as cardiovascular disease and autoimmune disorders result in an elevated and blunted CAR (Clow et al., 2010). However, factors such as age, gender, and smoking have no significant effect on CAR (Fries et al., 2009).

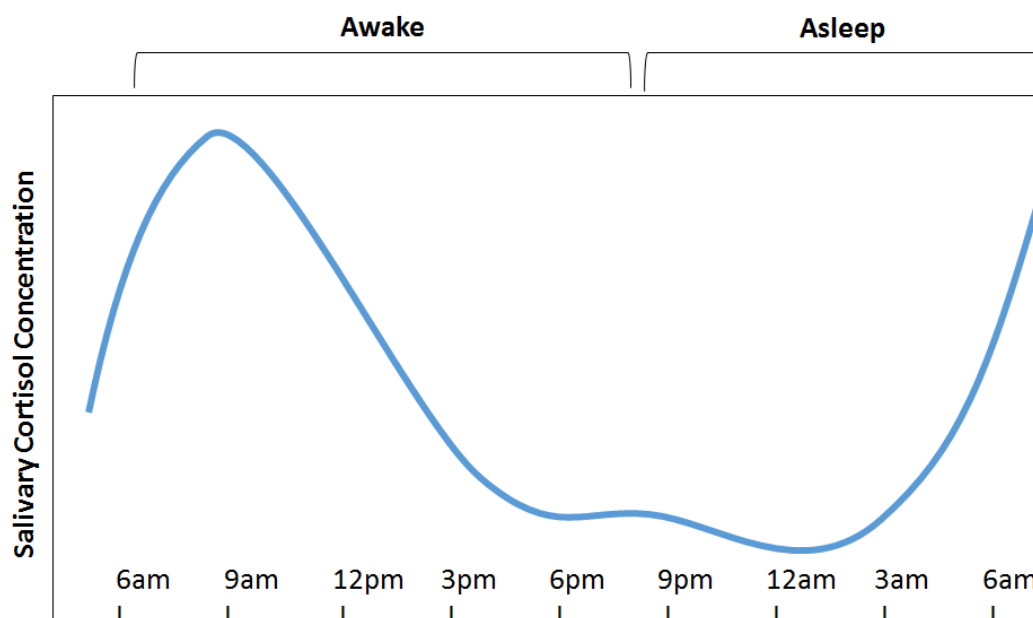


Figure 1.3 Typical diurnal cortisol variation illustration. In healthy individuals, a 24-hour cycle of cortisol activity exists. Cortisol levels are lowest around midnight and rise gradually starting at 2:00am to 3:00am, peaking within 30 minutes of waking (Based on Lightman and Conway-Campbell, 2010).

1.4.3 Measurement of cortisol awakening response

There are several established methods for assessing cortisol activity in humans. These include measurement through hair samples for assessment of chronic stress (Russell et al., 2012), cortisol in serum samples for measurement of circulating total cortisol content at a specific time point, and also saliva samples for measurement of bioactive or “free” cortisol levels. Measuring cortisol awakening response requires serial samples within the first hour of wakening, which can pose logistical issues if using the serum method. Additionally, cortisol measurement in serum accounts for a combination of protein-bound and free cortisol. The amount of free cortisol in serum can be affected by changes in cortisol-binding globulin, for example in the use of birth control pills, therefore measurements of serum cortisol do not always reflect elevation due to stress (Vining et al., 1983; Russell et al., 2012). Because of these pitfalls of cortisol measurement in serum, serial saliva samples are often a more appropriate option in research as they are less invasive and can be completed in one’s home rather than in a clinic or research facility.

Measuring the CAR based on saliva samples requires a series of timed collections within the first hour of wakening, and often includes evening time points for assessment of diurnal variation. The CAR can be described through various calculations that describe the dynamic of cortisol concentration changes after wakening including area under the curve (AUC) measurements and calculations that describe reactivity (wakening cortisol concentration subtracted from the final morning cortisol concentration) and peak levels of cortisol (Pruessner et al., 2003). Two main AUC calculations are typically used. The first is AUC with respect to increase (AUC_i), and the other is AUC with respect to ground (AUC_g) [Figure 1.4], though the consensus is that AUC_i is a more appropriate measure of CAR dynamics rather than total post-wakening free cortisol output represented by AUC_g (Stalder et al., 2016). Additionally, a regression line fitted through the morning cortisol data is often used to describe the dynamic of the awakening response via calculation of the slope and intercept of that line (Kurina et al., 2004; Fekedulegn et al., 2007). It has been shown that the use of objective monitoring devices to record the absolute times of sample collection could be essential, as any delays in collection of samples or inaccuracy of the time of collection could greatly effect calculation of AUC values and misrepresent HPA activity. Therefore, adherence to sample collection instructions is critical, and assessment of non-compliance would greatly benefit studies that measure the CAR (Stalder et al., 2016). Moreover, the CAR is affected by whether it is a weekday or weekend; therefore multi-day sample collection has been suggested to enhance the quality of CAR assessment (Stalder et al., 2016).

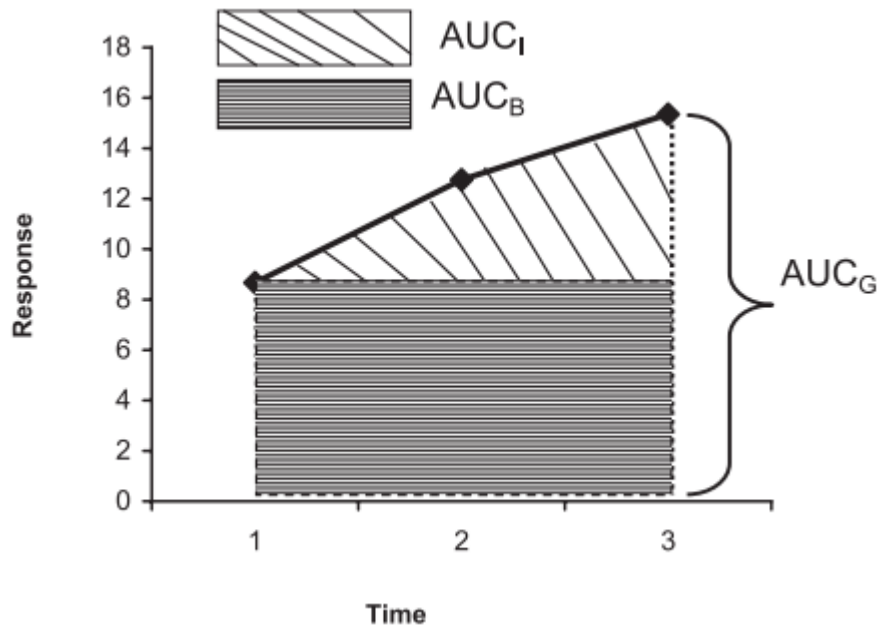


Figure 1.4 Area under curve calculation. Illustration of an example area under curve (AUC) measurement. The AUC can be calculated from ground (AUC_G) or from increase (AUC_I). AUC_G represents the entire area under the curve from ground or zero, whereas AUC_I is the differences between AUC_G and the area between baseline and ground. AUC_G is representative of total cortisol output post-wakening, while AUC_I is representative of the dynamic CAR. (From Fekedulegn et al., 2007).

There are some difficulties associated with at-home participant saliva sampling, such as participant compliance and accuracy of reported sampling time (Clow et al., 2004). Delays between waking and completing the first saliva sample collection can cause significant misrepresentation of CAR. Delaying the waking sample time point tends to result in a higher cortisol concentration measurement for the first sample, and a reduced AUC_I (Okun et al., 2010; Smith and Dougherty, 2014). Because of this, compliance with sampling instructions is essential for proper interpretation of CAR results as a measure of stress response.

1.4.4 Altered HPA axis functioning in MDD

Hyperactivity of the HPA axis is one of the consistent findings in the search for biological aetiology in MDD. Elevated cortisol in plasma and urine were some of the earliest indicators of HPA dysregulation in depression (Sachar et al., 1970). Raised levels of CRH in

cerebrospinal fluid also gave evidence of hyperactivity of this axis (Nemeroff et al., 1984). Furthermore, non-suppression during the dexamethasone suppression test was reported in 44% of depressed patients in another early study of HPA dysfunction in MDD, indicating a faulty feedback system (Arana et al., 1985).

Altered CAR patterns have been detected in studies of depressed patients. Patients with MDD tend to have elevated morning salivary cortisol with a flattened or blunted appearance to their CAR (Holsboer et al., 2000; Huber et al., 2006; Vreeburg et al., 2009). Previous studies showing increased AUC_i and AUC_g values in depressed patients are believed to indicate elevated dynamic responses and elevated post-wakening cortisol output respectively (Aubry et al., 2010; Dienes et al., 2013; Vreeburg et al., 2009). Moreover, elevated CARs have also been reported in cohorts of remitted depressed patients, indicating that HPA axis disturbance could be a trait marker for depression (Vreeburg et al., 2009). In contrast to this, treatment with SSRIs has been associated with normalised morning salivary cortisol concentrations in depressed patients who had previously exhibited hypercortisolemia, though normalisation was not strongly associated with reduced depressive symptoms (Hinkelmann et al., 2011). It has also been reported that depressed patients exhibit raised cortisol concentrations in the evening (O'Brien et al., 2004).

The enzyme 11 β -HSD1 has been shown to be dysregulated in depression. Subjects with the rs11119328 polymorphism of the gene that encodes for 11 β -HSD1, HSD11 β 1, were found to have higher cortisol levels and increased rates of depression (Dekker et al., 2011). Ablation of the HSD11 β 1 gene in mice results in anti-depressant effects during the forced swim test (Slattery et al., 2016). Higher expression of HSD β 11 has been associated with increased inflammation and an elevated, blunted CAR which are often observed in depressed patients (Carter et al., 2009; Yang et al., 2009; Wyrwoll et al., 2011).

1.4.5 Glucocorticoid resistance in depression

It has been hypothesized that the hyperactivity of the HPA axis in MDD patients is due to reduced efficacy of central glucocorticoid and glucocorticoid receptor (GR) function, also known as glucocorticoid resistance (Pariante, 2009). Circulating glucocorticoid levels including CRH, ACTH, and cortisol each increase as a result of the inability of glucocorticoids to exert their effects on target tissues (Chrousos et al., 1993; Anacker et al., 2011). Impaired GR function results in a broken feedback network within the HPA axis (Anacker et al., 2011;

O'Keane, 2012; Farrell and O'Keane, 2016). GR resistance is believed to occur in depression as a result of persistently elevated glucocorticoid levels associated with chronic stress. Another consequence of glucocorticoid resistance is reduced immune suppression which is one of the functions of glucocorticoids in health (Coelho et al., 1992). It is not known whether HPA axis dysfunction is a consequence of depression or rather is an active force in the pathophysiology of MDD, though GR resistance is believed to be the cause of hypercortisolemia in depression (Pariante, 2009).

1.5 Immune system and depression

1.5.1 Innate versus adaptive immunity

The immune system is the body's defence system in response to stress, injury, and pathogens. It detects pathogens and initiates a defensive response to eliminate them from the host system. The immune system is comprised of tissues including blood, the lymphatic system, the thymus, spleen, and bone marrow. In humans, the immune system is further classified into two subsystems which include the "innate" and "adaptive" responses which act together to maintain homeostatic balance (Janeway, 2005). Innate immunity entails nonspecific defence mechanisms while adaptive immunity refers to antigen-specific responses. The innate immune response is a more immediate response from the body that occurs within 24 hours of detecting a pathogen, while the adaptive immune system responds more slowly with a lag between time of exposure to a pathogen and the maximum response of the immune system that can be over a period of days to weeks (Janeway, 2005).

The adaptive immune system is the body's highly specific response to a particular pathogen, such as viruses or toxins. The goal of this system is to destroy invading pathogens and any molecules that they produce, which sometimes leads to the destruction of harmless host cells as well (Flajnik and Kasahara, 2010). The adaptive immune response can provide long-term protection against certain pathogens, as pathogen-specific receptors are developed over the lifetime after exposure to a virus or bacteria (Haapakoski et al., 2016). The work of the adaptive immune system is carried out by lymphocytes such as B and T cells. In a typical example of adaptive immunity in response to insult, activated B cells secrete antibodies which travel through the blood stream and bind to the antigen, preventing it from binding to the recognition elements within the host, and ultimately resulting in the destruction of the invading pathogen (Janeway, 2005).

The innate immune system serves to protect the body from infection in a different manner. The innate immune system includes physical barriers such as skin that protect the body from pathogens, phagocytes such as macrophages that engulf microbes, and toll-like receptors which exist on the surface of macrophages, dendritic cells, and more to detect pathogens and signal an immune response. Each of these structures assists in preventing infection and eliminating pathogens. When an immune response is activated, cytokines and chemokines are generated by monocytes, macrophages, and T cells (Janeway, 2005; Cray et al., 2009). The response is less specific than that of the adaptive immune system, and is more involved with the acute phase response.

1.5.2 Acute phase response

The acute phase response is the body's physiological reaction in response to inflammation that occurs as a result of acute illness (Baumann and Gauldie, 1994). Inflammatory cytokines produced during the innate immune response travel through the blood and stimulate protein production in the liver [Figure 1.6]. The proteins produced in response to inflammation are acute phase reactants, which then have an increased concentration in the plasma. Meanwhile, other proteins are decreased. The proteins altered in this process are the acute-phase proteins (APPs) of this biological mechanism. APPs include C-reactive protein (CRP) and cytokines, which act to inhibit the proliferation of microbes and to destroy pathogens [Figure 1.6].

1.5.3 Cytokines

Cytokines are a category of small cell signalling proteins that play a role in activation of the inflammatory response (Connor and Leonard, 1998). Cytokines are produced by monocytes, macrophages, dendritic cells, B lymphocytes, T lymphocytes, and mast cells. They are released by cells in both the brain including microglia and oligodendritic cells (Duque and Descoteaux, 2014). The role of cytokines in the immune system is to direct specific immune responses by signalling immune cell trafficking. Cytokines induce growth, differentiation, and activation functions of immune cells depending on the type of immune insult that has occurred (Dinarello, 2007; Stow and Murray, 2013). Depending on the cytokines produced, responses can be cytotoxic, cell-mediated, humoral, or allergic (Borish and Steinke, 2003; Commins et al., 2010). In many cases, the presence of multiple cytokines is required to recruit the optimal immune response to a pathogen. Cytokines include interleukins, interferons, and tumour necrosis factors.

Cytokines can be produced by CD4-positive T-helper lymphocytes. T-helper cells are classified into "T-helper" type 1 (Th1) and type 2 (Th2) cells, which each promote unique immune responses. Th1 cells promote pro-inflammatory responses via cytokines including interleukin 1 (IL-1) alpha and beta, interferon (IFN) gamma, and tumour necrosis factor (TNF) beta. Th2 cells promote antibody and allergic responses via IL-4, IL-6, and IL-10. Immune homeostasis relies upon a balance of Th1 and Th2 cell activity (Kronfol and Remick, 2000; Yarlagadda et al., 2009).

Peripheral cytokines communicate with the CNS despite the existence of the blood brain barrier (BBB) (Quan and Banks, 2007). It has been shown that some cytokines can rapidly cross the BBB, and that the permeability of the BBB can be increased by cytokine activity (Yarlagadda et al., 2009; Pan et al., 2011). The involvement of altered immune activity via cytokine activation is likely to play a role in depression pathophysiology (Dantzer, 2007; Yarlagadda et al., 2009; Anisman, 2009). The roles of individual cytokines in the immune system are detailed in the following sections.

1.5.3.1 Interleukin-1 β

Interleukin-1 β (IL-1 β) is produced by immune cells, primarily macrophages but also neutrophils and endothelial microglia, in response to pathogens recognised by pattern recognition receptors (Dinarello, 1988). When IL-1 β binds to its receptor, IL-1R1, it activates transcription factor NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and transcription of IL-1 β inducible genes (Li and Qin, 2005; Dantzer, 2009). Expression of IL-1 β is known to be associated with sickness behaviour including fever, fatigue, and appetite changes (Konsman et al., 2002; Dantzer, 2006; Goshen and Yirmiya, 2009). Additionally, increased secretion of brain IL-1 in response to immune challenges has been shown to induce the secretion of CRH from the paraventricular nucleus of the hypothalamus and subsequently activate all components of the HPA axis, including elevation of glucocorticoids (Turnbull and Rivier, 1999). Furthermore, excessive activation of IL-1 β may relate to dysregulation of serotonergic transmission that may result in altered mood including depressive episodes (Zhu et al., 2010).

1.5.3.2 Interleukin-6

Interleukin-6 (IL-6) is secreted from macrophages and T-cells in response to infection or insult and induces the acute phase response. It is known to have both pro- and anti-inflammatory effects and is important to gene activation within as well as growth and differentiation of immune cells (Heinrich et al., 1998). IL-6 is known to play a role in immune-mediated HPA axis activation, as infusion of IL-6 increases circulating corticosteroids in rats and human (Lenczowski et al., 1999; Steensberg et al., 2003). The effects of IL-6 occur through the Janus-Kinase signal transducer and activator of transcription (JAK-STAT) signalling pathway [Figure 1.5]. Binding of IL-6 to its receptor results

in dimerization of the signal transducer glycoprotein 130 subunit, which in turn results in activation of IL-6 receptor associated JAK1 and JAK2. This produces a phosphotyrosine docking site for STAT3, and STAT3 dimers translocate to the nucleus to activate transcription from IL-6 gene promoters (Aaronson and Horvath, 2002). Dysregulation of IL-6 cytokine signalling is thought to contribute to the onset of several diseases such as rheumatoid arthritis, inflammatory bowel disease, osteoporosis, multiple sclerosis, and several cancers (Heinrich et al., 2003).

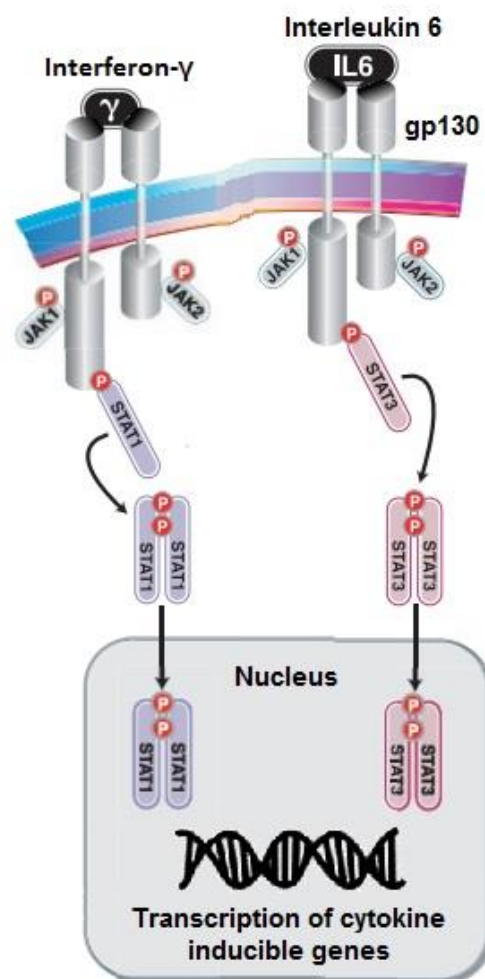


Figure 1.5 JAK-STAT signalling complex for IFN-γ and IL-6. Cytokine IFN-γ binds to its receptor which results in phosphorylation of JAK1 and JAK2, signalling STAT1. STAT1 is dimerized and translocates to the nucleus, resulting in transcription of IFN-γ inducible genes. IL-6 binds to gp130 subunit of its receptor which results in phosphorylation of JAK1 and JAK2, signalling STAT3. STAT3 dimerizes and translocates to the nucleus, resulting in transcription of IL-6 inducible genes. (Adapted from Aaronson and Horvath, 2002).

1.5.3.3 Tumour necrosis factor- α

Tumour necrosis factor- α (TNF- α) is a cytokine involved in the acute phase response and is produced by mononuclear phagocytes (Duque and Descoteaux, 2014). TNF- α receptors are found on nearly all cells in the body (Horiuchi et al., 2010). Binding of TNF- α to its receptor induces complex signalling pathways that ultimately promote inflammation by stimulating expression of IL-1 β and IL-6 via NF- κ B activation, and also promotes lymphocyte proliferation (Yokota et al., 1988; Driscoll et al., 1997). TNF- α also functions by inducing programmed cell death and cytotoxic immune responses to tumour cells (Janeway, 2005). This cytokine acts to increase expression of endothelial cell adhesion molecules which assist in trafficking immune cells to sites of inflammation. Chronically elevated levels of circulating TNF- α are associated with inflammatory diseases such as multiple sclerosis and Crohn's disease, and contribute to the pathogenesis of these disorders (Chen and Goeddel, 2002).

1.5.3.4 Interferon- γ

Interferon- γ (IFN- γ) is a cytokine considered part of both the innate and adaptive immune systems. It is predominantly produced by CD4⁺ Th1 T-cells, although it can also be produced by macrophages and natural killer cells (Duque and Descoteaux, 2014). Similar to the signalling pathway of IL-6, IFN- γ binds to its receptor and engages in the JAK-STAT signalling pathway via cross phosphorylation of JAK1 and JAK2 proteins [Figure 1.5]. The phosphorylation of these JAK proteins results in phosphorylation and dimerization of STAT1 which translocates into the nucleus, promoting transcription of IFN-inducible genes (Aaronson and Horvath, 2002). The anti-viral actions of IFN- γ are particularly essential to the adaptive immune response as this cytokine appears to directly inhibit viral replication. It also plays a role in differentiation of immune response and stimulates macrophage activity (Young and Hardy, 1995). Dysfunction of the IFN- γ signalling pathway has been linked to autoinflammatory and autoimmune diseases (Skurkovich and Skurkovich, 2007).

1.5.4 C-reactive protein

C-reactive protein (CRP) is found in blood plasma and is produced in response to inflammation and immune stimuli. CRP is an acute phase protein that is synthesised in the liver and increases in response to elevated levels of IL-6 or IL-1 β produced by macrophages or T-cells (Du Clos, 2000). Once secreted by the liver, CRP participates in opsonisation, as it

binds to phosphocholine on the surface of dying cells or bacteria [Figure 1.6]. It activates the complement system by binding to Fc receptors and inducing phagocytosis. When CRP binds to inflamed cells, it signals further immune activation and a pro-inflammatory response (Rhodes et al., 2011). Levels of CRP increase rapidly in response to trauma or infection, and decrease quickly when the condition has resolved, therefore CRP is often used to assess inflammatory states and is considered to have an integral role in the innate immune system (Du Clos, 2000).

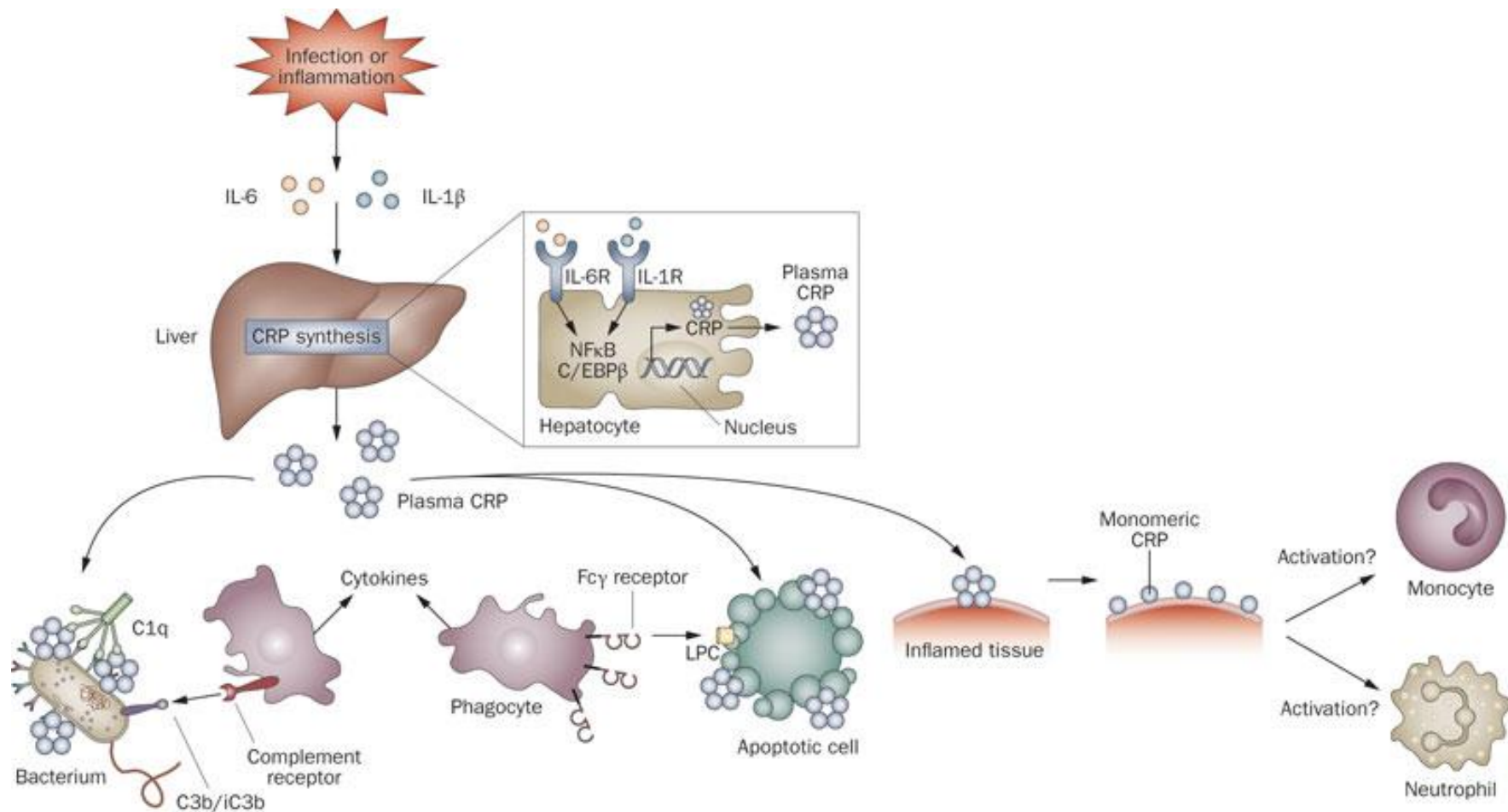


Figure 1.6 Inflammatory response involving IL-6, IL-1 β , and CRP. In response to cytokines such as IL-6 and IL-1 β , hepatic expression of CRP increases dramatically. Circulating CRP opsonizes bacteria and apoptotic cells, facilitating their clearance and phagocytosis. CRP ligation might contribute to the release of immunomodulatory cytokines from phagocytic cells. Evidence is mounting that plasma CRP deposited onto inflamed tissue breaks into biologically active monomeric subunits, to which have been attributed a range of pro-inflammatory effects. (Rhodes et al., 2011).

1.5.7 Inflammation in depression

The first studies to investigate the involvement of cytokines in depression were following from evidence that increased inflammation was associated with sickness behaviour (Hart, 1988; Dantzer and Kelley, 1989). Subsequently, the link was made between sickness behaviour, depression, and inflammation as the macrophage theory of depression was proposed by Smith (Smith, 1991). Sickness behaviour is exhibited in MDD including common symptoms of fatigue, altered sleep patterns, lack of appetite, inability to concentrate, and social withdrawal. The 1990s yielded a large amount of research focusing on inflammation and depression, as the innate immune system was shown to be activated in models of depression (Maes et al., 1993; Maes et al., 1995). Supporting evidence of involvement of inflammation in MDD was detected when immunotherapy such as the use of cytokine interferon alpha injections in the treatment of certain cancers and Hepatitis C was observed to induce depressive-like behaviour (Valentine, 1998; Cassidy and O'Keane, 2000). Others showed that administration of cytokines elicited depressive symptoms and that subsequent treatment with antidepressant medications relieved these symptoms (Yirmiya et al., 2000; Musselman et al., 2001; Capuron et al., 2001).

Activated inflammatory pathways have been repeatedly observed in MDD patients, as demonstrated by increases in specific inflammatory cytokines and C-reactive protein (CRP) (Raison et al., 2006). Most consistently reported is the increase of interleukin (IL)-6 and CRP concentrations in circulating serum or plasma of depressed patients compared to healthy controls (Maes, 1999; Lanquillon et al., 2000; Danner, 2003 et al.; Ford and Erlinger, 2004; Alesci et al., 2005). Increases in plasma IL-1 β and tumour necrosis factor (TNF)- α have also been described in depressed patients (Maes, 1999; Tuglu et al., 2003; Thomas et al., 2005). Furthermore, it has been shown that severity of symptoms is highly correlated to increased inflammation within depressed populations (Thomas et al., 2005).

Numerous studies have reported on the anti-inflammatory properties of antidepressant medication (Sluzewska et al., 1995; Kenis and Maes, 2002). Moreover, the use of non-steroidal anti-inflammatory drugs (NSAIDs), typically used for treatment of pain, is associated with an improved treatment response in depression according to a meta-analysis of clinical trials (Köhler et al., 2014). The overwhelming evidence of a bidirectional relationship between depression symptomatology and inflammation provides a strong rationale for the involvement of the immune system in depression pathophysiology.

1.6 Limbic system and brain volume changes in depression

1.6.1 Anatomy of the limbic system

The limbic system is a set of interconnected brain structures that are believed to be primarily responsible for emotional, learning, and memory processes (Sitoh and Tien, 1997). The structures of the limbic system are located below the cerebellum and sit above the brain stem [Figure 1.7]. The limbic system includes both cortical and subcortical regions. While there is some debate about the complete list of structures that are included in the limbic system, it is accepted that the central components of the limbic system include the amygdala, hippocampus, thalamus and hypothalamus. The basal ganglia, cingulate gyrus, mammillary bodies, fornix, and olfactory bulb are also connected to this system and influence its function (Rajmohan and Mohandas, 2007). The primary circuit of connectivity of the limbic system was identified in the 1930s and is known as the “Papez circuit” which begins and ends in the hippocampus. The hippocampal subiculum connects to the fornix, which connects to the mammillary bodies of the hypothalamus. This projects to the mammillothalamic tract, then the anterior thalamic nucleus, and carries on to the cingulum. Finally, the Papez circuit follows on to the entorhinal cortex of the medial temporal cortex, and feeds back into the hippocampus (Rajmohan and Mohandas, 2007) [Figure 1.7].

1.6.2 Function of the limbic system

The limbic system is responsible for influencing the endocrine system and the autonomic system (Barrett and Ganong, 2010). It is known to play a role in emotional processing, motivation, learning, and memory function (Rajmohan and Mohandas, 2007). It is interconnected with the brain’s pleasure centre, the nucleus accumbens, and has been shown to play a role in sexual arousal as well as addiction (Kalivas and Volkow, 2005). The limbic system is also highly connected to the prefrontal cortex, and therefore is believed to contribute to motivation for problem solving (LaBar and Cabeza, 2006). The function of specific regions of the limbic system will be detailed in the following sections.

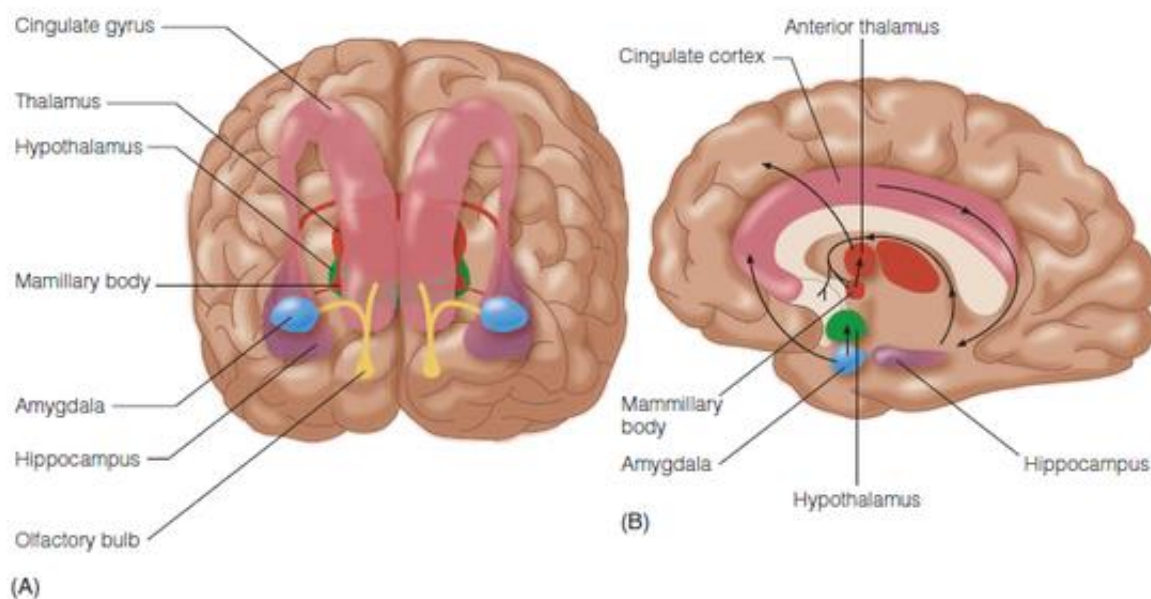


Figure 1.7 Anatomy of the limbic system. Illustration of the main structures of the limbic system including the hippocampus in purple, the hypothalamus in green, the amygdala in blue, and the thalamus in red. (A) is a coronal perspective and (B) is a sagittal perspective showing directionality of connectivity (Image from Blausen Medical, 2014).

1.6.3 Amygdala

The amygdala is an almond shaped group of nuclei located bilaterally in the medial temporal lobes of the brain. It is a region of the limbic system involved with processing memories, emotions, and responses to the environment (Rasia-Filho et al., 2000). The amygdala is involved with endocrine activity and has been shown to regulate stress effects on memory. For example, stress hormones such as glucocorticoid receptor agonists and stress activated neurotransmitters have been shown to enhance memory consolidation in response to emotionally charged experiences via mechanisms of the amygdala (McGaugh and Roozendaal, 2002; Roozendaal et al., 2009). When glucocorticoid receptor antagonists are administered to the amygdala in animal models, memory recall has been shown to be impaired (Donley et al., 2005). The amygdala is highly active in response to emotionally charged stimuli according to functional magnetic resonance imaging (fMRI) studies, and bilateral amygdala damage in humans has been shown to reduce the ability of humans to recognise fear in facial expressions (LaBar et al., 1995; Rasia-Filho et al., 2012). Because of this evidence, it is proposed that the amygdala may be involved in disorders of emotional

impairment. Moreover, the GR resistance theory of depression could be related to impaired amygdala function which may relate to poorer memory recall which is observed in depressed patients (Roozendaal et al., 2009; Wang et al., 2014).

1.6.4 Hippocampus

The hippocampus, one of the primary structures of the limbic system, is a subcortical curved structure resembling the shape of a seahorse, hence its name. The hippocampal formation includes subfield regions identified as the dentate gyrus, cornus ammonis (CA) 1-4, the subiculum, and the parahippocampal gyrus (Mueller et al., 2007) [Figure 1.8]. The hippocampus is known to play a role in short-term and long-term memory consolidation and spatial navigation (O'Keefe and Speakman, 1987; Burgess et al., 2002). This region is also known to contain a high concentration of glucocorticoid receptors (Sinclair et al., 2011). Increased presence of glucocorticoids in the hippocampus can result in excitability of hippocampal neurons, inhibit neurogenesis, and cause atrophy of dendritic cells in its CA3 region (Rogalska, 2010). The hippocampus exerts inhibitory control on the HPA axis which is essential for proper stress response functioning in addition to cognitive functions such as learning (Herman and Cullinan, 1995; Jankord and Herman, 2008; Rogalska, 2010).

Individual subfields of the hippocampus seem to be responsible for specific roles in cognitive function. The CA1 cells of the hippocampus are thought to be "place" cells, or neurons that are activated during recognition of a particular location in space (Jung and McNaughton, 1993). It has been suggested that the CA3 plays a role in associative memory recall and memory sequence (Jensen and Lisman, 1996; Nakazawa et al., 2002). The exact function of the CA4 region has not yet been established, but neurogenesis in the CA region in general has been shown to be suppressed during stress (McEwen and Magarinos, 2001). The role of the hippocampal subiculum is thought to be two-fold. The dorsal subiculum is hypothesised to be involved in the processing of spatial, mnemonic and movement information while the ventral subiculum plays a role in the mediation of the hippocampal formation's inhibitory control of the HPA axis (O'Mara, 2005). Through these mechanisms, the hippocampus serves an essential role in cognitive functions and in neuroendocrine regulation.

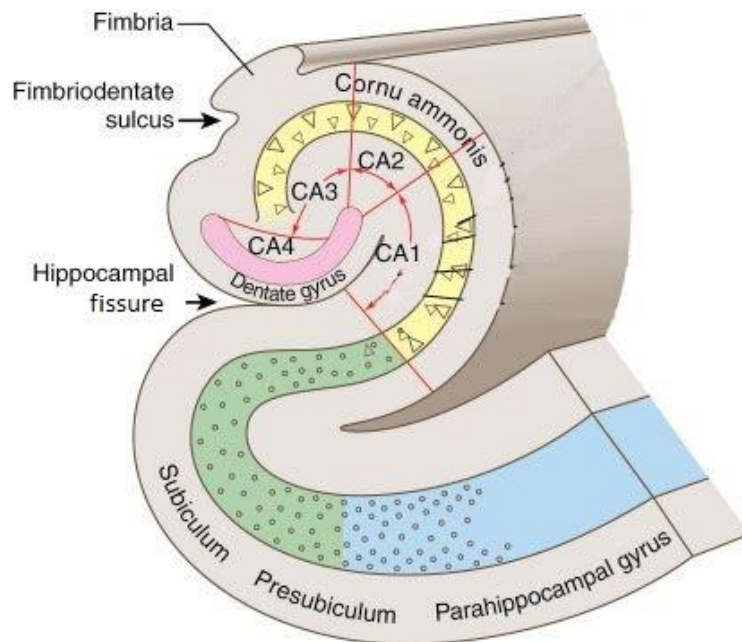


Figure 1.8 Illustration of hippocampus structure. The hippocampus is comprised of the dentate gyrus represented in pink, the CA region represented in yellow, the subiculum marked in green, and the parahippocampal gyrus illustrated in blue. The hippocampal fissure is the gap between the folds of the hippocampus. (Based on Mueller et al., 2007).

1.6.5 Neurodegeneration in aging and disease

As natural aging occurs, the brain endures several changes to its structure and function. Brain volume declines with age at a rate of about 5% total volume reduction per decade after the age of 40 (Svennerholm et al., 1997). Specific areas of the brain reduce in volume at different rates. For example, after the age of 55 the hippocampus reduces at a rate of between 1% to 2% per year (Jack et al., 1998), while the prefrontal cortex, caudate nucleus, and cerebellum, see a reduction between 0.5% and 2% per year in healthy adults (Fjell et al., 2009). Meanwhile the striatum, primary motor, and sensorimotor regions seem to retain their volume in healthy aging (Fjell et al., 2009).

There are numerous hypothesised explanations for brain atrophy in healthy aging involving both grey and white matter loss. Because of the lack of uniformity in volume loss across brain regions, it is likely that multiple mechanisms are simultaneously contributing to brain volume reduction in aging. Shrinkage of grey matter is reported to mainly be a result of increased cell death or apoptosis (Kolb and Whishaw, 1998; Peters, 2006). Reduced dendritic

branching, reduction of dendritic spines, and fewer synapses could also be responsible for grey matter reduction. In response to this cell death, dendritic sprouting could occur to compensate, though decreased dendritic synapse and reduced synaptic plasticity have also been found in healthy aging populations (Anderton, 2002; Barnes, 2003). White matter on the other hand may deteriorate in healthy aging via myelin sheath degradation, particularly within the frontal lobes (Bartzokis et al., 2003; Tullberg et al., 2004). Other mechanisms of brain atrophy in humans include decreased cell proliferation, axonal degeneration, shrinkage of cells, reduced vascularity, or any combination of these factors with those previously described (von Bohlen und Halbach, 2010).

There is mounting evidence that hippocampal volume reduction could be related to immune activation (Goshen and Yirmiya, 2008; Frodl and Amico, 2014; Marsland et al., 2015). For example, inflammatory cytokines such as IL-6 have been shown to be inversely correlated to hippocampal volume (Marsland et al., 2008). Proinflammatory cytokines produced by inflammatory cells such as macrophages, CD4 positive T cells, and B cells surrounding grey matter might result in cell death that is at the source of grey matter reduction in aging or disease [Figure 1.9]. Finally, hyperactivity of stress hormones such as cortisol has also been suggested to play a role in hippocampal volume reduction (Dedovic et al., 2009), linking alterations of brain matter volume to the stress and immune systems described in previous sections of this chapter (1.4 and 1.5).

1.6.6 Evidence of altered brain volume in depression

Many studies have reported volumetric brain differences between MDD patients and healthy controls through the use of neuroimaging techniques, particularly structural MRI (Drevets, 2001; Fitzgerald et al., 2008; Rigucci et al., 2010). Reduction of grey matter has been observed repeatedly in MDD patients, with volume loss specifically occurring in limbic regions (Sacher et al., 2012). Hippocampal volume reduction is the most frequently reported structural brain change associated with MDD (Campbell and Macqueen, 2004; Frodl et al., 2008; Mak et al., 2009). Analysis of hippocampal subfield volumes has shown even more specific brain changes in MDD, such as deformation of the subiculum and CA1 regions compared to healthy controls (Cole et al, 2010). Hippocampal volumes appear to be restored or at least significantly increased following remission of a depressive episode (Kempton et al., 2011). On the other hand, increased amygdalar volumes have been observed in depressed patients, indicating that separate parts of the limbic system are altered differently

in MDD patients (Hamilton et al., 2008). These findings suggest that brain volume integrity plays a role in the pathophysiology of MDD, and that the structure of the limbic system is altered during depression.

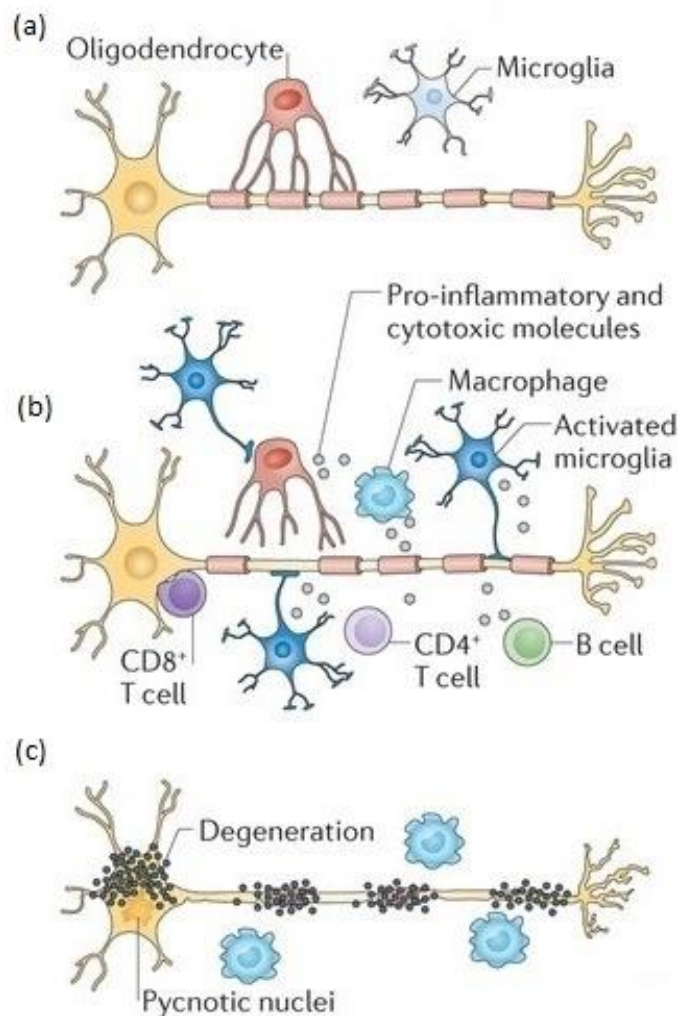


Figure 1.9 Neuronal damage caused by inflammation. (a) In the healthy and undisturbed grey matter, microglia survey the environment for protrusions but are generally in a resting state. (b) Pro-inflammatory cytokines released by macrophages, CD4 positive T cells, or B cells may lead to microglia or macrophage activation and cause damage to the oligodendrocyte. (c) This process may result in neuronal cell death or morphological alterations such as pycnotic nuclei, shrinkage of dendrites, or axonal degeneration. (Adapted from Calabrese et al., 2014)

1.7 Kynurenine pathway of tryptophan metabolism

1.7.1 Tryptophan metabolism

Tryptophan is an essential amino acid of the human body which must be acquired through external sources such as diet. It is found in protein based foods such as meat, dairy, and fruit (Jenkins et al., 2016). After absorption, it circulates in the blood stream either bound to albumin (90%) or unbound (10%) (Richard et al., 2009). The unbound or “free” tryptophan competes with other amino acids to cross the blood brain barrier through L-type amino acid transporters. Tryptophan is the precursor to two metabolic pathways. About 10% of tryptophan is metabolised into serotonin, the monoamine neurotransmitter, while the other 90% is catabolised into kynurenine (Sainio et al., 1996). Tryptophan is the sole precursor to serotonin, therefore reduction of tryptophan is believed to result in subsequent reduction of serotonin (Crockett et al., 2012). Because of serotonin’s role in maintenance of mood, disturbances in tryptophan metabolism are of interest regarding the underlying pathophysiology of psychiatric disorders. The other major branch of tryptophan catabolism, the kynurenine pathway, results in the production of kynurenine metabolites with opposing functions in the central nervous system and periphery (Schwarcz and Stone, 2017). The breakdown of tryptophan into kynurenine metabolites is detailed in the following sections.

1.7.2 Tryptophan 2,3-dioxygenase and Indolamine 2,3-dioxygenase

Tryptophan is oxidized by two rate limiting enzymes, tryptophan 2,3-dioxygenase (TDO) and indolamine 2,3-dioxygenase (IDO), which catalyse the formation of kynurenine (Thackray et al., 2008). However, TDO and IDO function through different mechanisms and have different functions. TDO expression is primarily found in the liver and is activated by tryptophan or corticosteroids such as elevated cortisol induced by the HPA axis stress response (Chen and Guillemin, 2009). Activation of the adrenal cortex and elevated cortisol has been shown to increase TDO activity and deplete tryptophan (Oxenkrug, 2010; Gibney et al., 2014). TDO and IDO catalyse the same reaction, but TDO is substrate-specific, only acting to dioxygenate L-tryptophan in the 5- and 6- location of its chemical structure (Sono et al., 1996).

Despite the fact that TDO and IDO catalyse the same reaction of tryptophan conversion to kynurenine, each enzyme plays a unique biological role. IDO plays an active role in the body’s immune response, whereas there is no evidence that TDO is involved in this system. IDO can be found in numerous cell types including macrophages, microglia, neurons, and

astrocytes, and is upregulated by cytokines such as IFN- γ and TNF- α (Dai and Gupta, 1990; Babcock and Carlin, 2000; Guillemin et al., 2003; Guillemin et al., 2005; Fujigaki et al., 2012). Inflammatory cytokines can induce both increased enzymatic activity and increased gene expression of IDO, which is encoded by genes IDO1 and IDO2 (Dai and Gupta, 1990; Metz et al., 2007). The known difference between IDO1 and IDO2 genes is that IDO2 is preferentially inhibited by the immunomodulatory drug 1-Methyl-D-Tryptophan (Metz et al., 2007; Opitz et al., 2011).

The involvement of IDO in immune mechanisms is complex, but two main tasks of IDO within the immune system are hypothesised. First, it assists in inducing appropriate immune responses with a balance between attack and tolerance (Thackray et al, 2008). Second, it is believed to be involved in the adaptive immune response, as IDO has shown to enable cancerous tumours to induce tolerance from the host's immune system by reducing tryptophan and cell proliferation (Mellor et al., 2003). Because of these mechanisms, IDO has become a drug target of interest for some cancers and immunosuppression following organ transplantation (Liu et al, 2009; Opitz et al., 2011). IDO and TDO play a central role in induction of the kynurenine pathway and mediate tryptophan depletion through different mechanisms that link kynurenine metabolism to both HPA axis activation and the immune system.

1.7.3 Kynurenines

As described previously, kynurenine is derived from tryptophan via IDO and TDO induction. Kynurenine is an intermediate step in the pathway which has multiple trajectories resulting in the production of several other metabolites with specific functions [Figure 1.10]. The two main pathways following kynurenine production are toward the production of kynurenic acid, and toward the production of quinolinic acid and nicotinamide adenine dinucleotide (NAD) (Oxenkrug, 2009) [Figure 1.10]. The conversion of kynurenine to NMDA receptor antagonist kynurenic acid occurs via kynurenine aminotransferases (KATs) (Guidetti, 2007). Meanwhile, the NAD pathway of kynurenine metabolism is more complex. Kynurenine is converted to 3-hydroxykynurenine (3-HK) via kynurenine 3-monooxygenase (KMO). 3-HK is known to lead to the production of free radicals. It is subsequently converted to 3-hydroxyanthranilic acid, another free radical producing metabolite, via the enzyme kynureninase (Oxenkrug, 2007). Continuing on along this pathway, quinolinic acid, an NMDA receptor agonist is produced. Further reactions result in the production of NAD [Figure 1.10].

Notably, the two main branches of kynurenine metabolism (kynurenine to kynurenic acid and kynurenine to quinolinic acid) occur in localized, separate cell types in the central nervous system in humans (Schwarcz and Stone, 2017) [Figure 1.11]. Brain kynurenines are degraded in glial cells rather than in neurons. The branch resulting in quinolinic acid is contained in microglial cells, while kynurenic acid is formed in astrocyte cells (Chiarugi et al., 2001; Kiss et al., 2003; Chen et al., 2011).

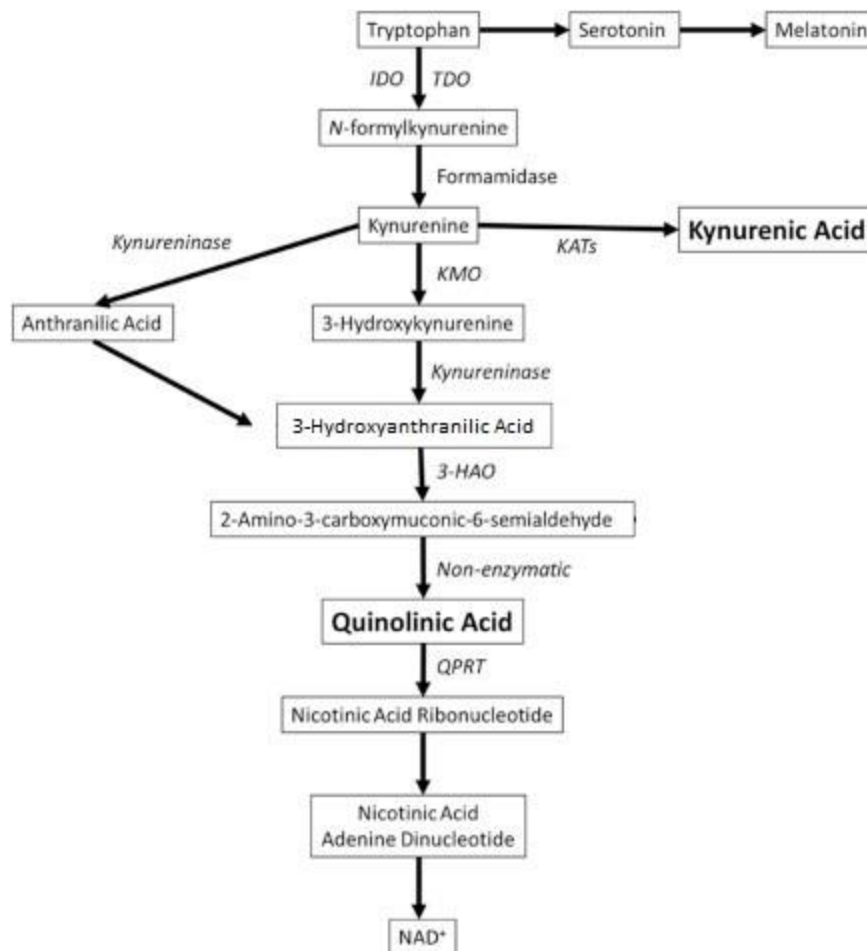


Figure 1.10 Tryptophan metabolism to serotonin and kynurenine. Diagrammatic illustration of the kynurenine pathway of tryptophan metabolism. Enzymes are italicized and metabolites are in boxes. The metabolites discussed in detail are in bold. (IDO=indoleamine-2,3-dioxygenase; TDO=tryptophan-2,3-dioxygenase; KATs=kynurenine aminotransferases; KMO=kynurenine 3-monooxygenase; 3-HAO=3-hydroxyanthranilate-3,4-dioxygenase; QPRT=quinolinate phosphoribosyltransferase; NAD=nicotinamide adenine dinucleotide. (Adapted from Bryleva and Brundin, 2016).

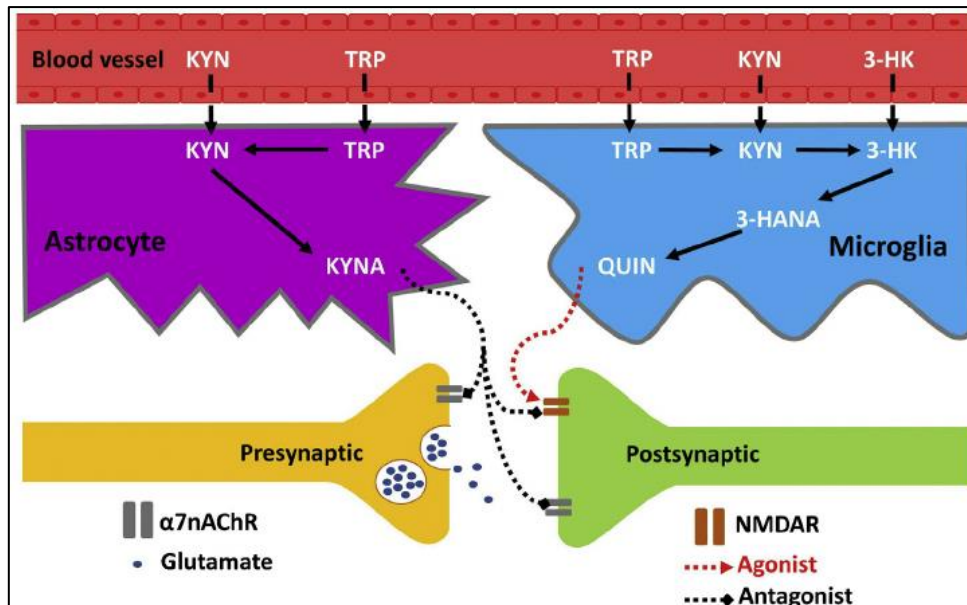


Figure 1.11 Illustration of the effect of kynurenines on NMDA receptors. Illustration of the location of kynurenine pathway metabolites in the brain. Only tryptophan, kynurenine, and 3-HK are able to cross the blood brain barrier. Kynurenic acid is produced within astrocytes, while quinolinic acid is produced within microglia. Kynurenic acid antagonises the NMDA receptor while quinolinic acid is a NMDA receptor agonist. Kynurenic acid also binds to α -7 nicotinic acetylcholine receptors. (Maddison and Giorgini, 2015).

1.7.3.1 3-hydroxykynurenine and quinolinic acid

3-Hydroxykynurenine and quinolinic acid are known to have negative effects on the central nervous system (Guillemin, 2012; Lugo-Huitrón et al., 2013; Reyes-Ocampo et al., 2015). The intermediate metabolite 3-HK has been shown to be responsible for the release of free hydroxyl radicals (Eastman and Guilarte, 1989). Free radicals are unpaired, unstable, charged molecules that destabilize surrounding molecules and have the potential to cause cell death (Pham-Huy et al., 2008). When accumulated free radicals cannot be destroyed by the body, disease occurs (Pham-Huy et al., 2008). 3-HK is the precursor to several other kynurenines including quinolinic acid.

Quinolinic acid is an amino acid that activates the NMDA subtype of glutamate receptors (Stone, 1993) [Figure 1.11]. Excess stimulation of the NMDA receptor causes excitotoxicity that can result in damage to the structure of neurons and cytoskeletons of astrocytes and potentially cell death (Lugo-Huitron et al., 2013). It exists in lower concentrations in the brain than in the blood, though at times of immune response, its levels increase dramatically (Flanagan et al., 1995). It was first shown to have neurotoxic effects by Lapin (Lapin et al.,

1996) who observed convulsions in mice after quinolinic acid injections, and later it was shown to induce oxidative stress (Stone, 1993; Cabrera et al., 2000). Moreover, increased levels of quinolinic acid have been shown to induce neuronal cell death in animal models (Schwarcz et al., 2010). Additionally, the link between inflammation and quinolinic acid is evident. Administration of pro-inflammatory cytokine IL-1 β to the hippocampus in rats potentiates the excitotoxicity of quinolinic acid via induction of IDO, while administration of anti-inflammatory IL-4 reduces quinolinic acid concentrations and its effects via suppression of IDO expression (Musso et al., 1994; Stone and Behan, 2007). Upregulation of quinolinic acid is believed to play a role in many CNS diseases including human immunodeficiency virus (HIV) associated atrophy (Heyes et al., 1998; Kandaneeratchi and Brew, 2012), Huntington's disease (Guidetti et al., 2006; Rahman et al., 2009) and Alzheimer's disease (Kincses et al., 2010; Schwarcz et al., 2010). It is clear that this branch of the kynurenine pathway contributes to harmful biological events.

It is noteworthy that quinolinic acid has varying affinity for differing NMDA receptor subunit combinations in the brain, thus interpretation of levels in the plasma are difficult to interpret. For example, it is selective for NMDA receptors in the neocortex and hippocampus, yet has a weak affinity for receptors in the cerebellum and spinal cord (Perkins and Stone, 1983; Schwarcz and Stone, 2016).

1.7.3.2 Kynurenic acid

Research has shown that kynurenic acid exerts neuroprotective effects on the central nervous system (Chen and Guillemin, 2009; Bryleva and Brundin, 2016). Kynurenic acid is the only endogenous NMDA receptor antagonist, and exhibits anti-excitotoxic properties (Birch et al., 1988; Stone et al., 2013). At low concentrations, it acts on the glycine modulatory site of the NMDA receptor, while at higher concentrations it acts on the glutamate recognition site of the NMDA receptor (Stone and Addae, 2002) [Figure 1.11]. Kynurenic acid acts on other targets too, as it is an antagonist of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and α -7 nicotinic acetylcholine receptors, and selectively activates the GPR35 G-protein coupled receptor (Hilmas et al., 2001; Stone and Addae, 2002; Wang et al., 2006). Early research revealed that increases in kynurenic acid in the brain results in sedative and anticonvulsant effects which have subsequently been shown to be protective against brain ischemia (Carpenedo et al., 1994; Cozzi et al., 1999). Kynurenic acid has also been shown to significantly decrease release of TNF- α in animal

models of septic shock (Riedemann et al., 2003). Decreased levels of circulating kynurenic acid have been reported in depression and suicidality (Myint et al., 2007).

There is evidence that challenges the concept of kynurenic acid as a neuroprotectant. Increased kynurenic acid has been measured in schizophrenia and psychosis patients (Erhardt et al., 2001; Erhardt et al., 2009). It has also been shown that cognitive impairment is associated with elevated kynurenic acid levels (Erhardt et al., 2009; Kozak et al., 2014). While these findings do not enhance the narrative of the neuroprotective effects of this substance, they indicate a role for kynurenic acid in psychiatric and cognitive wellbeing.

A synthetic antagonist of the NMDA receptor, ketamine, is believed to be an analogue to kynurenic acid because of its similar molecular mechanisms and effects on behaviour (Miller, 2013). Administration of ketamine, an NMDA receptor antagonist, results in both antidepressant and psychotomimetic effects (Krystal et al., 1994; Zarate et al., 2005). The similarities between kynurenic acid and ketamine may indicate a role for ketamine in the treatment of disorders where kynurenic acid is shown to be reduced.

1.7.4 Kynurenine pathway alterations in depression

The altered serotonergic transmission witnessed in MDD is hypothesised to be partly due to depletion of its precursor, tryptophan (Maes et al., 2011). Tryptophan reduction alone or in conjunction with shunting of tryptophan metabolism toward the kynurenine pathway branch and away from serotonin production has been suggested as causes (Maes, 2011). Moreover, imbalances of downstream kynurenines have been shown to have negative effects on the central nervous system and immune system, and have also been implicated in depressive behaviour (Mackay et al., 2009; Maes, 2011). Mounting evidence points to the involvement of the kynurenine pathway of tryptophan metabolism in the pathophysiology of depression.

First, depletion of tryptophan is associated with low mood (Van der Does, 2010). Reduced tryptophan has been shown in patients with MDD repeatedly (DeMyer et al., 1981; Hughes et al., 2012; Young, 2013). The susceptibility to low mood following tryptophan depletion has been shown to be greater in women than in men, which is noteworthy considering women are far more likely to experience an episode of depression than men (Richard et al., 2009; Kessler et al., 2010). Clinical trials have shown that tryptophan could be as effective as tricyclic antidepressants in relieving symptoms of MDD (Coppen et al., 1972). Furthermore,

tryptophan and serotonin depletion are induced by IDO and immune mechanisms which are known to play a role in depression pathophysiology (Muller and Schwarz, 2008).

Next, increased circulating levels of 3-HK and quinolinic acid have been observed in depressed patients in previous studies, while reduction of kynurenic acid has also been observed (Maes et al., 2011; Lopresti et al., 2014; Savitz et al., 2015). Increased quinolinic acid has been measured in post-mortem subgenual anterior cingulate cortices of patients with severe depression (Steiner et al., 2011). Other studies found both reduced kynurenic acid and reduced kynurenic acid to kynurenine concentration ratios (Myint et al., 2007). Ratios of kynurenine pathway metabolites are more frequently being used to describe the imbalance of kynurenines. Studies have shown that the quinolinic acid to kynurenic acid concentration ratio is doubled in cerebrospinal fluid (CSF) of suicidal patients compared with healthy controls (Erhardt et al., 2013). Increases of quinolinic acid to kynurenine ratio or reduction in kynurenic acid to kynurenine ratio in MDD patients reflects a preferential metabolism of kynurenine to quinolinic acid while the production of kynurenic acid is neglected (Muller and Schwarz, 2007). Additionally, it is hypothesised that increased production of quinolinic acid could result from imbalances in astrocyte and microglial activation which may contribute to depression pathophysiology (Muller and Schwarz, 2007).

In the central nervous system, quinolinic acid and kynurenic acid are agonists and antagonists of the NMDA receptor respectively, therefore disruption of homeostasis of these metabolites is indicative of disturbed NMDA receptor function (Maddison and Giorgini, 2015). Activation of NMDA receptors could lead to excitatory transmission and could ultimately contribute to emotional disturbances. Research has shown that overstimulation of the NMDA receptor is related to agitated mood, and memory and learning impairments, which are each related to depression (Newcomer, et al., 2000). This excessive activation of NMDA receptors has been shown in animal models of depression, along with observation of increased activity of the glutamatergic system that would result from NMDA receptor activation (Mitani et al., 2006; Ghasemi et al., 2014). Nuclear magnetic resonance spectroscopy studies have shown increased glutamatergic activity in brains of un-medicated depressed patients, too (Sanacora et al., 2004). Furthermore, NMDA receptor antagonism has been shown to raise serotonin concentrations in the brain (Yan et al., 1997). This evidence reveals that disruption of NMDA receptor activity as a consequence of altered kynurenines is likely to play a role in depression pathophysiology. Taken together, dysfunction at each level of the kynurenine pathway observed in patients with MDD indicates its potential involvement in the disorder's pathophysiology.

Table 1.1 Evidence of altered biological systems in Major Depressive Disorder.

System	Change in Depression	Evidence
<i>HPA axis</i>	Increased morning cortisol	Holsboer et al., 2000; Huber et al., 2006; Vreeburg et al., 2009; Dienes et al., 2013; Ulrike et al., 2013
	Blunting of CAR	Stetler and Miller, 2005
	Raised levels of CRH in the CSF	Nemeroff et al., 1984
	Non-suppression during the dexamethasone suppression test	Arana et al., 1985
<i>Inflammation</i>	Increased circulating cytokines (IL-6, IL-1 β , IFN- γ , TNF- α) and CRP	Meta-analyses: Dowlati et al., 2010; Howren et al., 2009
<i>Structural brain changes</i>	Reduced grey matter	Drevets, 2001; Fitzgerald et al., 2008; Rigucci et al., 2010; Sacher et al., 2012
	Reduced hippocampal volumes	Campbell and Macqueen, 2004; Frodl et al., 2008; Mak et al., 2009
<i>Kynurenine pathway</i>	Reduced tryptophan	Mackay et al., 2009; Van der Does, 2010; Maes et al., 2011
	Imbalance of circulating quinolinic acid and reduced kynurenic acid	Myint et al., 2007; Maes et al., 2011; Steiner et al., 2011; Erhardt et al., 2013; Lorpresti et al., 2014; Savitz et al., 2015

Summary of relevant evidence of disrupted HPA axis, inflammation, structural brain changes, and circulating kynurenine pathway alterations in depressed patients.

1.8 Biomarkers for Major Depressive Disorder

Major depressive disorder is a heterogeneous disorder with many proposed aetiologies, though none fully account for the symptoms and subtypes of the disease. Despite the high prevalence of the disorder and number of treatments available, antidepressants only resolve symptoms in some patients (Sinyor et al., 2010). Treatment of MDD becomes a process of trial and error, which patients often endure while remaining severely depressed and sometimes at risk of suicide. Even when antidepressants are effective, several weeks of treatment are required before the full response is achieved. What is more, relapse is more common than not and could be a result of inadequate antidepressant treatment (American Psychiatric Association, 2000). Development of improved diagnostic approaches or development of subtype specific treatments would lead to improved treatment strategies.

Biological markers, also known as biomarkers, are objective, measureable indicators associated with a condition that are used to assess risk, diagnosis, disorder severity, or treatment response (NIH, 2001; Strimbu and Tavel, 2010). Differences in HPA axis, immune system, brain volume, and kynurenine pathway measures have each been observed in depressed populations, yet they are not specific enough to assist in diagnostic processes, especially when considering the distinct subtypes and range of severity that can be exhibited within a diagnosis of depression. Moreover, depression is primarily a disorder of the central nervous system, and the relationship between peripheral markers and CNS is not always clear. Biological samples that are practical to collect, such as blood and saliva, do not necessarily reflect the endocrine, immune, or metabolic occurrences in the brain. On the other hand, collection of cerebrospinal fluid by lumbar puncture is associated with risks (Evans, 1998).

For measurements of neuroendocrine activity, especially the cortisol awakening response, saliva samples have been found to be the most practical type of sample to assess because of the ability of participants to collect samples in different environments and without supervision (Jessop and Turner-Cobb, 2008). The blood brain barrier (BBB) separates the central nervous system from peripheral tissues but does not prevent hormones from entering the brain, which would strengthen the argument for use of saliva as a biomarker for depression (Banks, 2012). Also, the cortisol detected in saliva samples is free cortisol which represents the portion of cortisol that is biologically active, rather than in serum where the total cortisol (bound and free) is measured. For this reason, saliva samples have been considered superior to serum samples in measurement of cortisol (Vining et al., 1983).

However, cortisol levels in saliva are not always correlated to levels of paraventricular CRH, arginine vasopressin, ACTH, or cortisol in blood or urine, leading to poor covariance between perceived stress and measurement of salivary cortisol at times (Hellhammer et al., 2008). While saliva is still the standard sample for measuring free cortisol, these factors must be considered and the use of salivary cortisol alone may be limited as a biomarker.

Most studies reporting immune or metabolic differences in depression use peripheral plasma or serum to measure proteins or metabolites. For example, peripheral cytokines are frequently reported as a measure of inflammation in depression. It has been shown that cytokines can pass the BBB directly and rapidly due to axonal transport systems or simple diffusion (Dantzer and Kelley, 2007). Moreover, cytokines are known to damage the BBB and increase its permeability by damaging tight junctions (Pan et al., 2011). In the case of cytokine measurement, peripheral plasma may be reflective of cytokine activity in the brain. However, when using plasma to measure metabolic processes, the parallels are not so clear. For kynurenine pathway measurement, the concentrations of catabolites in plasma may not be as directly reflective of their concentration in the brain. Kynurenine catabolites such as quinolinic acid and kynurenic acid cannot cross the BBB, yet their precursors, tryptophan, kynurenine and 3-HK, can. Once tryptophan, kynurenine, or 3-HK cross the brain barrier the synthesis of quinolinic acid and kynurenic acid occurs in microglia and astrocytes respectively. While only a fraction of tryptophan crosses the BBB, changes frequently reported in peripheral kynurenine metabolism of depressed patients have been observed similarly in the CSF of patients receiving cytokine immunotherapy (Raison et al., 2010). Also, an earlier study reported that the concentration of quinolinic acid in plasma was correlated to the concentration of quinolinic acid in CSF in rats (Basile et al., 1995). For these reasons, plasma may be a reasonable sample for assessment of CNS immune and metabolic alterations in depression.

PAXgene whole blood mRNA expression has also been used to assess biological markers for MDD in recent research. Peripheral blood has the potential for use as a diagnostic tool for psychiatric conditions as 81.9% of all genes expressed in the brain are co-expressed in human blood cells (Liew et al., 2006). Furthermore, 17 specific brain regions including the hypothalamus and amygdala have been shown to have similar gene expression profiles as whole blood (Sullivan et al., 2006). It has been established that a tissue such as whole blood could in fact give surrogate information on expression in the brain for genes co-expressed between the two tissues (Davies et al., 2009). In particular, whole blood mRNA markers for

inflammation have been a focus of depression research (Hepegul et al., 2013; Hughes et al., 2016).

Finally, neuroimaging studies have also assessed biomarkers in depression. Structural brain changes have been shown repeatedly in depressed patients, including reduced grey matter and hippocampal volumes (Campbell et al., 2004; Rigucci et al., 2010; Sacher et al., 2012). Since structural integrity of brain tissue is related to immune function, measurement of peripheral inflammatory markers in conjunction with volumetric analysis presents an opportunity for development of imaging biomarkers. Each of the tissues discussed have value as indicators of depression pathophysiology. An improved understanding of the specific alterations of these measures in association with depression severity, subtype, or responsivity to antidepressant medication would have significant clinical value in the treatment of MDD.

1.9 Aims and structure of thesis

Despite the prevalence of MDD and the abundance of research that has been conducted to further understand its aetiology, a biological mechanism that accounts for all aspects of the disorder has not yet been substantiated. This study aims to investigate the role of three pathways hypothesised to be involved in depression pathophysiology, and to evaluate the dysfunctions of each of these pathways in relation to each other. The methods of patient recruitment, biological sample collection, laboratory analysis, and statistical analysis are detailed in **Chapter 2** of this thesis. In **Chapter 3**, the activity of the HPA axis is assessed in depressed patients and healthy controls by measurements of salivary cortisol and cortisone concentrations over the course of a day, and through gene expression of 11 β -HSD1 which converts inert cortisone to cortisol. Glucocorticoid resistance associated with chronically elevated cortisol is believed to lead to impaired immune suppression, thus differences in immune system activation between depressed patients and healthy controls are analysed in **Chapter 4**. The circulating concentrations and relative gene expressions of several inflammatory cytokines and C reactive protein are measured in depressed patients and healthy controls as an indicator of immune response. To investigate the consequence of immune dysfunction on brain matter integrity, volumetric analysis of limbic system regions including the amygdala and the hippocampus are explored. Since the HPA axis and the immune system both stimulate the breakdown of tryptophan into kynurenine, **Chapter 5** compares the balance of circulating products of the kynurenine pathway in depressed patients and healthy controls. Associations between measurements of the kynurenine pathway and the previous pathways observed, including the HPA axis, immune system, and brain volumes, are then reported. Furthermore, because of the heterogeneity of MDD, this thesis aims to assess the differences observed in these pathways between varying subtypes and symptom profiles of depression in **Chapter 6**. Finally, **Chapter 7** provides a discussion of this thesis's findings in relation to existing literature, and recommends a direction for future research based on the novel results obtained.

Chapter 2

Materials and Methods

2.1 Materials

Clinical Sample Collection

Blood Collection Set & Holder 19cm, 23Gx3/4"	Cruinn Diagnostics, IRL
Ethylene Diamine Triacetic Acid (EDTA) 9ml vacutainer	Sarstedt, IRL
PAXgene blood RNA tube	Qiagen, UK
Salivette cortisol tubes with synthetic saliva swab	Sarstedt, IRL
Urine container 2L	Sarstedt, IRL

General Laboratory Chemicals

β -Mercaptoethanol	Sigma Aldrich, IRL
2-Propanol	Sigma Aldrich, IRL
3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma Aldrich, IRL
Bovine Serum Albumin 96% (BSA)	Sigma Aldrich, IRL
Potassium Chloride (KCl)	Sigma Aldrich, IRL
Dihydrogen phosphate (KH_2PO_4)	Sigma Aldrich, IRL
Sodium dihydrogen phosphate (NaH_2PO_4)	Sigma Aldrich, IRL
Sulphuric Acid 98% (H_2SO_4)	VWR International, IRL
Tween-20	Sigma Aldrich, IRL

General Laboratory Plastics and Hardware

Eppendorf tubes	Sarstedt, IRL
Falcon tubes (15 ml)	Sarstedt, IRL
Falcon tubes (50 ml)	Sarstedt, IRL
Glass Inserts	Labquip, IRL
Glass Vials	Labquip, IRL
Hypodermic needle 13mm 26G	Becton and Dickenson, UK
Mini tubes (0.5 ml)	Sarstedt, IRL
P20, P200, P1000 Micropipettes	Fisher Scientific, UK
Microtubes (1.5 ml)	Sarstedt, IRL
Multichannel Pipette (30-300 μL)	Fisher Scientific, UK
Nylon filters (0.45 μm)	Nalgene, UK
Para-film Laboratory Rolls	Sarstedt, IRL
Pasteur Pipettes	Sarstedt, IRL
Pipette tips (10, 200, 1000 μL)	Sarstedt, IRL

Plastic Syringe (1 ml)	Becton Dickenson, UK
Salivette tubes	Sarstedt, IRL
ELISA	
Adhesive plate covers	ThermoFisher Scientific, UK
Cortisol ELISA kit	ENZO Life Sciences, UK
CRP ELISA Duoset	R&D systems, UK
Human IFN- γ ELISA MAX [™] Deluxe Kit	Biolegend, UK
Human IL-1 β ELISA MAX [™] Deluxe Kit	Biolegend, UK
Human IL-6 ELISA MAX [™] Deluxe Kit	Biolegend, UK
Human TNF- α ELISA MAX [™] Deluxe Kit	Biolegend, UK
Nunc-Immuno MicroWell 96 well solid plates	Sigma Aldrich, IRL
HPLC	
3-Hydroxyanthranilate	Sigma Aldrich, IRL
3-Hydroxykynurenine	Sigma Aldrich, IRL
Acetonitrile	Sigma Aldrich, IRL
Glacial Acetic Acid	Fisher Chemicals, UK
HPLC Grade Water	Fisher Chemicals, UK
Kynurenic Acid	Sigma Aldrich, IRL
L-Kynurenine	Sigma Aldrich, IRL
L-Tryptophan	Sigma Aldrich, IRL
N-methy-5-HT	Sigma Aldrich, IRL
Perchloric Acid	VWR International, IRL
Quinolinic Acid	Sigma Aldrich, IRL
Zinc Acetate	Sigma Aldrich. IRL
PCR	
Fast 96 well optical reaction plates	Applied Biosystems, UK
Filter pipette tips (10, 200, 1000 μ l)	Sarstedt, IRL
High capacity cDNA archive kit	Applied Biosystems, UK
Molecular grade water	Sigma Aldrich, IRL
Molecular grade Absolute Ethanol	Sigma Aldrich, IRL
Optical adhesive covers	Applied Biosystems, UK

PAXgene blood RNA kit	Qiagen, UK
RA1 lysis buffer	Macherney-Nagel, GER
RNA extraction kit	Macherney-Nagel, GER
RNase Zap Wipes	Ambion, UK
RNase-free 1.5 and 2 ml microfuge tubes	Ambion, UK
RNase-free H2O	Sigma Aldrich, IRL
Taqman® Fast Advanced master mix	Applied Biosystems, UK
Taqman® gene expression assays	Applied Biosystems, UK

2.2 Participant Recruitment

2.2.1 Tallaght Hospital Psychiatric Services

Depressed patients were recruited from the psychiatric outpatient clinic at Sheaf House in Tallaght Hospital, Tallaght, Dublin 24 which is the headquarters for the Tallaght area Community Mental Health team. Study participants were also recruited from the Mary Mercer Health Centre, Jobstown, Dublin 24, which is a primary care clinic for the outpatient department of psychiatric services in Tallaght. Both centres are administrated by the Health Services Executive (HSE), the national public health service for Ireland. Patients who presented to these clinics with depression were considered for the study. They were evaluated for study eligibility based on fulfilment of the Diagnostic and Statistical Manual 5 (DSM-5) criteria for a Major Depressive Disorder on a score of 17 or above on the Hamilton Depression (HAM-D) Rating Scale which were assessed during a clinical interview by consultant psychiatrist, Dr. Veronica O'Keane. The patients received information regarding the study and were encouraged to ask questions (Appendix 2a). If eligible and willing to participate, a consent form was signed. (Appendix 2b).

2.2.2 Inclusion criteria for recruitment of MDD patients

Males and females ≤ 45 years of age were included in this study. A diagnosis of Major Depressive Disorder according to the DSM-5 using the Mini-International Neuropsychiatric Interview (MINI) was required for inclusion. Additionally, a score of 17 or above on the HAM-D was mandatory. Patients were required to be fluent in English to give fully informed consent.

2.2.3 Exclusion criteria for recruitment of MDD patients

Patients were excluded from participation in this study if they were known to have any chronic medical conditions or were on any medication other than antidepressants and the oral contraceptive pill. No participant was undergoing psychological therapy at the time of recruitment. Patients with substance abuse disorders or psychotic illnesses were also excluded from participation in the study. Medical records as well as psychiatric interview

were used to rule out a history of these conditions. No standardized assessment was used to determine other chronic health disorders or psychiatric disorders, which is a limitation.

2.2.4 General participant information form

Upon recruitment, all participants completed a form that included contact information, demographic details such as education level, marital status, and race. Education level was scored based on the most recent version of the International Standard Classification of Education (IESCD) standardised scale that designates a score from 0-8 based on level of completed education. Clinical data such as height, weight, and medication use were also collected on this form (Appendix 3a).

2.2.5 Hamilton Depression Rating scale

The severity of depression was assessed in patients using the 21-question HAM-D (Appendix 3b). This scale has been validated and standardised as a measure of depression, and includes questions that address a range of symptoms and experiences including suicidal ideation, insomnia, anxiety, somatic symptoms, and more (Hamilton, 1960; Hamilton, 1980). The total HAM-D score is calculated as a sum of the first 17 questions, while the remaining four provide supplementary clinical information. This rating scale was completed by the researcher during a clinical interview of the patient, and validated by a clinician.

2.2.6 Mini-International Neuropsychiatric Interview (MINI) Modules A-C

The MINI Interview of DSM Disorders is a brief, structured interview developed for the major Axis I psychiatric disorders including MDD (Sheehan et al., 1998). For this study, the researcher completed modules A-C which are (A) Major Depressive Episode and Major Depressive Episode with Melancholic Features, (B) Dysthymia, and (C) Suicidality in the evaluation of each participant. For participation in this research study, the patient was required to meet diagnostic criteria for a Major Depressive Episode. (Appendix 3c).

2.2.7 Classification as atypical subtype of MDD

Each patient was assessed for criteria that would further classify their depression as the atypical subtype or not. To be defined as “atypical subtype”, the participant would have to have shown mood reactivity, or brightening of mood in response to potential or actual or positive events, and at least two of the following: weight gain or increase in appetite, hypersomnia, leaden paralysis, and/or longstanding interpersonal rejection sensitivity. This was assessed by psychiatric interview.

2.2.8 Recruitment of healthy controls

Healthy controls were recruited to the study by advertisement on the Trinity College campus and by word of mouth in local communities. Males and females ≤ 45 years of age were eligible to participate in the study if they had no prior history of mental illness, were medically healthy, and were not on any medications other than the oral contraceptive pill. Participants were required to be fluent in English to give informed consent.

2.2.9 Power calculation and recruitment outcome

A statistical power analysis was performed for sample size estimation during the recruitment process based on data from a pilot study within the REDEEM group (N=48), comparing wakening salivary cortisol concentrations measured by ELISA between depressed patients and healthy controls. The effect size (ES) in this study was 1, which is considered to be a large ES using Cohen's (1988) criteria. With an $\alpha=0.05$ and power=0.80, the projected sample size needed with this effect size (GPower 3.1) is approximately N=100 (50 per group) for this simplest between group comparison.

In total, 72 patients with MDD and 60 healthy controls were recruited for participation in this study per the preceding inclusion and exclusion criteria. Consistent with recruitment criteria, the mean HAM-D score of the depressed group was significantly higher than that of the healthy controls [Table 2.1]. Each chapter's analysis focuses on a subset of the total recruitment's depressed patients and healthy controls, depending on which biological samples they provided to the study. Therefore, clinical and demographic data will be presented with each chapter that specifically corresponds with each type of analysis.

2.3 Self-rating scales

Patients were asked to complete three self-rating scales to further quantify their depressive symptoms and other factors that might be related to mood. All rating scales were completed under the supervision of a researcher.

2.3.1 Centre for Epidemiological Studies – Depression

The Centre for Epidemiological Studies – Depression (CES-D) scale is a 20-item self-report Likert scale (Appendix 3d). It is completed by the participant at the time of recruitment to assess the perceived severity of their depression symptoms (Radloff, 1977). Questions refer to symptoms associated with depression, including restless sleep, poor appetite, and feelings of loneliness. Response options range from 0 to 3 for each item (0 = “Rarely or None of the Time”; 1 = “Some or Little of the Time”; 2 = “Moderately or Much of the time”; 3 = “Most or Almost All the Time”). Scores range from 0 to 60, with higher scores indicating greater depressive symptoms.

2.3.2 Pittsburgh Sleep Quality Index

The Pittsburgh Sleep Quality Index (PSQI) is a questionnaire pertaining to the participant’s sleep habits over the course of the last month (Buysse et al., 1989). It contains 19 questions that generate seven “component” scores including subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use sleep medication, and daytime dysfunction. The sum of the seven “component” scores results in a global score ranging from 0 to 21, for which a higher score indicates lower sleep quality. (Appendix 3e).

2.3.3 Childhood Trauma Questionnaire

The Childhood Trauma Questionnaire (CTQ) is a standardised, 28-item self-report instrument which assesses five categories of childhood maltreatment including emotional, physical, and sexual abuse, and emotional and physical neglect (Bernstein et al., 2003). The questionnaire is comprised of five questions for each subscale of childhood maltreatment, in addition to 3 items meant to evaluate minimization and denial to identify participants who might be

under reporting traumatic events. Participants scoring in the moderate to severe range in at least one of the items were classified as having a positive history of childhood adversity. The CTQ global scores which combine the scores of all five categories are also used in analysing the study population (Appendix 3f).

Table 2.1 Demographic data for all patients with MDD and all healthy controls recruited to the study.

	Patients (n=72)	Controls (n=60)	Statistics (p-value)
Age (years)	27.69 (8.42)	28.02 (5.95)	Z=-0.815, p=0.415
Gender (female/male)	48/24	37/23	$\chi^2=0.357$, p=0.550
BMI	24.47 (5.77)	23.32 (3.54)	Z=-0.714, p=0.475
Smoking (Yes/No)	30/42	12/48	$\chi^2=7.082$, p=0.008*
ISCED Education	3.43 (1.43)	6.25 (1.51)	Z=-7.439, 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) where appropriate. *p $\leq$ 0.05 vs. controls. ***p $\leq$ 0.001 vs. controls.

Table 2.2 Clinical data for all patients with MDD and all healthy controls recruited to the study.

	Patients (n=72)	Controls (n=60)	Statistics (p-value)
HAM-D	22.92 (5.02)	2.76 (2.68)	Z=-9.606, p<0.001***
CES-D	39.20 (9.41)	6.43 (6.29)	Z=-9.606, p<0.001***
PSQI	13.14 (3.55)	4.16 (2.49)	Z=-9.114, p<0.001***
CTQ Global Score	44.67 (16.44)	30.14 (7.49)	Z=-5.418, p<0.001***
Early Life Adversity (Yes/No)	45/23	11/46	$\chi^2=27.555$, p<0.001***
Medication (free/SSRI/SNRI/other)	30/26/9/7		

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (HAM-D, CES-D, PSQI, and CTQ Global Score), and Chi squared (χ^2) test (ELA) where appropriate. ***p $\leq$ 0.001 vs. controls.

2.4 Sample collection and storage

2.4.1 Saliva sample collection and storage

Saliva samples were collected in Salivette® tubes (Sarstedt) by the study participant at five time points throughout the course of one day. These time points were 0, 30, and 60 minutes after waking, and 12 and 12.5 hours after waking. Sample collection always took place on a weekday. The completed sets of samples were returned in person or by post as soon as possible. Once the completed set of saliva samples was received, they were centrifuged for 10 minutes at 3,000rpm and room temperature. The insert was then removed discarded from the salivette. The saliva was aliquoted into 500µL samples and stored in microtubes at -80°C until further analyses were carried out.

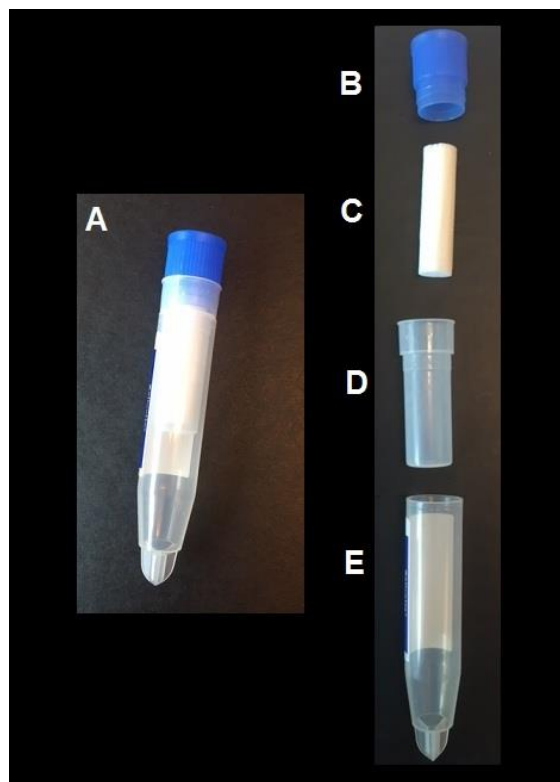


Figure 2.1 Diagram of salivette tubes for saliva sample collection. Participants were given five salivettes (A) with time points labelled on the outside of the tube. At the appropriate times, participants were instructed to remove the cap (B) and the swab (C), then place the swab in their mouth and under their tongue for 30 seconds or until the swab was saturated. The swab was then replaced into the inner tube (D). Centrifuging the salivettes resulted in extraction of the saliva from the swab, through the inner tube which acts as a filter, and into the outer tube (E). Finally, the saliva was aliquoted from the outer tube into microtubes and subsequently frozen.

2.4.2 EDTA blood collection for plasma

Two 9 ml Ethylenediaminetetraacetic acid (EDTA) tubes of blood were collected from all participants at the time of recruitment or on the day of their MRI scan. Within two hours of collection, the EDTA tubes were centrifuged for 15 minutes at 3,500rpm and room temperature to generate plasma. The plasma was then aliquoted into amounts of 500µL and stored in labelled microtubes at -80°C until required for ELISA, HPLC, or LC-MS analysis.

2.4.3 PAXgene whole blood collection

Collection of 2.5 ml of blood was taken into PAXgene RNA tubes. These tubes contain a proprietary solution which allows for the preservation of RNA in whole blood. Filled PAXgene tubes were stored at room temperature (20°C) for 48 hours, moved to -20°C for 48 hours and finally to -80°C in order to gradually reduce temperature. Labelled tubes were stored at -80°C until RNA extraction was performed.

2.4.4 Magnetic Resonance Imaging (MRI)

Structural MRI scans were acquired at Trinity College Institute of Neuroscience within a week of recruitment to the study. Participants were screened for MRI safety using a checklist provided by the institution and then scheduled for a one-hour scan. Magnetic resonance images from each participant were obtained with a Philips Achieva MRI scanner (Philips Medical System, Netherland BV, Veenphuis 4-6, 5684 PC Best, Netherlands) operating at 3 Tesla. 180 axial high-resolution T1-weighted SPGR images (TE=3.8ms, TR=8.4ms, FOV 230mm, 0.898 x 0.898 mm² in-plane resolution, slice thickness 0.9mm, flip angle=8°) were acquired for brain volume analysis. The approximate acquisition time was 6.5 minutes. The MRI sequence also included a resting state scan, diffusion tensor imaging (DTI), and a functional MRI (fMRI) task. Functional imaging and DTI results are not presented in this thesis.

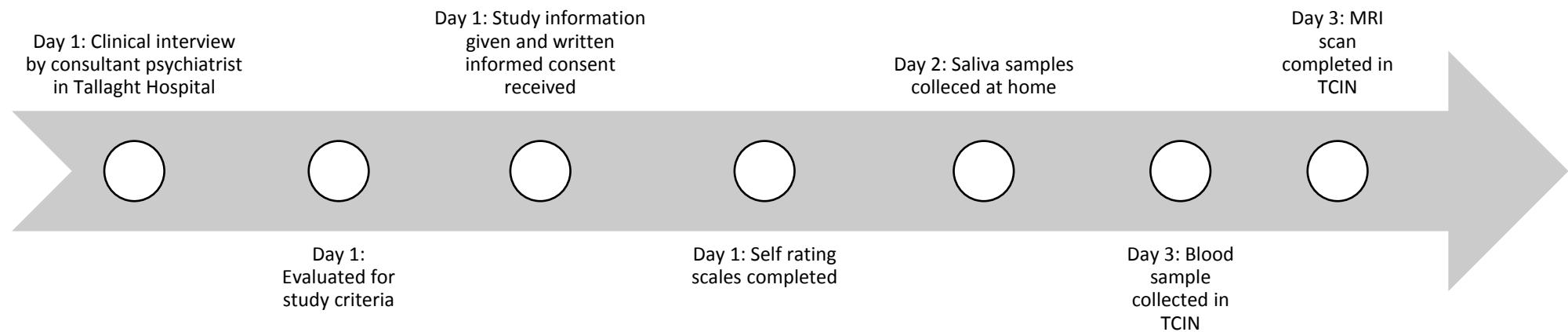


Figure 2.2 Timeline of individual patient recruitment and biological sample collection. Timeline used for completing patient recruitment and biological sample collection. Participants were evaluated for study criteria based on their clinical interview on Day 1, and then given detailed information regarding the purpose and protocols of the study. After informed consent was received, saliva sample collections were completed by the participant in their home on Day 2, and returned in person to TCIN on Day 3. Finally on Day 3, a blood sample was obtained and MRI scan completed.

2.5 Salivary stress hormone quantification

2.5.1 Enzyme-linked immunosorbent assay (ELISA) for cortisol

Saliva samples were analysed for cortisol content using a Cortisol ELISA Kit (Enzo Life Sciences). This kit is used for the quantitative measurement of cortisol by using a monoclonal antibody that cortisol in the saliva sample becomes bound to. Standards dilutions were prepared within 60 minutes of performing the assay from a 100,000 pg/ml cortisol standard solution to the following concentrations: 10,000, 5,000, 2,500, 1,250, 625, 313 and 156 pg/ml with assay buffer as the diluent. Saliva samples were diluted with 1 part a steroid displacement reagent for every 99 parts sample, and then samples were diluted 1:3 with assay buffer. 100 µL of standards and samples were added to the appropriate wells of plates coated with goat antibody specific to mouse immunoglobulin G. Next, 50 µL of assay buffer was added to the nonstandard blank (NSB) wells, while 50 µL of blue conjugate is added to all cells except blanks and the total activity (TA) well. Subsequently, 50 µL of a mouse monoclonal antibody was added to each well except blanks, NSB, and TA wells and then plates were incubated for 2 hours at room temperature and 500 rpm on a plate shaker. Three washes were performed using 400 µL wash solution in each well. Then 5 µL of alkaline phosphatase conjugate was added to the TA wells, and 200 µL of pNpp Substrate solution to every well. Plates were then incubated at room temperature for 1 hour without shaking. Finally, 50 µL of a stop solution composed of trisodium phosphate in water was added to every well. The plate absorbance was read at 405 nm using a microplate reader (Sunrise Tecan, Reading, UK) and Gen5 Microplate Reader Software (BioTek Instruments, Inc.), then recalculated as a concentration (pg/ml) using standard curves derived using GraphPad Prism Software Version (6.0) (GraphPad Software, Inc).

2.5.2 Liquid Chromatography – Mass Spectrometry for salivary cortisol and cortisone

Liquid Chromatography – Mass Spectrometry (LC-MS) was performed at the University of Manchester in the Department of Clinical Biochemistry. Work was conducted by members of Professor Brian Keevil's laboratory at the University Hospital of South Manchester. The following protocol (sections 2.5.2.1. through 2.5.2.3.) has been adapted from LJ Owens et. al. 2010 which describes these methods.

2.5.2.1 Sample preparation for LC-MS

Stock solutions were prepared by dissolving hydrocortisone (Sigma, UK) in methanol (Sigma, UK) and were stored at -20°C. A separate stock solution was prepared for standards and quality controls (QCs), both 10 mg/ml. Working standards and QCs were prepared by diluting the stock solution with phosphate buffered saline (PBS) pH 7.4 (Sigma, UK) to give concentrations of 0, 3.4, 17, 34, 67 and 100 nM and 5.6, 28 and 56 nM, respectively. Aliquots of these were stored at -30°C. Deuterated cortisol (D2) was used as internal standard (CDN Isotopes, Canada) at a working concentration of 30 mg/L in methanol. Standard, QC or sample (200 µl) was manually pipetted directly to the well of a 96-deep-well block (Abgene, UK). To this, 25 µl of working strength internal standard was added. The block was thermosealed (Abgene, UK) and vortexed for one minute, then centrifuged at 1500g for 10 min. Following centrifugation, the block was transferred directly to the autosampler for analysis; 50 µl of sample was injected into the liquid chromatography (LC) system using partial loop mode. (Owen et al., 2010).

2.5.2.2 Chromatography

A Shimadzu Prominence LC system (Shimadzu, Milton Keynes, UK) was used for chromatography. The mobile phases utilized were: A, water with 2 mmol/L ammonium acetate (Sigma, Poole, UK) and 0.1% formic acid (VWR International Ltd, Leicestershire, UK); and B, methanol (LC-MS grade, Riedel-de Haen, Hanover, Germany) with 2 mmol/L ammonium acetate and 0.1% formic acid. The sample was injected onto a Phenomenex C8 4 x 2 mm guard cartridge (Phenomenex, Macclesfield, UK) using 10% mobile phase B at a flow rate of 1 ml/min for 0.6 min. The eluate from the guard cartridge during this time was diverted to waste to allow sample clean-up and removal of saliva matrix before elution onto the analytical column.

After 0.6 min, the mobile phase composition was stepped up to 60% B and flow rate reduced to 0.4 ml/min to elute the cortisol from the guard cartridge. The eluate was directed onto the analytical column, a Phenomenex 30 x 3 mm 4 µm Synergy Hydro-RP C18. The flow was maintained at 60% B until 2.5 min when it was stepped up to 100% B for 0.7 min before returning to 60% B for the final 0.8 min. The small delay between injections (40 s) on this system was enough to re-equilibrate the guard cartridge with 10% B while maintaining the

analytical column at 60% due to the initial divert to waste. Both guard and analytical columns were maintained at ambient temperature. (Owens, 2010).

2.5.2.3 Mass spectrometry

The eluate was injected directly into a Quattro Micro™ tandem mass spectrometer (Waters, Manchester, UK) with a Z spray ion source. MassLynx NT 3.5 software was used for system control, the MassLynx QuanLynx programme allowed data processing. This software used the height of the detected peaks, $1/x$ weighting and linear least-squares regression to produce a standard curve. The $1/x$ weighting gives greater accuracy at lower concentrations. The mass spectrometer was operated in electrospray positive mode, the capillary was maintained at 1 kV and the source temperature was 140°C. The desolvation temperature and gas flow were 400°C and 1000 L/h, respectively. The most abundant transitions identified for cortisol and D2-cortisol using 1 mg/L tuning solutions of each compound in 50% mobile phases were m/z 363.2 > 121.1 and 365.1 > 122.2, respectively. The cone and collision energies were 26 V and 22 eV for cortisol and 22 V and 22 eV for D2-cortisol. A confirmatory ion transition for cortisol was monitored at m/z 363.2 > 97.0. Transitions were monitored in multiple reaction monitoring (MRM) mode, with a dwell time of 0.15 s. (Owens, 2010).

2.5.3 Calculation of Cortisol Awakening Response parameters

Data cleaning was carried out by eliminating values that were above or below two standard deviations from the mean; however there were no outliers found in the morning time point LCMS data. Cortisol awakening response (CAR) parameters were derived from both ELISA and LC-MS concentration data using Area Under the Curve (AUC) and other calculations (Fekedulegn, 2007). These parameters included 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). Equations used for calculation are further described in Table 2.3. All CAR parameters were calculated using data from the first three saliva collection time points at 0, 30, and 60 minutes after waking.

Table 2.3 Cortisol awakening response parameter calculations.

Parameter	Definition	Formula
AUCg	Area under the curve with respect to ground	$= \{[(T30 \text{ cortisol value} + T0 \text{ cortisol value})/2] \times \text{time difference}\} + \{[(T60 \text{ cortisol value} + T30 \text{ cortisol value})/2] \times \text{time difference}\}$
AUCi	Area under the curve with respect to increase	$= \text{AUCg} - [T0 \text{ cortisol value} \times (\text{total time between first and last measurement})]$
Peak	Highest concentration of cortisol recorded	$= \text{Maximum cortisol value}$
TBP	Time from baseline to peak	$= \text{Minutes until peak cortisol measurement occurred}$
Reactivity	Change in cortisol between first and last morning measurement	$= T60 \text{ value} - T0 \text{ value}$
Slope	Slope of the regression line fitted through raw cortisol data	$= \text{correlation coefficient } r \times [\text{SD of } y \text{ (cortisol) values} / \text{SD of } x \text{ (time) values}]$
Intercept	Intercept of the regression line fitted through raw cortisol data	$= \text{the mean of cortisol values} - (\text{slope} \times \text{mean of } x \text{ values})$

Description of cortisol awakening response parameters and their calculations. SD=Standard deviation.

2.5.4 Selecting a method for glucocorticoid measurement: ELISA versus LC-MS

A methodology study comparing ELISA and LCMS measurement of salivary cortisol performed on 24 healthy control samples revealed the benefit of using LC-MS for glucocorticoid concentration determination. While there are advantages to each methodology (Baecher, 2013), the ELISA method of cortisol measurement is known to include unspecific binding of molecules with a similar structure, such as the glucocorticoid cortisone (ENZO). Cortisol concentration data from each method was highly correlated ($r=0.449$, $p=0.024$), however, due to unspecific binding, the concentrations derived from the ELISAs were significantly higher at every time point compared to the concentrations derived from the more specific LC-MS method [Figure 2.3]. It was not possible to discern what percentage of the detected corticosteroid represents cortisol versus non-specifically bound cortisone or other glucocorticoids with the ELISA method, while the LC-MS method is capable of measuring concentrations of cortisol and cortisone independently. The uncertainty of the extent of unspecific binding in the ELISA method resulted in the decision to proceed with the LC-MS method for analysis of cortisol for the remainder of this study.

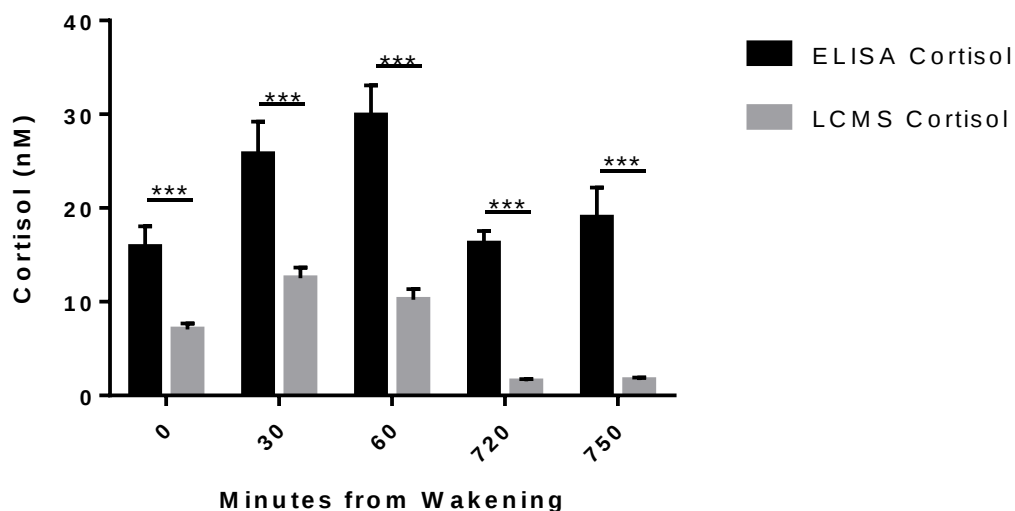


Figure 2.3 Cortisol concentrations measured by ELISA and LC-MS. Concentrations of cortisol measured in the same healthy controls by both ELISA and LCMS are depicted above ($n=24$). Paired t-test carried out on log transformed cortisol data ($***p<0.001$).

2.6 Enzyme-linked immunosorbent assays for protein measurement

2.6.1 Cytokine analysis by Enzyme-linked immunosorbent assay (ELISA)

Human IL-6, IL-1 β , TNF- α , and IFN- γ were quantified by sandwich ELISA using BioLegend ELISA MAX™ Deluxe kits as per manufacturer's instructions for EDTA plasma. The capture antibodies for IL-6, IL-1 β , TNF- α , and IFN- γ were diluted 1:200 in 1X Coating buffer. Then 100 μ L of this capture antibody solution was added to all wells of the 96-well maxisorb ELISA plates then incubated overnight at 4°C. Plates were washed 4 times with 300 μ L/well PBS + 0.05% Tween 20 and blocked with 200 μ L/well 1 X assay diluent for 1 hour at room temperature with shaking at 200 rpm, to eliminate non-specific binding. Plates were washed 4 more times and 100 μ L of either samples or standards were added to the appropriate wells to incubate for 2 hours at room temperature with shaking.

Plasma samples were run neat, and standards were prepared as per kit instructions. For IL-6, TNF- α , and IFN- γ , six two-fold serial dilutions of the 500pg/ml top standard will be carried out with 1X assay diluent. For IL1- β , a top standard of 125pg/ml was used. The detection limits for these assays were 1.95 pg/ml.

Following 4 more washes, 100 μ L of detection antibody, diluted 1:200 in 1X assay diluent, was added to each well and incubated for 1 hour at room temperature with shaking. This was followed by four additional washes and the addition of 100 μ L/well Avidin-Horseradish peroxidase (HRP), diluted 1:10,000 in 1X assay diluent and incubated for 30 minutes at room temperature with shaking. Five final washes were completed and 100 μ L of freshly mixed TMB substrate solution added to each well and incubated in the dark until the wells turn blue in colour (approximately 10-20 minutes depending on which cytokine). The addition of 100 μ L/well 2NH₂SO₄ was used stop the reaction. Absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and Gen5 Microplate Reader Software (BioTek Instruments, Inc.), then recalculated as a concentration (pg/ml) using standard curves derived using GraphPad Prism Software Version (6.0) (GraphPad Software, Inc).

2.6.2 ELISA for CRP

Human CRP was quantified by sandwich ELISA using Quantinine ELISA kits (R&D Systems, UK). Plasma samples were diluted 1:10,000. Then 100 μ L/well of capture antibody was diluted 1:180 in 1X PBS and incubated at room temperature overnight. Following 3 washes

with PBS + 0.05% Tween 20 the plates were blocked for 1 hour at room temperature with 300 µl/well of 1% bovine serum albumin (BSA). After 3 washes, 100µl of diluted samples or standards were added to each well and incubated for 2 hours at RT. Two-fold serial dilutions were prepared from a top standard of 1,000 pg/ml. This was followed by 3 washes as before and 100 µl/well of detection antibody diluted 1:180 in 1% BSA was incubated for 2 hours at RT. Next, 3 additional washes were completed and 100 µl avidin-HRP, diluted 1:200 in 1% BSA, was added to each well and incubated for 20 minutes in the dark. Following 3 washes, the plates were incubated in the dark for 20 minutes with TMB (100 µl/well). The addition of 100 µl per well of 2NH₂SO₄ stopped the reaction. The absorbance was read at 450 nm using a microplate reader (Sunrise Tecan, Reading, UK) and Gen5 Microplate Reader Software (BioTek Instruments, Inc.), and was then recalculated as a concentration (mg/L) using standard curves derived using GraphPad Prism Software Version 5.0 (GraphPad Software, Inc). The detection limit for this assay was 0.8 pg/ml.

2.7 Kynurenine pathway metabolite quantification

2.7.1 High performance liquid chromatography (HPLC)

Tryptophan and kynurenine pathway metabolites were measured by HPLC coupled with UV and fluorometric detection from plasma. Kynurenine was detected at a wavelength of 250 nm by PDA-UV detection (SPD-M10AVP, Shimadzu). Tryptophan, kynurenic acid (KYNA) and 3-hydroxyanthranilic (3-HAA) concentrations were measured fluorometrically (RF-10A XL, Shimadzu) at an excitation wavelength of 254 nm and an emission wavelength of 405 nm.

2.7.1.1 HPLC sample and buffer preparation

Mobile phase buffer consisted of 50 nM glacial acetic acid, 100 mM zinc acetate (Sigma Aldrich) and 3% acetonitrile dissolved in double-distilled NANO-pure HPLC grade water (Sigma Aldrich) at pH 4.9. Homogenisation buffer was prepared using mobile phase buffer with the addition of 6% perchloric acid to deproteinise the samples and 20 µg/20µl N-methyl 5-hydroxy-tryptamine (Sigma Aldrich) as internal standard. Plasma was centrifuged for 10 minutes at 12,000 rpm and 4°C to clean the samples. Then 300 µl of plasma was added to 300 µl homogenisation buffer, which was vortexed vigorously and then centrifuged at 14,000 rpm for 20 minutes and 4°C. The supernatants were then extracted with a hypodermic needle (Becton and Dickinson, UK) and passed through syringe filters (non-sterile 0.45 µm nylon, Nalgene, UK) and transferred to new 1.5 ml tubes on ice. Samples were analysed immediately.

The standards used for HPLC were N-methyl 5-HT (internal standard), L-Kynurenine, L-Tryptophan, KYNA, 3HK, 3-HAA, and Quinolinic Acid (Sigma Aldrich). Standard stock solutions of 10 mg/10 ml were prepared by dissolving the standards in 10 ml of HPLC mobile phase. These solutions were stored at 4°C. Using the stock solutions, a 10 ml standard mix containing 200 ng/20µl of each standard was prepared.

2.7.1.2 Measurement of kynurenine pathway metabolites

Samples and standards mixed with homogenisation buffer were transferred into individual 0.2 ml conical inserts (Labquip, IRL) within 2 ml glass vials (Labquip, IRL). HPLC analysis was conducted with an automated HPLC system (Shimadzu ADVP module). A 20 µl volume of

sample was injected onto a reverse phase analytical column (Kinetex Core Shell Technology, Phenomenex, UK) with specific area of 100 mm x 4.6 mm and particle size of 2.6 μ m and fitted with a guard column (Lichrosorb RP18, specific surface area 30 x 4 mm, Phenomenex, UK) for separation of metabolites. The flow rate was set to 0.8 ml/min (LC-10AT pump, Shimadzu) and the acquisition time was 18 minutes. A standard mix was injected every seven samples to recalibrate the system and reduce divergences in the retention times during sample runs. The auto-sampler was also pre-programmed to self-rinse with methanol between each sample injection. CLASS-VP software (Shimadzu) was used to produce chromatographs, and for data collection and processing. Retention times and peak heights were recorded from the chromatographs generated and then used to calculate concentrations of the metabolites in the samples. Example chromatographs for a standard mix and a sample are displayed in Figure 2.4. Concentrations were quantified using relative response factor calculations in reference to the internal standard and expressed as nM concentrations.

2.7.2 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Frozen 50 μ L plasma samples were shipped on dry ice from TCIN to Boehringer Ingelheim, laboratory of Dr. Andre Liesener for measurement of kynurenine pathway metabolites by LC-MS/MS. Quantification of plasma levels of tryptophan and its catabolites kynurenine, 3-hydroxykynurenine (3-HK), quinolinic acid, and kynurenic acid was performed 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 was quantified separately. Both assays comprised sample clean-up by 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, namely [D5] tryptophan, [D3] 3-hydroxykynurenine, [D5] kynurenic acid, [D4] kynurenine and [D3] quinolinic acid as internal standards.

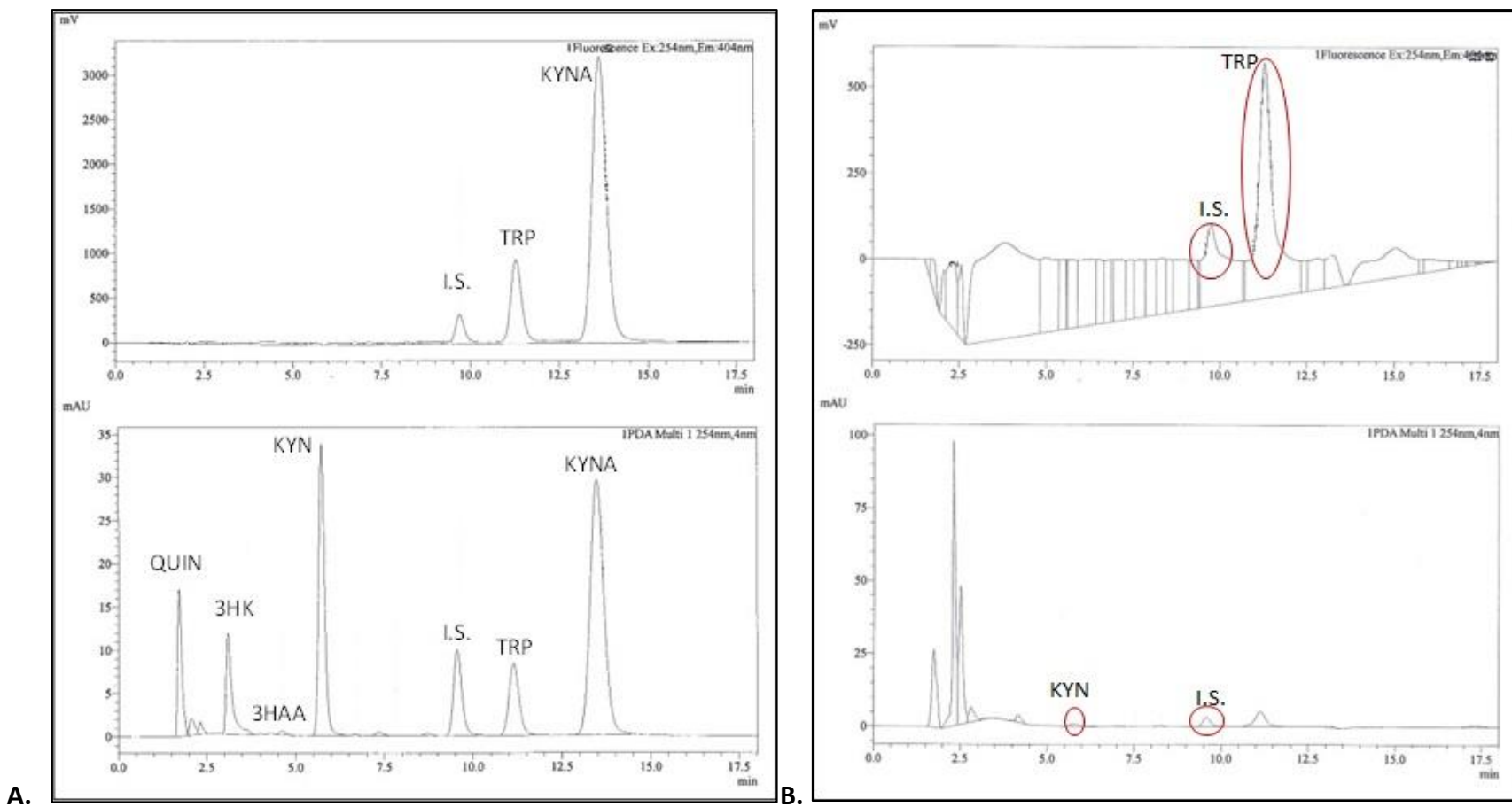


Figure 2.4 Example HPLC chromatographs. An example of fluorescent and PDA chromatographs derived from a standard mix (A), and an example of fluorescent and PDA chromatographs derived from a random control sample (B). Identified sample peaks used for concentration calculations circled in red. QUIN= quinolinic acid, 3HK=3-hydroxykynurenine, 3HAA=3-hydroxyanthranilic acid, KYN=kynurenine, I.S.=internal standard, TRP=tryptophan, KYNA=kynurenic acid.

2.7.3 Selecting a method for measurement of KP metabolites: HPLC or LC-MS/MS

Initially, HPLC was used to assess tryptophan and kynurenine pathway activation between depressed patients (n=59) and healthy controls (n=33). This method was effective for determining tryptophan and kynurenine concentrations, and revealed significant depletion of tryptophan among depressed patients (MDD: 8602.14 ± 221.49 ng/ml; HC: 9611.54 ± 376.56 ng/ml) ($Z=-1.965$, $p=0.049$). Concentrations of kynurenic acid, 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), and quinolinic acid were not possible to derive from the chromatographs generated by this method due to inconsistent or absent retention peaks [Figure 2.4], therefore the LC-MS/MS method was implemented for all future kynurenine pathway analyses in order to gain insight into the activity of the entire pathway. The LC-MS/MS method quantified tryptophan, kynurenine, 3-HK, quinolinic acid, and kynurenic acid concentrations and enabled a more thorough assessment of tryptophan catabolism. LC-MS/MS methods have been validated for quantification of kynurenine pathway metabolites in plasma, and have been shown to produce accurate, precise, and sensitive results (Huang et al., 2013; Fuertig et al., 2016).

2.8 Real-time polymerase chain reaction (RT-PCR) for whole blood mRNA quantification

2.8.1 Isolation of total RNA from human whole blood

Prior to starting, the RNA preparation area was wiped down using RNase Zap wipes to eliminate contamination by the presence of other RNases. A PAXgene Blood RNA kit was used to isolate RNA as per manufacturer's instructions. The PAXgene Blood RNA tubes were removed from the -80°C freezer and left on the lab bench for 4 hours to thaw and equilibrate to room temperature before being centrifuged at 3,000 x g for 10 minutes at 22°C. The supernatant was discarded and 4 ml RNase-free water was added to the pellet. The pellet was vortexed until visibly dissolved and centrifuged at 3,000 x g for 10 minutes at 22°C.

After discarding the supernatant, 350 µl resuspension buffer (BR1) was added to each pellet and vortexed until dissolved. To ensure complete protein digestion, the sample was transferred into a 1.5ml microcentrifuge tube and 300 µl binding buffer (BR2) and 40 µl Proteinase K was added. Each tube was then vortexed and incubated for 10 minutes at 55°C in a 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 350µl 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. The flow-through was discarded and the process was repeated with the remaining sample. To remove any remaining contaminants, 350 µl wash buffer 1 (BR3) was added to the PAXgene spin column and centrifuged for 1 minute at 19,000 x g. The flow-through was discarded and the spin column was placed in a new 2 ml processing tube.

To ensure complete DNA elimination from the column-bound RNA, a stock solution containing 10 µl DNase per sample and 70 µl DNA digestion buffer per sample 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 (BR3), centrifuged for 1 minute at 19,000 x g and the flow through was discarded. 500 µl wash buffer 2 (BR4) was then added

to the column, centrifuged for 1 minute at 19,000 x g, the flow-through was discarded. This was then followed by a second 500 µl addition of BR4 which was centrifuged for 3 minutes at 19,000 x g. The PAXgene RNA spin column was then transferred to new 2 ml processing tube and centrifuged for 1 minute at 19,000 x g to completely dry the column. The PAXgene RNA spin column was then placed in a 1.5 ml microcentrifuge tube to which 40 µl elution buffer (BR5) was added and centrifuged for 1 minute at 19,000 x g. This elution step was then repeated with another 40 µl BR5. The RNA was aliquoted into 20 µl aliquots and subsequently stored at -80°C until required for quantification and equalisation.

2.8.2 RNA quantification and equalisation

The RNA yield and quality of each sample was quantified based on optical density (OD) using the NanoDrop®ND-1000 UV-Vis spectrophotometer (Thermo Fischer Scientific). After blanking the machine with 1 µl BR5, 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 with a range of 1.8-2.1 was an indicating a good quality RNA sample. The spectrophotometer was wiped with tissue paper after each individual sample to prevent contamination. Based on the concentrations recorded from the spectrophotometer, RNA was equalised to 10 ng/µl using RNase-free H₂O as diluent.

2.8.3 cDNA synthesis

Synthesis of cDNA was carried out using a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). A master mix solution was made up of 2 µl 10X buffer, 0.8 µl 25X dNTP mix 100 mM, 2 µl 10X random primers, 1 µl Multiscribe™ Reverse Transcriptase, and 4.2 µl RNase free H₂O per sample. Then 10 µl per sample of the master mix was added to an equal volume of RNA. The samples were then vortexed and centrifuged briefly before being placed in the thermocycler (Peltier Thermal Cycler PTC-200) and the program was set according to protocol which included 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 seconds. The final step inactivated the reverse transcriptase. Upon completion, the samples were centrifuged for 30 seconds, aliquoted into 10 µl volumes and diluted 1 in 5 with molecular grade water before being stored at -20°C.

2.8.4 Multiplex RT-PCR

The StepOnePlus™ Real-Time PCR system (Applied Biosystems) in the 96-well format was used for quantitative (Q)-PCR. Taqman® gene expression assay's (Applied Biosystems) were used to quantify expression of target genes of interest, each of which contained a specific set of forward and reverse primers and a FAM-labelled MGB probe for the target of interest [Table 2.4]. PCR reactions were in a duplex format also containing a Taqman® gene expression assay (primer limited) containing a VIC labelled probe for the endogenous control gene glyceraldehyde-3 phosphatedehydrogenase (GAPDH). A 10 µl volume was added to each well, the components of which are as follows: 4 µl of diluted cDNA, 0.5 µl of FAM-labeled Taqman® gene expression assay for the target of interest, 0.5 µl VIC-labeled Taqman® gene expression assay for GAPDH and 5 µl of Taqman®Fast Advanced Master Mix. GAPDH was chosen as an endogenous control gene in the amplification system for these analyses as it demonstrated a consistent expression profile.

Samples were assayed over 40-50 cycles using ABI's 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). The high temperature of 95°C for 1 second is applied initially to separate the target DNA strands from each other. As the temperature lowers to 60°C, the complimentary 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. At 60°C, the exponential accumulation of PCR product over 40-50 cycles was measured cycle by cycle based on the fluorescent emission.

At the end of the reaction, data analysis was performed with the StepOnePlus™ System Software (Applied Biosystems) and ExpressionSuite Software (Taqman) for inter-plate normalisation. 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.

Table 2.4 List of genes used with GenBank sequence ref numbers

Target Symbol	Target Name	TaqMan Gene Assay ID
Cytokines		
IL-1 β	Interleukin-1beta	Hs01555410_m1
IFN- γ	Interferon gamma	Hs00989291_m1
TNF- α	Tumour necrosis factor alpha	Hs00174128_m1
KP Catalysts		
IDO1	Indolamine 2,3 dioxygenase 1	Hs00984148_m1
IDO2	Indolamine 2,3 dioxygenase 2	Hs01589373_m1
TDO2	Tryptophan 2,3-dioxygenase 2	Hs00194611_m1
KP Enzymes		
KMO	Kynurenine-3-monooxygenase	Hs00175738_m1
KAT1	Kynurenine aminotransferase 1	Hs00187858_m1
KAT2	Kynurenine aminotransferase 2	Hs00212039_m1
Kynureninase	Kynureninase	Hs01114099_m1
Monoamine Transporter		
SLC6A4	Serotonin transporter	Hs00169010_m1
HPA Axis		
HSD11B1	11 β -hydroxysteroid dehydrogenase	Hs00419449_g1

2.8.5 RT-PCR analysis

The $\Delta\Delta CT$ method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression for all real-time PCR analysis. This method was used to assess relative gene expression by comparing gene expression of patient samples to healthy control samples, rather than quantifying the exact copy number of the target gene. In this manner, the relative quantification of gene expression can be assessed between depressed patient and healthy control samples. The relative quantification (RQ) was assessed using the cycle number (CT) difference between samples. The CT is measured against a set threshold. To accurately assess differences between gene expression the threshold was set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus, samples with low CT reading demonstrate high fluorescence, indicating greater amplification and hence greater gene expression. To measure RQ in relation to controls, the CT of the endogenous control (GAPDH) is subtracted from the CT of the target gene for each sample, accounting for any difference in cDNA quantity that may exist. This normalised CT value and is referred to as the ΔCT . The CT difference or ΔCT of the control is subtracted from itself to give 0, and subtracted from all other samples, to calculate the $\Delta\Delta CT$ value. The $\Delta\Delta CT$ (cycle difference corrected for GAPDH) is then converted into the RQ value. 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 RQ of gene expression between the control and treated samples.

2.9 Magnetic resonance imaging

2.9.1 Voxel based morphometry for Grey Matter quantification

The MRI images were processed using the VBM8 toolbox (Christian Gaser; <http://dbm.neuro.uni-jena.de/vbm/download/>) within SPM12 (Wellcome Department of Cognitive Neurology, London, UK). Each MRI image was first displayed in SPM8 to screen for artefacts or gross anatomical abnormalities. MR images were segmented into grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) using the standard unified segmentation model in SPM8 (Ashburner and Friston, 2005). Next, GM population templates were generated from the entire image dataset using the diffeomorphic anatomical registration using exponentiated Lie algebra (DARTEL) technique (Ashburner, 2007). This deformation technique has superior normalization power across individuals by improving inter-subject alignment, especially for small inner structures (Yassa and Stark, 2009) and it is more sensitive to regional differences, such as those that appear in the hippocampus (Bergouignan et al., 2009). After an initial registration of the GM DARTEL templates to the tissue probability maps in Montreal Neurological Institute (MNI), non-linear warping of GM images was performed to the DARTEL GM template in MNI space. The covariance between all normalized modulated images was visualized using a boxplot and covariance matrices to check for homogeneity and, thus, inspect for outliers. Images were then modulated to ensure that relative volumes of GM were preserved following the spatial normalisation procedure. Finally, the normalized modulated images were smoothed with a standard Gaussian kernel of 8 mm, full width at half maximum. Smoothing rendered the data more normally distributed so that the assumption of parametric statistical comparisons was not violated (Worsley et al., 1996). After spatial pre-processing, the smoothed, modulated, normalised GM datasets were used for statistical analysis.

2.9.2 Hippocampal subfield measurement

Further volumetric analysis was performed to determine the volumes of left and right hippocampal subfields in depressed patients and controls. This was carried out using T1-weighted anatomical SPGR images in Free Surfer 5.3.0 image analysis suite (<http://surfer.nmr.mgh.harvard.edu/>) (Fischl and Dale, 2000; Fischl, 2012). Cortical reconstruction and volumetric segmentation was performed with Free Surfer using technical methods described in previous publications (Dale et al., 1999; Desikan et al., 2006; Fischl et

al., 2002; Fischl, 1999; Fischl et al., 2004; Reuter et al., 2012). Hippocampal subfield analysis tools from FreeSurfer (v6.0) were employed, as this version of the software features a novel atlas algorithm and ex vivo MRI data from autopsy brains. Motion effects are eliminated in this tool, yielding images with extremely high resolution and signal-to-noise ratio, which can accurately identify more sub-regions with improved delineation (Iglesia et al., 2015). This method was used to calculate volumes of the left and right alveus, parasubiculum, presubiculum, subiculum, CA1, CA3, CA4, granule cell layer of dentate gyrus, hippocampus-amygdala-transition-area, fimbria, molecular layer, hippocampal fissure, tail, and whole hippocampal volume (Iglesia et al., 2015). It was hypothesised that specific subfields associated with metabolic processes may exhibit decreased volumes in depressed patients compared to controls, therefore analysis focused on particular regions of interest. The areas associated with the metabolic processes that we examined were the CA3 and CA4. It was also hypothesised that the subiculum, a hippocampal subfield suggested to play a role in HPA axis activity, may display volumetric reduction in the depressed cohort. Finally, the hippocampal fissure was assessed since a decrease in volumes of other hippocampal subfields has the potential to increase the size of the hippocampal fissure. These regions of interest are depicted in Figure 4.8. Hippocampal subfield volume calculation was conducted with the assistance of Dr. Erik O'Hanlon.

2.10 Statistical Analysis

Graphs and statistics were generated using GraphPad Prism Software Version 5.00 (GraphPad software, Inc). All data were analysed using SPSS (Version 16). Data are presented as mean with standard error of the mean (SEM) or standard deviation (SD) where appropriate. SEM was used for all biological data while SD was used for demographic data. All data were tested for normality using the Shapiro-Wilk test. Normally distributed data were analysed using the student's t-test or two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test, where appropriate. Homogeneity of variance was also assessed when using ANOVA which requires that this assumption is met. Non-parametric data were analysed using the Mann-Whitney U test for independent sample comparisons, the Wilcoxon-Signed Rank test for paired comparisons or Kruskal-Wallis one-way ANOVA, where appropriate.

Correlational analysis was carried out using Spearman's rho correlation statistics. A correlation was considered "strong" if the r value was above 0.60, and "moderate" strength if between 0.40 and 0.59. A "weak" correlation was reported if the r value fell below 0.40. All statistical analyses were considered significant when $p \leq 0.05$ if hypothesis driven. Correction for multiple comparisons was carried out after large correlational analyses by adjusting the p-value with the Bonferroni method.

Cluster analysis was performed with CHAID decision tree using all biological variables. Linear regression analysis of biological variables was performed as a predictor of CES-D score in depressed patients and healthy controls to determine the relationship between key variables and depression severity. Binary logistic regression analysis to predict diagnosis status was performed to determine an equation that best predicted a diagnosis of MDD using biological variables.

Smoking was ruled out as a covariate for the analyses in this thesis based on a CHAID tree analysis followed by a stepwise backward elimination logistic regression analysis. The tree analysis revealed that the most significant biological variables remained the same for both smoking and non-smoking groups, despite the significant association between diagnosis and smoking. Logistic regression analysis was then performed to examine the relationship between biological parameters with smoking. The result of this analysis showed that no biological parameters were associated with smoking, therefore it was determined that it was not necessary to utilise smoking as a covariate in analyses of this dataset.

Chapter 3

Hypothalamic pituitary adrenal axis alterations in Major Depressive Disorder

3.1 Introduction

Major Depressive Disorder (MDD) is a serious psychiatric disorder that has been identified as the leading cause of suicide (Arsenault-Lapierre et al., 2004), and has a high lifetime prevalence rate of 16% (Kessler et al., 2005). Despite its prevalence, the biological aetiology of depression remains elusive. Stress has been shown to be a major risk factor in developing depression (Kessler, 1997), therefore further investigation of biological pathways relating to stress in a depressed population are necessary.

The hypothalamic pituitary adrenal (HPA) axis is a major part of the neuroendocrine system which controls stress responses to the environment. It is composed of the hypothalamus which produces corticotropin releasing hormone (CRH), the pituitary gland which secretes adrenocorticotrophic hormone (ACTH), and the adrenal glands which secrete cortisol. The hypothalamus responds to levels of cortisol by either increasing or reducing CRH production accordingly. Hyperactivity of the HPA axis exhibited as elevated cortisol concentrations is one of the most consistent findings in the search for biological aetiology in MDD (Pariante et al., 2008). It has been hypothesized that this hyperactivity is due to reduced efficacy of central glucocorticoid and glucocorticoid receptor function which results in dysfunctional HPA axis feedback (O'Keane et al., 2012; Farrell and O'Keane, 2016). Other frequently reported findings concerning HPA axis dysregulation in depression include elevated CRH (Holsboer, 2000), non-suppression on the dexamethasone suppression test (Arana et al., 1985), a blunted ACTH response to CRH (Owens and Nemeroff, 1993), and hippocampal volume reduction (Sheline et al., 1999). Evidence of HPA axis activation appears to have value as a predictor of increased risk of depression relapse and even suicide (Varghese and Brown, 2001).

One measure commonly used to observe HPA hyperactivity is the cortisol awakening response (CAR) (Fries et al., 2009). This rapid increase in cortisol secretion occurs daily; roughly within the first 30 minutes of wakening, signifying the physiological stress response to waking (Wüst et al., 2000). In both current and remitted depressed populations, it has been reported that peak cortisol awakening levels are higher compared to controls (Vreeburg et al., 2009).

Other markers of HPA axis activity are also of interest in the investigation of depression pathophysiology. The enzyme 11-beta-steroid dehydroxygenase (11 β -HSD) catalyses the conversion of cortisol to cortisone, and vice versa, in peripheral tissue. Cortisone is another glucocorticoid and is relatively inert due to poor binding to the glucocorticoid receptor, but

when available in it can be readily enzymatically converted inside cells to the active steroid cortisol by 11 β -HSD1 (Coutinho and Chapman, 2011). Therefore, injection of cortisone is used to suppress inflammatory conditions such as arthritis by increasing available cortisone to be converted to cortisol in peripheral tissue. Injections of cortisone for treatment of inflammatory conditions have a known side effect of increasing anxiety and depression (Brown and Chandler, 2001), indicating that it too may play a role in pathophysiology of depression.

There are two types of 11 β -HSD enzyme: type 1 which converts cortisone to cortisol, and type 2 which performs the opposite function. 11 β -HSD1 has also been implicated in human HPA axis regulation and susceptibility to depression. Subjects with the rs11119328 polymorphism of the gene that encodes for 11 β -HSD1, HSD11 β 1, were found to have higher cortisol levels and increased rates of depression (Dekker et al., 2011). Furthermore, ablation of the gene that encodes for 11 β -HSD1 in mice results in anti-depressant effects during the forced swim test (Slattery et al., 2016). Also, specifically 11 β -HSD1 rather than 11 β -HSD2 has been linked to inflammatory conditions (Coutinho et al., 2012). These findings indicate that the involvement of the HPA axis in depression may be characterised by more than an increase in cortisol concentration alone, and that each of these markers should be measured in the assessment of HPA axis activity in depressed patients.

Study Aims

1. To evaluate differences in cortisol awakening responses between depressed patients and healthy controls.
2. To evaluate differences in salivary cortisone concentrations between depressed patients and healthy controls
3. To determine whether whole blood mRNA expression of HSD11 β 1 is related to differences in CAR parameters.

3.2 Methods

In this study, activity of the HPA axis was assessed in 57 patients with MDD and 41 healthy controls. Depressed patients were recruited from the psychiatric outpatient clinic at Sheaf House in Tallaght Hospital and at the Mary Mercer Healthy Centre in Jobstown, Dublin 24. Recruitment of depressed patients was based on criteria for a Major Depressive Episode (DSM-5, American Psychiatric Association, 2013). Additional inclusion and exclusion criteria for depressed patients and healthy controls were as stated in Chapter 2. Psychiatric rating scale data was collected from participants to measure severity of depressive symptoms, sleep disturbance, and childhood trauma. These scales included the HAM-D, CES-D, PSQI, and CTQ (Appendices 3a through e).

The primary biological measures in this study were salivary cortisol and cortisone concentrations across five time-points within a day. Participants used Salivette® tubes (Sarstedt) to collect saliva samples in the morning at 0, 30, and 60 minutes after waking, and in the evening at 12 and 12.5 hours after waking as described in Chapter 2.

Saliva samples were assessed for cortisol and cortisone content by liquid chromatography – mass spectrometry (LC-MS) at the University of Manchester in the Department of Clinical Biochemistry. These assays were conducted by members of Professor Brian Keevil's laboratory at the University Hospital of South Manchester. Detailed methodology of the LC-MS methods conducted is as described in Chapter 2. The cortisol to cortisone ratio was calculated to gain further insight into the HPA activity in depressed patients and healthy controls, as cortisone is the inert counterpart to cortisol.

The cortisol awakening response (CAR) was assessed in depressed patients and healthy controls by calculating Area Under the Curve (AUC) and other parameters using the morning cortisol measurements. These calculations are used to describe total morning free cortisol output and cortisol responsivity. Equations for deriving the CAR parameters discussed in this chapter are described in detail in Chapter 2.

PAXgene whole blood mRNA expression of HSD11 β 1, the gene that encodes for 11 β -hydroxysteroid dehydrogenase type 1, was also measured to further evaluate HPA activity in depressed patients and healthy controls. Collection of 2.5 ml of blood was taken into PAXgene mRNA tubes which were stored as described in Chapter 2. Total RNA was isolated from the whole blood and equalized before it was used for cDNA synthesis. Analysis of gene expression of the target gene was conducted using Real-Time PCR methods and Taqman®

Gene Expression Assays (Applied Biosystems, UK). Relative quantification data was calculated using the $\Delta\Delta C_t$ method and GAPDH as an endogenous control, as described at length in Chapter 2.

Data are presented as mean with standard error of the mean (SEM) or standard deviation (SD) where appropriate. All data were tested for normality using the Shapiro-Wilk test. Non-normal data was log transformed for analysis. Normally distributed data were analysed using the student's t-test. Non-parametric data were analysed using the Mann-Whitney U test for independent sample comparisons where appropriate. Univariate general linear model analysis was used when necessary to implement age and gender as covariates. Correlational analysis was carried out using Pearson's correlation statistics when variables were normally distributed and Spearman rho statistics for non-normal or categorical data. All statistical analyses were considered significant when $p \leq 0.05$. All data were analysed using SPSS (Version 16). Graphs and statistics were generated using GraphPad Prism Software Version 5.00 (GraphPad Software, Inc).

3.3 Results

3.3.1 Demographic data for patients with MDD and healthy controls

There were no differences in age, gender, BMI, or smoking between healthy controls and patients with MDD recruited to this study [Table 3.1]. Depressed participants displayed a significantly lower duration of education according to the International Standard Classification of Education (ISCED) scale ($p < 0.001$) compared to healthy controls [Table 3.1].

Table 3.1 Demographic data for patients with MDD and healthy controls for whom salivary cortisol and cortisone was measured by LC-MS.

	Patients (n=57)	Controls (n=41)	Statistics (p-value)
Age (years)	28.26 (8.41)	27.49 (5.54)	$Z = -0.036$, $p = 0.971$
Gender (female/male)	37/20	26/15	$\chi^2 = 0.070$, $p = 0.831$
BMI	24.96 (6.17)	23.00 (3.21)	$Z = -1.199$, $p = 0.230$
Smoking (Yes/No)	23/34	10/31	$\chi^2 = 2.720$, $p = 0.099$
ISCED Education	3.54 (1.54)	6.30 (1.45)	$Z = -6.194$, $p < 0.001^{***}$

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 MDD and healthy controls

Consistent with a diagnosis of MDD, patients scored significantly higher compared to healthy controls on the HAM-D and CES-D [Table 3.2], signifying increased depressive symptoms. Patients also exhibited significantly higher sleep disturbance through elevated PSQI scores [Table 3.2]. In the depressed group, a significantly greater number of participants had experienced Early Life Adversity (MDD: 61% vs. HC: 21%), with significantly higher Global CTQ scores than healthy controls [Table 3.2]. All clinical measures described were significantly higher at $p < 0.001$. Of the depressed cohort, 25 were medication free, 19 were prescribed SSRIs, 7 were prescribed SNRIs, and 6 were on other medications including benzodiazepines, melatonergics, anti-epileptics or a combination of medications.

Table 3.2 Clinical data for patients with MDD and healthy controls for whom salivary cortisol and cortisone was measured by LC-MS.

	Patients (n=57)	Controls (n=41)	Statistics (p-value)
HAM-D	22.81 (5.14)	3.00 (2.81)	$Z = -8.254$, $p < 0.001$ ***
CES-D	39.04 (10.09)	6.97 (6.64)	$Z = -8.123$, $p < 0.001$ ***
PSQI	13.58 (3.36)	4.11 (2.70)	$Z = -7.786$, $p < 0.001$ ***
CTQ Global Score	44.50 (17.20)	29.47 (5.93)	$Z = -4.319$, $p < 0.001$ ***
Early Life Adversity (Yes/No)	35/22	7/34	$\chi^2 = 19.136$, $p < 0.001$ ***
Antidepressant (Yes/No)	32/25	0/41	

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). *** $p \leq 0.001$ vs. control

3.3.3 Salivary cortisol concentrations in depressed patients and healthy controls

A Mann-Whitney U test performed on log transformed cortisol data revealed significantly higher concentrations of cortisol at waking ($Z=-2.275$, $p=0.023^*$) in MDD patients relative to control subjects [Figure 3.1, Figure 3.2]. There was no significant difference in cortisol concentration between depressed patients and healthy controls at time points T30, T60, and T720 relative to controls [Table 3.3].

Table 3.3 Raw cortisol data for MDD patients and healthy controls.

	Patients (n=57)	Controls (n=41)	Statistics (p-value)
Cortisol T0 (at waking)	10.92 (1.34)	7.49 (0.63)	$Z=-2.275$, $p=0.023^*$
Cortisol T30	12.93 (1.03)	10.91 (0.90)	$Z=-1.156$, $p=0.247$
Cortisol T60	9.58 (1.13)	9.05 (0.83)	$Z=-0.187$, $p=0.852$
Cortisol T720	2.56 (0.66)	2.49 (0.74)	$Z=-0.801$, $p=0.423$
Cortisol T750	2.12 (0.57)	2.38 (0.66)	$Z=-0.132$, $p=0.895$

Data are expressed as mean cortisol in nM with SEM in parentheses. Statistical analysis was performed using a Mann-Whitney U test on log transformed cortisol data. $*p\leq 0.05$ vs. control.

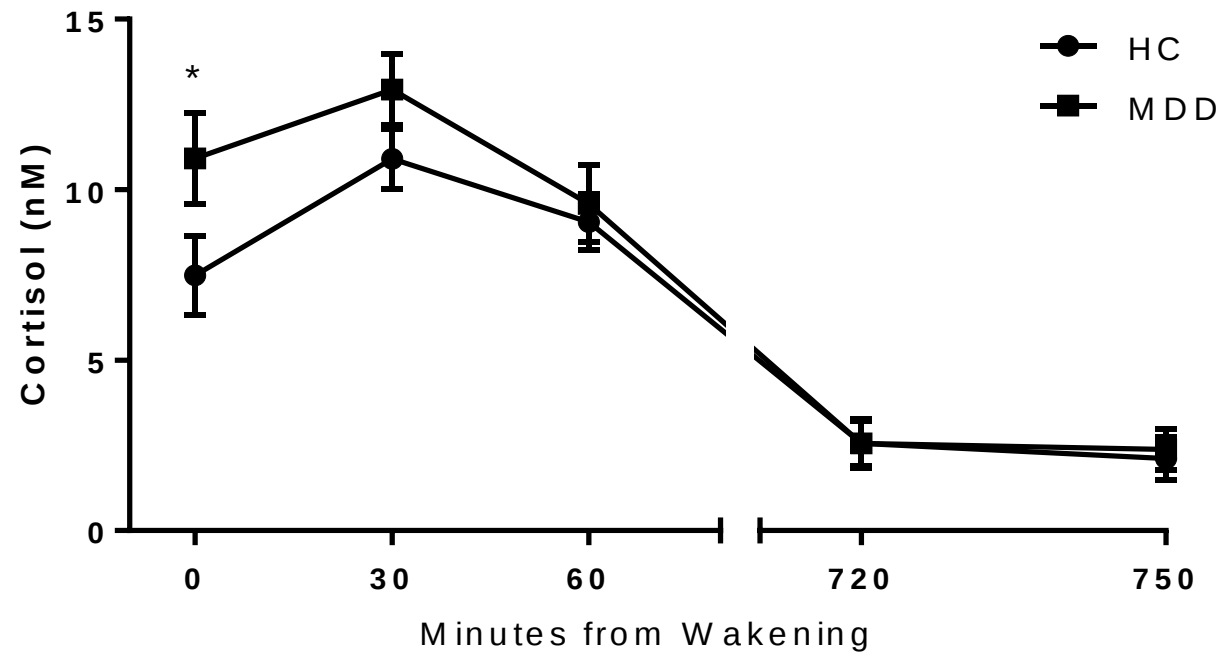


Figure 3.1 Salivary cortisol concentration data. Salivary cortisol concentrations at five time points from wakening in depressed patients (n=57) compared with healthy controls (n=41). Depressed patients exhibited a statistically significantly higher cortisol concentration at 0 minutes from wakening. Data are expressed as means and SEM. * $p \leq 0.05$ vs. control. (Mann-Whitney U test performed on log transformed data).

3.3.4 Cortisol Awakening Response in depressed patients and healthy controls

The cortisol awakening response (CAR) was assessed in depressed patients and controls using several established measures that describe the Area Under the Curve (AUC) of the morning time point cortisol concentrations [Figure 3.2], as well as parameters that describe the regression line fitted through the morning cortisol data. All parameters used to assess CAR are displayed in Table 3.4 with means and SEM of each study group. A Mann-Whitney U test performed on log transformed cortisol data revealed a trend towards a lower AUC_i in the depressed group and significantly lower reactivity in depressed patients compared to healthy controls [Figure 3.4a,b]. Additionally, the regression line fitted through the morning cortisol data had a significantly lower slope and higher intercept in depressed patients compared to healthy controls [Figure 3.5a,b]. Analysis revealed no significant differences in the average evening cortisol or diurnal variation between groups.

Table 3.4 Derived CAR parameters for MDD patients and healthy controls.

	Patients (n=57)	Controls (n=41)	Statistics (p-value)
Average AM Cortisol (nM)	11.46 (1.01)	9.09 (0.58)	Z=-1.300, p=0.194
Average PM Cortisol (nM)	2.36 (0.55)	2.45 (0.68)	Z=-0.658, p=0.510
AM-PM	8.99 (0.96)	7.05 (0.83)	Z=-0.689, p=0.491
AUC _G	681.96 (58.88)	569.29 (38.95)	Z=-0.902, p=0.367
AUC _i	28.38 (63.97)	128.78 (41.76)	Z=-1.295, p=0.195
Peak	15.41 (1.36)	12.68 (0.91)	Z=-1.120, p=0.263
Reactivity	-1.71 (1.41)	1.37 (1.00)	Z=-1.962, p=0.050*
Intercept	12.46 (1.24)	8.41 (0.61)	Z=-2.045, p=0.041*
Slope	-0.039 (0.02)	0.023 (0.02)	Z=-1.977, p=0.048*

Data are expressed as mean with SEM in parentheses. AUC_G=area under the curve with respect to the ground; AUC_i=area under the curve with respect to increase; Intercept=intercept of the regression line fitted through the raw cortisol data; Slope=slope of the regression line fitted through the raw cortisol data. Statistical analysis was performed using a Mann-Whitney U test on log transformed data. *p≤0.05 vs. control.

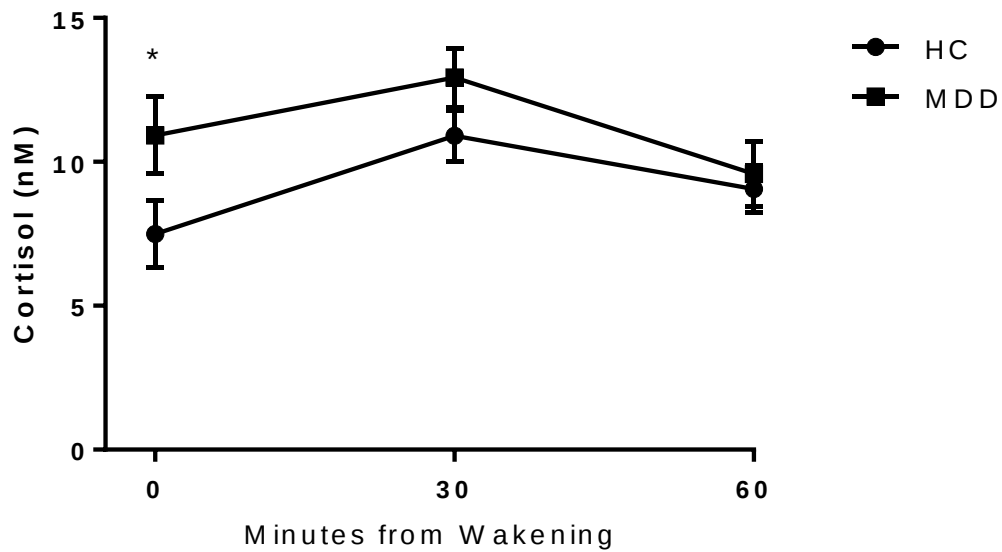


Figure 3.2 Cortisol Awakening Response (CAR) in MDD patients and healthy controls. Salivary cortisol concentrations at three morning time points in depressed patients (n=57) and healthy controls (n=41). Data are expressed as means and SEM. * $p \leq 0.05$ vs. control (Mann-Whitney U test performed on log transformed data).

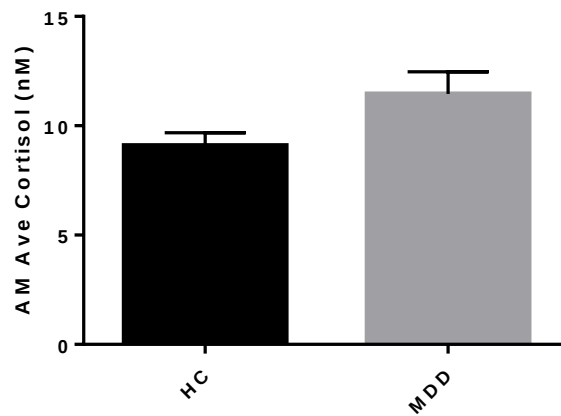


Figure 3.3 Average morning cortisol concentrations for patients and healthy controls. Average of T0, T30, and T60 salivary cortisol concentrations expressed as mean and SEM for depressed patients (n=57) and healthy controls (n=41) (Mann-Whitney U test performed on log transformed data).

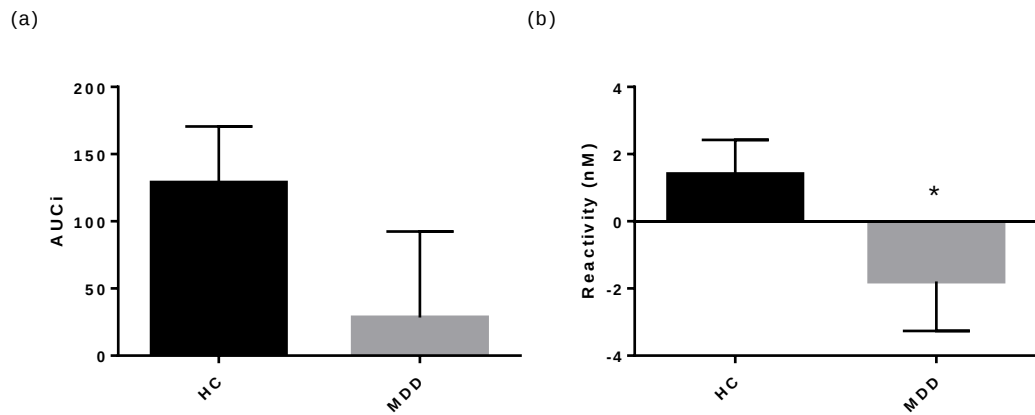


Figure 3.4 CAR responsivity measures in patients and healthy controls. (a) AUCi and (b) CAR reactivity expressed as mean and SEM in depressed patients (n=57) and healthy controls (n=41). * $p \leq 0.05$ vs. control. (Mann-Whitney U test performed on log transformed data).

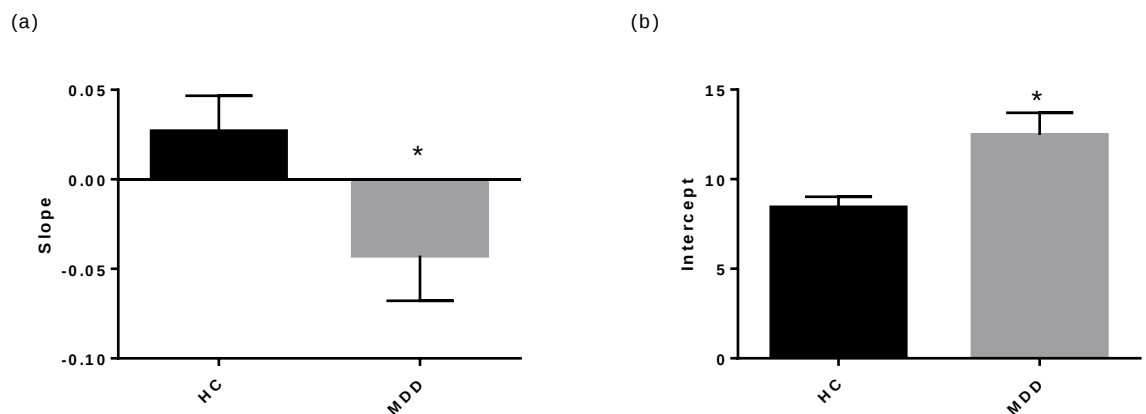


Figure 3.5 Parameters of regression line fit through morning cortisol data. Regression lines fitted through the morning cortisol data of each of the study group displayed significantly different parameters. (a) The slope of the regression line and (b) the intercept of the regression line fitted through morning cortisol concentrations in depressed patients (n=57) compared to controls (n=41) are displayed as means with SEM. * $p \leq 0.05$ vs. control (Mann-Whitney U test performed on log transformed data).

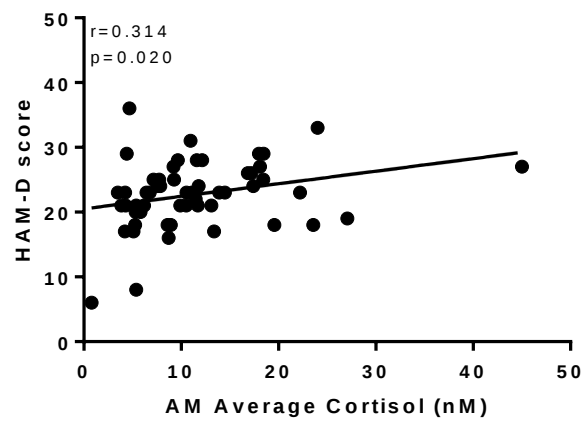
3.3.5 Relationship between morning salivary cortisol and depressive symptoms

A Spearman's rho correlation analysis performed on psychiatric rating scales and cortisol data revealed several significant positive correlations both within the depressed group and amongst the entire cohort, indicating a strong relationship between morning cortisol concentrations and psychiatric symptoms.

Cortisol concentration at T30 was significantly positively correlated to HAM-D within the depressed group ($r=0.352$, $p=0.013$). The average morning cortisol concentration was also correlated to HAM-D scores within the depressed group ($r=0.314$, $p=0.020$), in addition to peak morning cortisol concentration ($r=0.275$, $p=0.042$) [Figure 3.6a, b].

Correlational analysis of the entire cohort also revealed a significant positive relationship between average morning cortisol concentration and PSQI ($r=0.244$, $p=0.017$). Peak morning cortisol concentration was also positively correlated to PSQI ($r=0.253$, $p=0.017$).

(a)



(b)

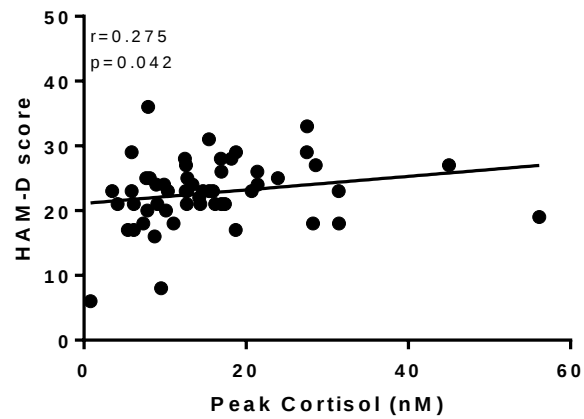


Figure 3.6 Correlational analysis between depression rating scales and cortisol awakening parameters in MDD patients. (a) A significant positive correlation exists between HAM-D scores of depressed patients ($n=55$) and average morning cortisol concentration. (b) A significant positive correlation exists between HAM-D scores of depressed patients ($n=55$) and peak morning cortisol (Spearman's rho).

3.3.6 Salivary cortisone concentrations in depressed patients and healthy controls

A Mann-Whitney U test performed on log transformed cortisone data revealed no significant differences in cortisone concentrations between MDD patients relative to healthy control subjects [Table 3.5]. In both depressed and control patients, mean salivary cortisone concentrations increase between T0 and T30, and decrease between T30 and T60, creating the anticipated awakening response curve [Figure 3.7]. Cortisone concentrations decrease sharply in the evening time points compared to the morning time points [Table 3.5].

Table 3.5 Raw cortisone data for patients with MDD and healthy controls.

	Patients (n=57)	Controls (n=41)	Statistics (p-value)
Cortisone T0	31.07 (2.03)	27.59 (1.76)	Z=-1.045, p=0.296
Cortisone T30	39.85 (2.28)	33.99 (1.76)	Z=-1.436, p=0.151
Cortisone T60	32.19 (2.22)	30.63 (1.54)	Z=-0.155, p=0.877
Cortisone T720	10.93 (1.28)	9.72 (1.33)	Z=-0.459, p=0.646
Cortisone T750	8.60 (1.00)	8.83 (1.14)	Z=-0.579, p=0.562

Data are expressed as mean cortisol in nM with SEM in parentheses. Statistical analysis was performed using a Mann-Whitney U test on log transformed cortisone data.

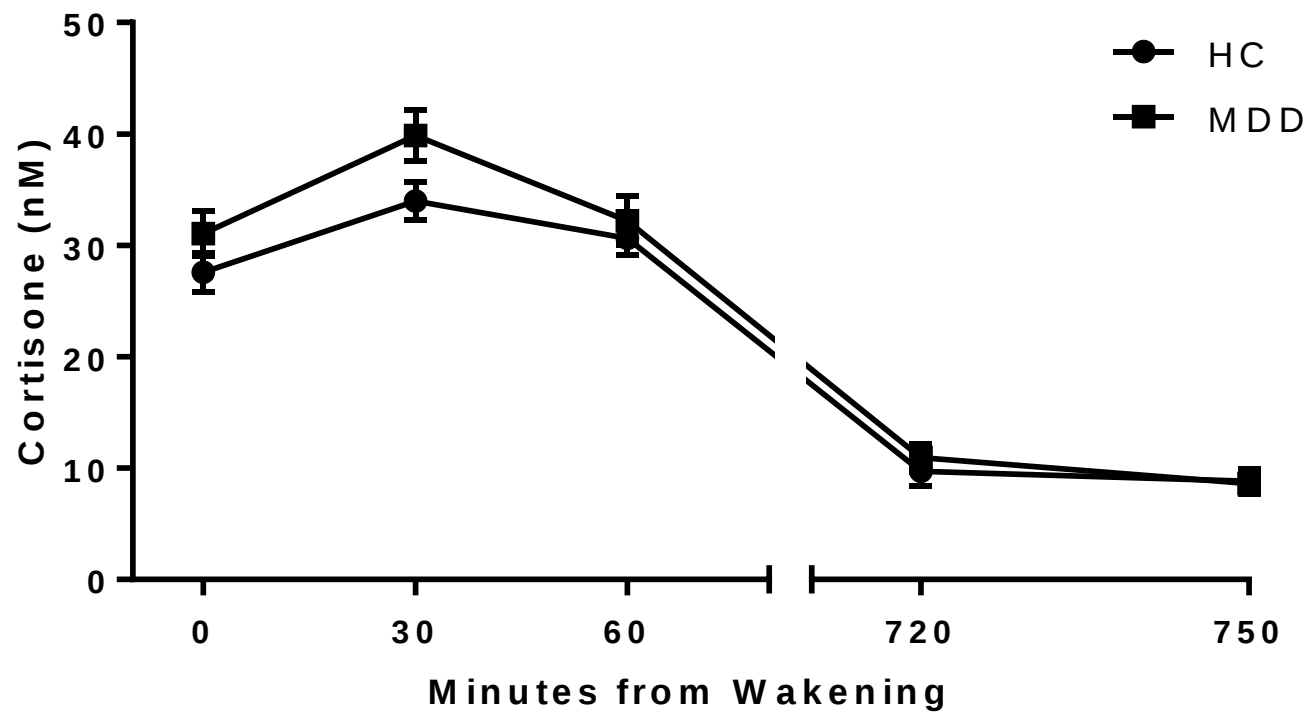


Figure 3.7 Salivary cortisol concentration data. Data are expressed as means and SEM. No significant differences in cortisol concentrations between MDD and HC groups were detected. (Mann-Whitney U test performed on log transformed cortisol data).

3.3.7 Cortisone Awakening Response parameters in depressed patients and healthy controls

No significant differences in cortisone awakening response parameters were found between depressed patients and healthy controls when performing a Mann-Whitney U test on log transformed data [Table 3.6].

Table 3.6 Derived parameters for cortisone awakening response (CAR) for patients with MDD and healthy controls.

	Patients (n=57)	Controls (n=41)	Statistics (p-value)
Average AM Cortisone	34.76 (1.68)	30.58 (1.27)	
Average PM Cortisone	10.08 (1.12)	9.39 (1.20)	
Cortisone PM-AM	-24.12 (1.69)	-21.12 (1.61)	
Cortisone AUC _G	2082.63 (125.23)	1918.42 (74.34)	
Cortisone AUC _I	277.68 (88.39)	233.40 (93.46)	
Cortisone Peak	42.90 (2.10)	38.16 (1.70)	
Cortisone Reactivity	2.24 (2.92)	2.47 (2.35)	
Cortisone Intercept	33.20 (2.30)	29.89 (1.67)	
Cortisone Slope	-0.0017 (0.04)	0.0393 (0.04)	

Data are expressed as mean with SEM in parentheses. AUC_G=area under the curve with respect to the ground; AUC_I=area under the curve with respect to increase; Intercept=intercept of the regression line fitted through the raw cortisone data; Slope=slope of the regression line fitted through the raw cortisone data. Statistical analysis was performed using an Mann-Whitney U test on log transformed data.

3.3.8 Cortisol/cortisone ratios in depressed patients and healthy controls

A univariate general linear model analysis of log transformed cortisol/cortisone ratio data revealed a significantly higher cortisol/cortisone ratio in depressed patients compared to healthy controls at waking [Figure 3.8]. However, the ratio of cortisol/cortisone was consistent between the depressed and control group at all subsequent times [Table 3.7].

Table 3.7 Cortisol/cortisone concentration ratios in depressed patients and healthy controls.

	Patients (n=57)	Controls (n=41)	Statistics (p-value)
Cortisol/Cortisone T0	0.40 (0.06)	0.27 (0.01)	t=-2.074, p=0.041*
Cortisol/Cortisone T30	0.33 (0.02)	0.33 (0.02)	t=-0.167, p=0.868
Cortisol/Cortisone T60	0.30 (0.03)	0.28 (0.02)	t=0.169, p=0.866
Cortisol/Cortisone T720	0.22 (0.02)	0.27 (0.07)	t=0.284, p=0.777
Cortisol/Cortisone T750	0.24 (0.04)	0.30 (0.08)	t=0.834, p=0.407

Data are expressed as mean ratio with SEM in parentheses. Statistical analysis was performed using a univariate general linear model analysis of variance on log transformed ratio data. *p $\leq$ 0.05 vs. control.

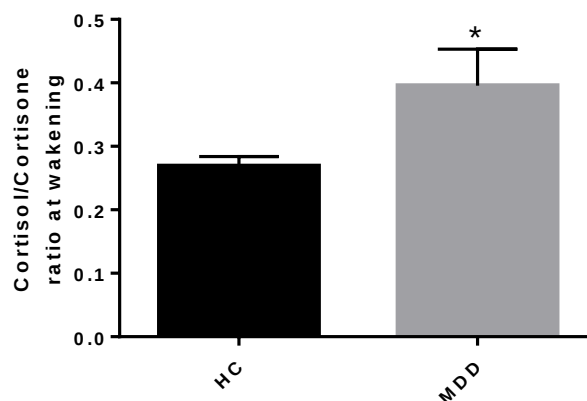


Figure 3.8 Wakening cortisol/Cortisone ratio in depressed patients compared with healthy controls. Cortisol/Cortisone ratio in depressed patients (n=57) compared with healthy controls (n=41). Data expressed as mean with SEM. *p<0.05 vs. control (Univariate General Linear Model Analysis of Variance performed on log transformed ratios).

3.3.9 Relationship between cortisol and cortisone

A Spearman's rho analysis between cortisol and cortisone concentration data of depressed patients and controls revealed a large positive correlation at each of the five saliva collection time points. The correlation was highly significant for each of the time points, with analysis revealing each p-value to be $p < 0.001$. The saliva collection time point with the largest positive correlation between cortisol and cortisone concentrations was T60 ($r = 0.845$, $p < 0.001$). The relationship between cortisol and cortisone at waking is depicted in Figure 3.9.

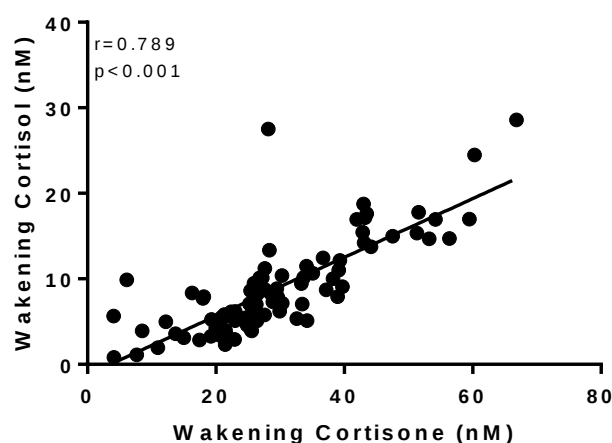


Figure 3.9 Correlational analysis between Cortisol and Cortisone at waking. A significant positive correlation exists between cortisol and cortisone at each of the time points over the course of the day. Above, the correlation between waking concentration (T0) of cortisol and cortisone is displayed (Spearman's rho).

3.3.10 Relationship between morning salivary cortisone concentrations and psychiatric rating scales

A Spearman's rho correlational analysis performed on psychiatric rating scales and cortisone concentration data revealed several significant positive correlations within the depressed group, indicating a strong relationship between morning cortisone concentrations and psychiatric symptoms.

A significant positive relationship exists between HAM-D score of depressed patients and T30 cortisone concentration ($r=0.323$, $p=0.023$) [Figure 3.10a]. Additionally, peak cortisone is significantly correlated to HAM-D scores of depressed patients ($r=0.284$, $p=0.037$).

PSQI scores were correlated to average morning cortisone ($r=0.302$, $p=0.031$) and peak cortisone ($r=0.372$, $p=0.007$) in depressed patients as well [Figure 3.10b].

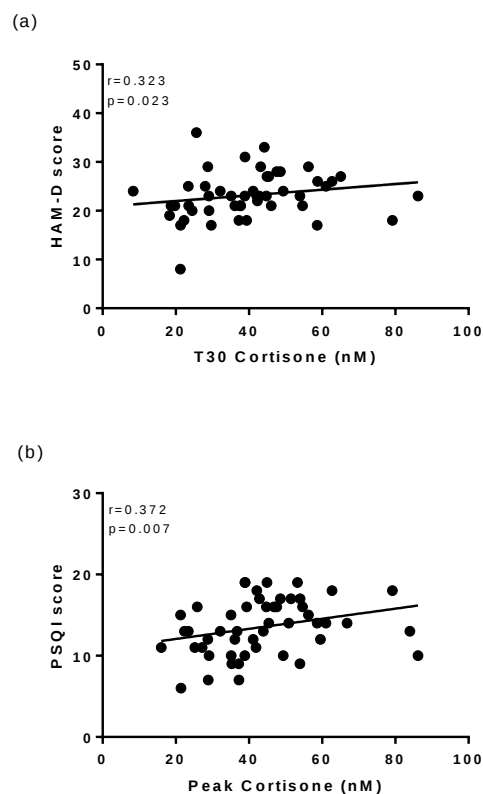


Figure 3.10 Correlational analysis between cortisone awakening parameters and psychiatric rating scales. (a) A significant positive correlation exists between HAM-D scores of MDD patients and T30 cortisone concentrations. (b) A significant positive correlation exists between PSQI scores of MDD patients and peak morning cortisol concentrations. (Spearman's rho correlation).

3.3.11 Relative quantification of whole blood HSD11 β 1 mRNA expression in depressed patients and healthy controls

Demographic and clinical data relating to depressed patients and healthy controls that had whole blood mRNA expression measured can be found in Appendix 4a. There were no differences in age, gender, or BMI between depressed patients and healthy controls. Consistent with a diagnosis of MDD, depressed patients had significantly higher depressive symptom and sleep disturbance scores, and had experienced more early life adversity than healthy controls.

Basal expression of HSD11 β 1 mRNA was observed in all whole blood samples collected and the average Ct in the control group was 35.1. The average Ct for the housekeeping gene GAPDH was 25.7, resulting in an average Δ CT of 9.4. A Mann-Whitney U test revealed no significant difference between whole blood transcriptional expression of HSD11 β 1 between depressed patients and healthy controls (MDD: 1.15 (0.16) vs. HC: 1.02 (0.11)) ($Z=-0.490$, $p=0.624$) [Figure 3.11].

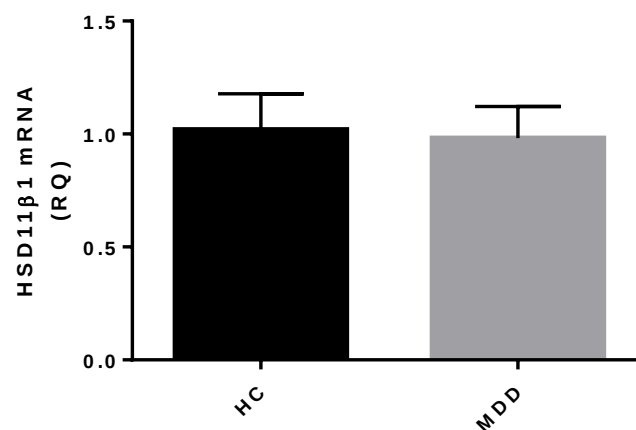


Figure 3.11 Relative quantification of HSD11 β 1 in MDD patients and healthy controls. Data displayed as means with SEM. There is no difference in gene expression of HSD11 β 1 between MDD patients ($n=55$) and healthy controls ($n=38$) (Mann-Whitney U).

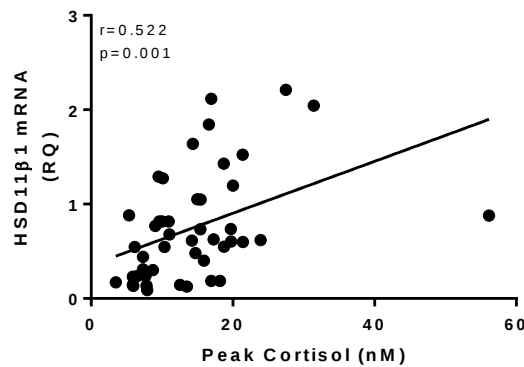
3.3.12 Relationship between morning corticosteroid concentrations and whole blood HSD11 β 1 mRNA

The relative quantification of whole blood mRNA expression of HSD11 β 1 was significantly correlated to cortisol and cortisone morning concentrations in healthy controls and depressed patients. A Spearman's rho correlational analysis revealed a significant positive relationship between transcriptional expression of HSD11 β 1 and T0 cortisol ($r=0.361$, $p=0.017$), T30 cortisol ($r=0.450$, $p=0.002$), T60 cortisol ($r=0.414$, $p=0.005$), and average morning cortisol concentrations ($r=0.533$, $p<0.001$) in the entire cohort [Figure 3.12a]. Additionally, HSD11 β 1 expression was significantly positively correlated to peak morning cortisol concentration ($r=0.522$, $p<0.001$) among all participants.

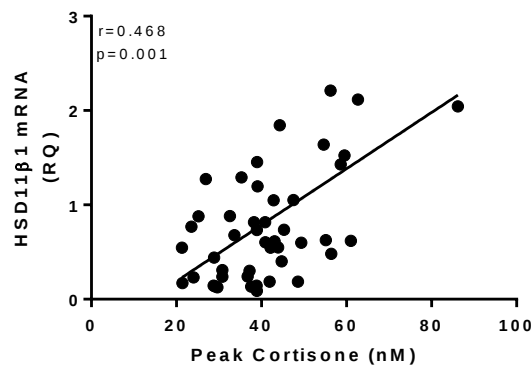
Correlational analysis between cortisone concentration data and relative quantification of HSD11 β 1 mRNA expression revealed several significant associations. Expression of HSD11 β 1 was significantly positively correlated to cortisone concentration at T30 ($r=0.418$, $p=0.004$), T60 ($r=0.358$, $p=0.015$), average morning cortisone ($r=0.419$, $p=0.003$), and peak cortisone concentration ($r=0.468$, $p=0.001$) [Figure 3.12b].

Interestingly, whole blood transcriptional expression of HSD11 β 1 was significantly correlated to cortisol/cortisone ratio at T0 ($r=0.318$, $p=0.038$) [Figure 3.12c], and no other time points. This is noteworthy since T0, or wakening, is the only time point at which there is a significant difference in cortisol/cortisone ratio between depressed patients and healthy controls.

(a)



(b)



(c)

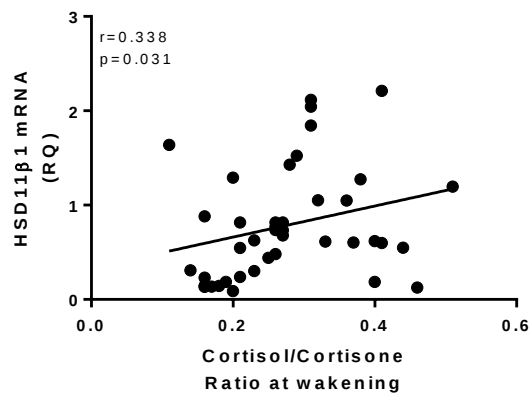


Figure 3.12 Correlational analysis between whole blood HSD11β1 mRNA and glucocorticoid concentrations. A significant positive correlation exists between mRNA expression of HSD11β1 and (a) peak cortisol concentration, (b) peak cortisone concentration, and (c) cortisol/cortisone ratio at waking according to a Spearman's Rho analysis.

3.4 Discussion

In the present study, the HPA axis activity of depressed patients and healthy controls was profiled by the quantification of salivary cortisol and cortisone at five time points throughout the day, as well as by relative quantification of whole blood HSD11 β 1 mRNA expression. The primary finding in this study was the significant elevation of waking cortisol concentrations in the depressed cohort. In addition to this, it was found that there were no differences in cortisone concentrations between depressed patients and controls at any time point. Because of the increase in cortisol and parity in cortisone concentrations, a significant difference in cortisol/cortisone ratio was discovered between groups at waking. Furthermore, transcriptional expression of HSD11 β 1, the enzyme that converts cortisone to cortisol, was significantly positively correlated to the cortisol/cortisone ratio at the waking time point only, indicating the possibility of involvement of the 11 β -HSD1 enzyme in HPA axis dysregulation. Taken together, these findings support the theory of HPA axis alterations in depression, while indicating that the involvement of this pathway may be more complex than elevated cortisol alone.

3.4.1 Altered cortisol awakening response in depression

The results of this study support the hypothesis of HPA axis dysregulation in depressed patients through evidence of an altered CAR. Waking cortisol concentration is a commonly used indicator of HPA axis activity and determination of a stress response, as it is representative of the body's response to the stressful biological event of waking up (Fries et al., 2009). In this study, the depressed group displayed significantly higher waking (T0) salivary cortisol concentrations than healthy controls [Figure 3.2], representative of a hyper-active stress system. This is a finding that has been reported widely in the literature, and the results from this study are in accordance with the existing belief that stress is highly involved in the pathophysiology of depression (Pariante and Lightman, 2008; Lopez-Duran et al., 2009).

Not only did the depressed group display higher cortisol waking concentrations, but their CAR activity over three morning time points was significantly different than that of the healthy control group. Established measurements of CAR activity were derived from these morning concentrations, including Area Under the Curve (AUC), reactivity, and the slope and intercept of a regression line fitted through the three measurements [Figure 3.4; Figure 3.5].

It was revealed that while there was no difference in AUC between depressed patients and healthy controls, the reactivity measure was significantly lower in depressed patients. In fact, the reactivity was a negative value for depressed patients, indicating that the mean final morning cortisol concentration measured was lower than the mean initial waking cortisol concentration, while the reactivity was a positive value in health controls, as the final morning cortisol concentration measured was higher than at waking. The regression lines fitted through morning cortisol data were significantly different between depressed patients and controls, as the depressed group exhibited a significantly higher intercept and lower slope. This reinforces the observation that depressed patients display higher initial concentrations of cortisol and lower fluctuation of cortisol concentration over the course of the morning. Since AUC_i has been considered the most robust parameter for assessing CAR alterations, the lack of difference in AUC_i between groups is a limitation of this study (Stalder et al., 2016). However, reactivity is a valid measure of CAR dynamics (Fekedulegn et al., 2007).

Both groups' cortisol levels decreased significantly between the morning and evening time points, with average evening cortisol concentrations falling below 3 nM in each group. This is a typical pattern of diurnal variation in cortisol content (Kirschbaum and Hellhammer, 1989; Edwards et al., 2001). There were no significant differences in diurnal variation between groups which has been reported in the literature (Hsiao et al., 2006). This could be due to inconsistencies in the time of saliva sampling, since sampling times were scheduled by their distance from waking time, rather than an absolute time (i.e. 12 and 12.5 hours from waking, rather than at midnight).

This study also revealed significant correlations between cortisol activity and depressive symptomatology. HAM-D scores within the depressed group were significantly positively correlated to peak morning cortisol [Figure 3.6]. This supports the hypothesis that HPA axis dysregulation is exacerbated with increased severity of depressive symptoms, which has also been reported in previous studies (Vreeburg et al., 2009; Unschuld et al., 2010).

3.4.2 Relationship between sleep disturbance and CAR

The results of this study suggest a link between disruption of sleep and the HPA axis. Several significant associations between morning cortisol concentrations and sleep disturbance as measured by the self-rated PSQI were found when correlating scores of the whole cohort.

While depressed participants scored significantly higher on the PSQI compared to healthy controls, the range of scores within each group and across the entire cohort was broad. Wakening cortisol concentration, average morning cortisol concentration, and peak morning cortisol were each significantly positively correlated to PSQI scores of the entire cohort. Cortisol activity is known to play an essential role in maintenance of normal circadian rhythms (Knutsson et al., 1997). These findings reiterate the relationship between HPA axis dysregulation and sleep disturbance that has been reported in previous research (Hori et al., 2011; Li et al., 2012). If HPA axis alterations are a result of depression or vice versa, these data aid in understanding why sleep dysregulation is one of the prominent physical symptoms associated with MDD. The association between sleep disruption and HPA axis regulation leads to questions concerning sleep disruption that occur independently of low mood. Previous research reveals that people with poor sleep are at risk of mental health consequences (Glozier et al., 2010; Fitzgerald et al., 2011). Based on the findings of this study and previous studies, poor sleep could be used in the future to identify an opportunity for early intervention with people who are at risk of developing depression. It is possible that treating sleep disruption of non-depressed patients could be useful in regulating the HPA axis in order to prevent low mood (Copinschi et al., 1995; Besnier et al., 2014).

3.4.3 Cortisone concentrations unaffected by a depression diagnosis

There were no differences in cortisone concentrations between depressed patients and healthy controls at any of the five saliva sample collection time points in this study [Figure 3.7]. This indicates that the disruption of the HPA axis witnessed in depressed patients is occurring as an increase of cortisol whilst maintaining similar levels of cortisone. The cortisol/cortisone ratio was calculated in order to assess the differences in the ratios of these corticosteroids at the five time points of saliva sample collection. It was determined that the wakening (T0) cortisol/cortisone ratio was significantly higher in depressed patients compared to healthy controls [Figure 3.8]. This indicated that while cortisone levels were the same in both healthy and depressed populations, the total corticosteroid concentration was increased only via increases in the active stress hormone cortisol. These results are novel and give justification for further research into the ratios of glucocorticoids associated with the stress system.

3.4.4 Relevance of 11 β -HSD1 to depression pathophysiology

The enzyme 11 β -HSD1, which converts cortisone to cortisol [Figure 3.13], has been implicated in human HPA axis regulation and susceptibility to depression as a certain polymorphism of HSD11 β 1 (rs11119328) has been associated with increased rates of depression (Dekker et al., 2011). Because of the implications identified in the literature, differences in whole blood HSD11 β 1 mRNA expression were anticipated between depressed patients and healthy controls. Contrary to the aforementioned findings, the present study detected no differences in mRNA expression of HSD11 β 1 between groups. Since the participants in this study were not genotyped to determine if they carried the polymorphism of this gene associated with depression, the lack of difference in relative quantification of this gene may not be relevant. However, several significant correlations were found between morning corticosteroid concentration and transcriptional expression of HSD11 β 1 [Figure 3.12]. Most noteworthy is the significant positive association with the cortisol/cortisone ratio at wakening, which was previously found to be the only time point containing a significantly elevated cortisol/cortisone ratio in depressed patients. This finding is in line with previous work which has demonstrated that increased cortisol is associated with increased mRNA expression of HSD11 β 1 (Stimson et al., 2009). The existing literature exploring the role of 11 β -HSD1 in depression is limited, yet according to the current study, this enzyme may play a part in the regulation of cortisol concentration and serve as a marker of cortisol activity.

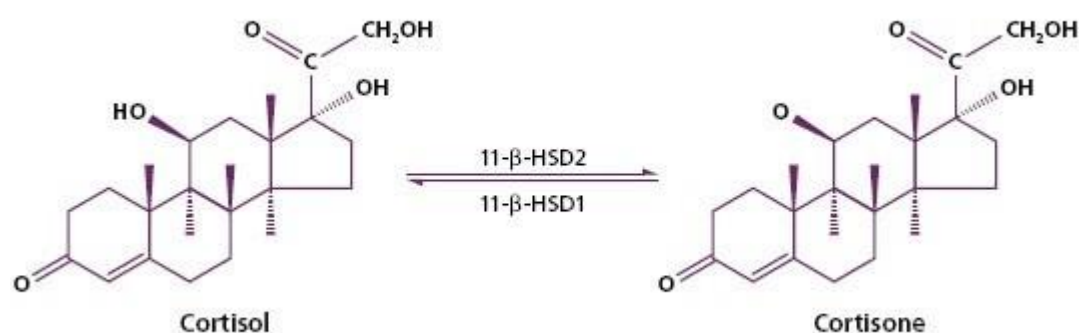


Figure 3.13 Conversion of cortisol to cortisone and vice versa by 11 β -HSD. Chemical structures of cortisol and cortisone. The enzyme that catalyses the conversion of cortisol to cortisone is 11 β -HSD2 and the enzyme that catalyses the conversion of cortisone to cortisol is 11 β -HSD1. (Quattropani et al., 2001).

3.4.5 Limitations and future directions

This study faced several limitations that should be addressed by future studies. For purposes of practicality and compliance, participants in this study were only asked to complete 5 saliva sample collections over the course of the day. An increased number of morning saliva samples and smaller time increments would have strengthened the ability to interpret the CAR. If saliva sample collections had extended to 90 or 120 minutes from wakening, the decline of cortisol concentration would have been visualised more completely and could have revealed further information about diurnal variation and the rate of decreased glucocorticoid activity. Similarly, a 45-minute sample collection would have given a more accurate indication of CAR variability between groups. Many previous studies have assessed HPA axis activity with a greater sampling timespan, but this study chose to reduce the number of saliva samples to 5 per person for compliance purposes.

The study would also have benefited from the addition of record keeping by the participants with regard to the actual times of saliva sample collection, instead times relative to wakening. For example, to denote that a participant woke up at 9:00am and took the saliva sample at this time specifically, rather than simply referring to the sample as being “0 minutes from wakening”. This could have identified samples which may have deviated from protocol and necessitated exclusion, as well as allowed for consideration of the effect of waking time on HPA axis activity. It has been shown that waking time can affect HPA axis activity (Fekedulegn, 2012), therefore this should have been a consideration in the study design.

In future studies, it would be of interest to measure whole blood mRNA expression of the gene encoding for the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) in addition to 11 β -HSD1. While 11 β -HSD1 catalyses the conversion of inert cortisone to active cortisol, 11 β -HSD2 catalyses the opposite reaction. In order to gain a more complete understanding of the role of the isoenzyme in mediating altered glucocorticoid concentrations, both enzymes should be taken into account. Previous studies have suggested the involvement of 11 β -HSD2 in anxiety and depression. One study showed that 11 β -HSD2 was significantly negatively correlated to maternal prenatal anxiety in humans (O'Donnell et al., 2011) while another study showed that a knock-out of the 11 β -HSD2 gene in the foetal brain of male mice caused depressive symptoms and cognitive dysfunction as they became adults (Wyrwoll et al., 2015). The potential involvement of 11 β -HSD2 in the dysregulation of the HPA axis should be explored in conjunction with the measures observed in this study.

3.4.6 Conclusions

This study gives strong evidence of HPA axis dysregulation during depression. While increased waking cortisol has been observed in previous research, this study confirms those findings and suggests the involvement of an altered waking cortisol/cortisone ratio in patients with major depressive disorder. Future research should continue to explore the possibility of involvement of 11 β -HSD enzymes in the dysregulation of the HPA axis.

Chapter 4

Immune system and hippocampal volume alterations in Major Depressive Disorder

4.1 Introduction

Inflammation is the body's biological response to harmful stimuli including infection or damaged cells, and ultimately serves to initiate tissue repair (Watkins et al., 1995). Elevated inflammation is observed in many illnesses including cancer, and is known to increase with age (Lin and Karin, 2007). Furthermore, it has been hypothesised that inflammation mediates grey matter loss through both adaptive and innate immune mechanisms (Glass et al., 2010; Calabrese et al., 2014). Evidence of both inflammation and brain matter loss has been observed in depressed populations and may be involved in its pathophysiology.

Activated inflammatory pathways have been repeatedly observed in MDD patients, as demonstrated by increased inflammatory cytokines and C-reactive protein (CRP) (Raison et al., 2006). Most consistently reported is the increase of interleukin (IL)-6 (Maes, 1999; Lanquillon et al., 2000; Alesci et al., 2005) and CRP (Maes, 1999; Danner et al., 2003; Ford and Erlinger, 2004) concentrations in circulating serum or plasma of depressed patients compared to healthy controls. Increases in plasma IL-1 β and tumour necrosis factor (TNF)- α have also been described in depressed patients compared to non-depressed people (Maes, 1999; Tuglu et al., 2003; Thomas et al., 2005). Within depressed populations, it has been shown that severity of symptoms is correlated to inflammation (Thomas et al., 2005). Despite the abundant findings of increased inflammation in depression, other studies have reported results inconsistent with this. Some studies have failed to find associations between depressive symptoms and inflammation, or found that associations were negated when analysis included covariates such as BMI and gender (Bouhuys et al., 2004). This indicates that inflammation may be one of many contributing factors in depression.

There are clinical findings that support the possible link between depression and inflammation. Numerous studies have reported on the anti-inflammatory properties of antidepressant medication (Sluzewska et al., 1995; Kenis and Maes, 2002). Moreover, it has been shown that cytokine immunotherapy can induce depression in patients being treated for Hepatitis C and certain cancers (Valentine et al., 1998; Capuron et al., 2001). Also, findings from a meta-analysis of clinical trials suggest that the use of NSAIDS is associated with an improved antidepressant treatment response (Köhler et al., 2014). This bidirectional relationship between depression and inflammation provides a strong rationale for further investigation into the relationship between inflammation and other biological pathways of depression.

Evidence points to a role for the immune system and inflammation in brain volume integrity, as hippocampal volume has been shown to be reduced in aging and disease including Alzheimer's and Multiple Sclerosis (Small, 2010; Glass et al., 2010). The presence of chronic inflammation in the subarachnoid and perivascular spaces might be responsible for an abnormal and microglial over-activation that can be harmful to neurons and subsequently lead to a dysregulation of astrocyte functions and glutamate metabolism, resulting in additional neuronal and synaptic damage (Calabrese et al., 2015; Sankowski et al., 2015). It has been shown that hippocampal volume reduction is associated with an increase in inflammatory cytokines including IL-6 (Marsland et al., 2008; Kesler et al., 2013) and TNF- α (Braskie et al., 2014; Kesler et al., 2013). While grey matter loss is considered to be part of natural aging, this process is accelerated in MDD patients compared to healthy controls (Grieve et al., 2013). Early life stressors have been shown to diminish cell proliferation and dampen the production of new neurons during adulthood in several animal models, reinforcing the possible involvement of stress in neurodegeneration (Lemaire et al., 2000; Coe et al., 2003).

Inflammation is known to be one of the biological consequences of stress (Miura et al., 2008). The major stress system, the HPA axis, is functionally linked to the immune system as glucocorticoids regulate inflammatory responses (Elenkov et al., 1999). The HPA axis has been found to be overactive in depression, resulting in glucocorticoid resistance. It has been shown that dysregulation of the HPA axis results in lack of suppression of the immune system (Sorrells, 2009). It is possible that these two pathways are both altered in depression as a result of their functional relationship.

The hippocampus is also linked to regulation of the HPA axis and dysregulation in MDD. The role of the hippocampus in limiting levels of circulating glucocorticoids is impaired following stress (Herman and Cullinan, 1995). Additionally, depressed patients with HPA dysregulation have shown hippocampal volume reduction in previous clinical studies (Vythilingam et al., 2002). This evidence connecting hippocampal dysregulation and volume reduction, immune alterations, and HPA axis activation supports the need for further investigation into the involvement of these pathways in major depressive disorder.

Study Aims:

1. To determine the inflammatory state of depressed patients compared to healthy controls in this cohort.
2. To investigate hippocampal volume integrity in depressed patients compared to healthy controls.
3. To determine the relationship between depressive symptom severity and measures for immune activation and/or hippocampal volume.
4. To investigate the relationship between HPA axis activation and immune response.
5. To investigate the relationship between the HPA axis and hippocampal subfield volumes.

4.2 Methods

In this study, inflammation and brain matter volumes were assessed in 62 depressed patients and 41 healthy controls. Inflammation and brain volume data were then correlated to HPA variables assessed in Chapter 3 to determine the relationship between these pathways in this cohort. Depressed patients were recruited from the psychiatric outpatient clinic at Sheaf House in Tallaght Hospital and at the Mary Mercer Healthy Centre in Jobstown, Dublin 24. Recruitment of depressed patients was based on criteria for a Major Depressive Episode (DSM-5, American Psychiatric Association, 2013). Additional inclusion and exclusion criteria for depressed patients and healthy controls were as stated in Chapter 2. Psychiatric rating scale data was collected from participants to measure severity of depressive symptoms, sleep disturbance, and childhood trauma. These scales included the HAM-D, MINI, CES-D, PSQI, and CTQ (Appendices 3a through e).

Circulating concentrations of inflammatory cytokines were measured in this cohort. Two 9 ml EDTA tubes of blood were collected from all participants at the time of recruitment or within one week, at the time of their MRI scan. Plasma was extracted from the EDTA blood for use in assays. Human IL-6, IL-1 β , TNF- α , and IFN- γ were quantified by sandwich ELISA using BioLegend ELISA MAX™ Deluxe kits as per manufacturer's instructions which are detailed at length in Chapter 2. Sandwich ELISA is a sensitive and robust method which measures the cytokine concentration in an unknown sample. The assay is called a "sandwich" ELISA because the cytokine of interest is quantified between two layers of antibodies, the capture and the detection antibody. C-reactive protein (CRP) was also assessed using the sandwich type Quantikine ELISA kit (R&D Systems, UK). Concentrations of circulating cytokines and CRP were calculated by using a standard curve with standards of known antigen concentrations.

Whole blood mRNA expression of genes encoding for cytokines IL-1 β , TNF- α and IFN- γ were also assessed. One 2.5 ml PAXgene DNA tube of blood was collected and stored as described in Chapter 2. Total RNA was isolated from the whole blood and equalized before it was used for cDNA synthesis. Analysis of gene expression of the target genes was conducted using Real-Time PCR methods and Taqman® Gene Expression Assays (Applied Biosystems, UK). Relative quantification data was calculated using the $\Delta\Delta C_t$ method and GAPDH as an endogenous control, as described at length in Chapter 2.

Magnetic resonance imaging (MRI) scans were collected from participants within a week of recruitment to the study to assess brain matter volumes. Structural T1-weighted scans were acquired at Trinity College Institute of Neuroscience using a Philips Achieva MRI scanner (Philips Medical System, Netherland BV, Veenphuis 4-6, 5684 PC Best, Netherlands) operating at 3 Tesla. Voxel based morphometry (VBM) analysis was employed to calculate grey matter volumes of the amygdala and hippocampus in depressed patients and healthy controls. For these calculations, MRI images were processed using the VBM8 toolbox within SPM12 as described in greater detail in Chapter 2 with the assistance of Dr. Leonardo Tozzi. In order to assess more discrete volumetric differences, hippocampal subfield volume measurements were calculated using Free Surfer (<http://surfer.nmr.mgh.harvard.edu/>). It was hypothesised that specific subfields associated with HPA activity, such as the subiculum, CA3, and CA4, would exhibit decreased volumes in depressed patients compared to healthy controls due to the previously observed HPA alterations in depressed patients (Chapter 3). Hippocampal subfield volume calculation was conducted with the assistance of Dr. Erik O'Hanlon and is described in greater detail in Chapter 2.

Data are presented as mean with standard error of the mean (SEM) or standard deviation (SD) where appropriate. All data were tested for normality using the Shapiro-Wilk test. Non-parametric data were analysed using the Mann-Whitney U test for independent sample comparisons. Univariate general linear model analysis was used to implement age, gender, and Total Intracranial Volume (TIV) as covariates for MRI data. Correlational analysis was carried out using Spearman's rho correlation statistics, or partial correlations correcting for age, gender, and TIV when correlating brain volume variables. All statistical analyses were considered significant when $p \leq 0.05$. All data were analysed using SPSS (Version 16). Graphs and statistics were generated using GraphPad Prism Software Version 5.00 (GraphPad Software, Inc).

4.3 Results

4.3.1 Demographic data for patients with MDD and healthy controls whose circulating cytokine concentrations were measured

Analysis revealed that there were no differences in age, gender, or BMI between depressed patients and healthy controls in this cohort [Table 4.1]. However, the depressed group had a significantly higher number of smokers ($p=0.002$) compared to healthy controls (MDD: 40.3% smokers vs. HC: 12.2% smokers), and healthy controls were more educated on the ISCED scale ($p<0.001$) compared to depressed patients (MDD: 3.44 (1.46) vs. HC: 6.17 (1.80)) [Table 4.1].

Table 4.1 Demographic data for patients with MDD and healthy controls whose circulating cytokine concentrations were measured.

	Patients (n=62)	Controls (n=41)	Statistics (p-value)
Age (years)	27.82 (8.34)	28.59 (6.86)	$Z=-0.722$, $p=0.470$
Gender (female/male)	43/19	25/16	$\chi^2=0.772$, $p=0.379$
BMI	24.69 (5.96)	23.20 (3.40)	$Z=0.903$, $p=0.366$
Smoking (Yes/No)	25/37	5/36	$\chi^2=9.459$, $p=0.002^{**}$
ISCED Education	3.44 (1.46)	6.17 (1.80)	$Z=-6.050$, $p<0.001^{***}$

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI) and Chi squared (χ^2) test (Gender, Smoking). $^{**}p<0.01$ vs. control, $^{***}p<0.001$ vs. control.

4.3.2 Clinical data for patients with MDD and healthy controls

Reflective of a diagnosis of MDD, patients scored significantly higher compared to healthy controls on the HAM-D and CES-D [Table 4.2], signifying elevated depressive symptoms. Patients also exhibited increased sleep disturbance through elevated PSQI scores [Table 4.2]. In the depressed group, a larger number of participants had experienced early life adversity (MDD: 64.5% vs. HC: 14.6%), with higher Global CTQ scores than healthy controls [Table 4.2]. All clinical measures described were higher at $p < 0.001$. Of the depressed cohort, 25 were medication free, 22 were prescribed SSRIs, 8 were prescribed SNRIs, and 7 were on other medications including benzodiazepines, melatonergics, anti-epileptics or a combination of medications. In accordance with recruitment criteria, healthy controls were not taking medication at the time of recruitment.

Table 4.2 Clinical data for patients with MDD and healthy controls whose circulating cytokine concentrations were measured.

	Patients (n=62)	Controls (n=41)	Statistics (p-value)
HAM-D	22.93 (5.12)	2.10 (2.29)	Z=-8.547, $p < 0.001^{***}$
CES-D	39.56 (9.44)	5.41 (5.48)	Z=-0.8437, $p < 0.001^{***}$
PSQI	13.22 (3.64)	3.73 (2.04)	Z=-8.183, $p < 0.001^{***}$
CTQ Global Score	43.93 (14.80)	29.44 (7.33)	Z=8.437, $p < 0.001^{***}$
Early Life Adversity (Yes/No)	40/22	6/35	$\chi^2=25.897$, $p < 0.001^{***}$
Antidepressant (Yes/No)	37/25	0/41	

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). $^{***}p < 0.001$ vs. controls.

4.3.3 Circulating concentrations of CRP and inflammatory cytokines in depressed patients and healthy controls

A Mann-Whitney U test revealed no differences in circulating concentrations of cytokines assayed including IL-1 β , IFN- γ , TNF- α or IL-6 [Table 4.3] [Figure 4.1a-d]. Fourteen of the 31 control samples and 27 of the 58 depressed patient samples assayed had IL-1 β concentrations that fell below the limits of detection. Three of the 37 control samples and 9 of the 57 depressed patient samples assayed had TNF- α concentrations that fell below the limits of detection. Fourteen of the 31 control samples and 19 of the 52 depressed patient samples assayed had IFN- γ concentrations that fell below the limits of detection. Finally, 4 of the 28 control samples and 13 of the 53 depressed samples assayed had IL-6 levels below the limits of detection. Samples with cytokine concentrations below the limits of detection were recorded as having a 0 pg/ml concentration.

CRP was measured in all of the samples assayed, including 55 depressed patients and 39 healthy controls. A Mann-Whitney U test revealed no significant differences in CRP concentration between MDD patients and healthy controls [Table 4.3]. There was no significant difference in circulating CRP concentrations between depressed patients and healthy controls [Figure 4.1e].

Table 4.3 Circulating cytokine and CRP concentration data.

	Patients (n=62)	Controls (n=41)	Statistics
IL-1 β (pg/ml)	5.19 (1.15)	6.58 (1.75)	Z=-0.508, p=0.612
IFN- γ (pg/ml)	12.33 (2.32)	16.78 (5.67)	Z=-0.553, p=0.581
TNF α (pg/ml)	16.78 (2.25)	23.94 (4.81)	Z=-0.860, p=0.390
IL-6 (pg/ml)	7.52 (0.96)	5.89 (1.12)	Z=-0.653, p=0.513
CRP (μ g/ml)	1.72 (0.30)	1.35 (0.25)	Z=-0.741, p=0.459

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

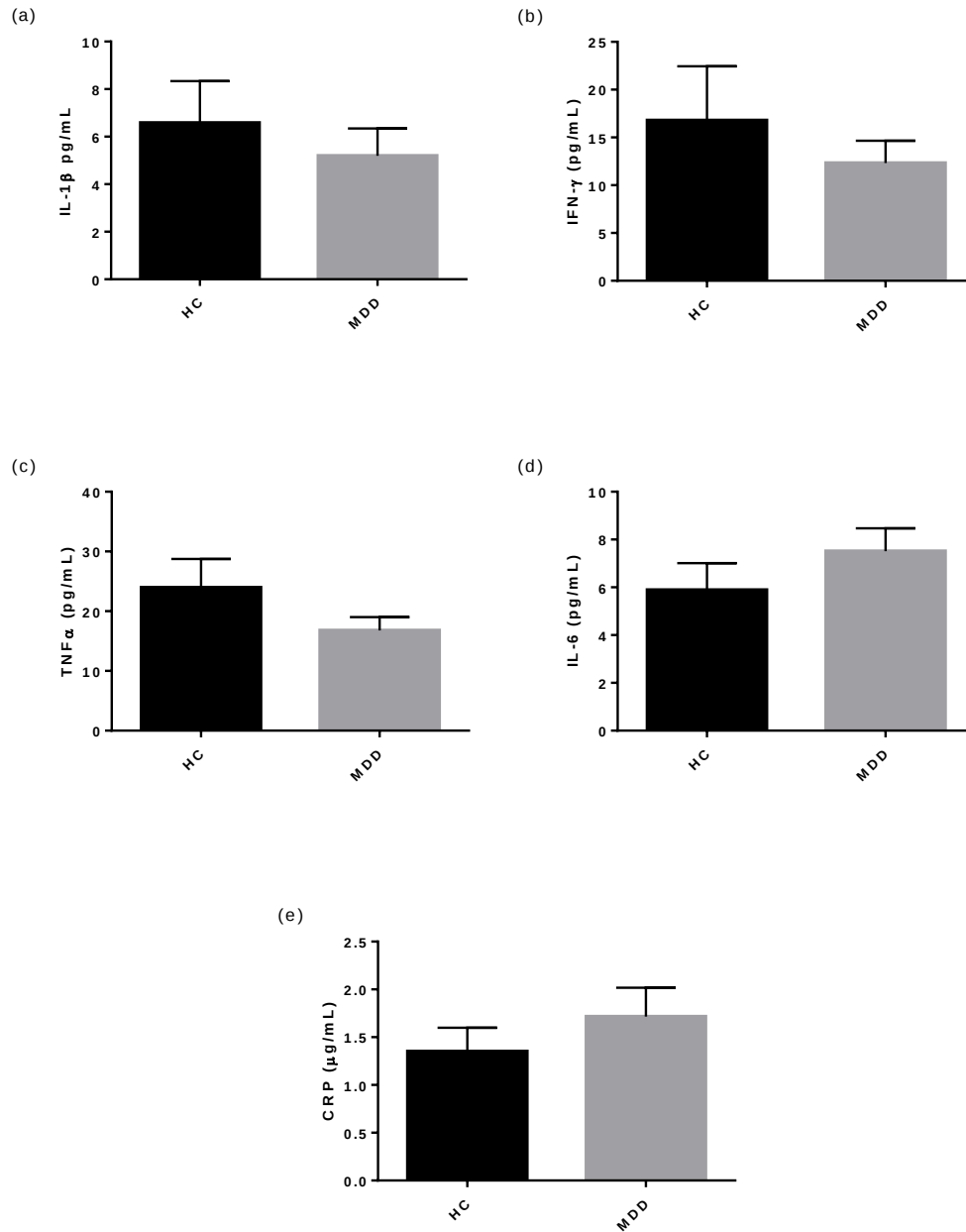


Figure 4.1 Circulating inflammatory cytokines and CRP in depressed patients and healthy controls. Circulating concentrations of (a) IL-1 β , (b) IFN- γ , (c) TNF α , (d) IL-6, and (e) CRP in depressed patients (n=62) and healthy controls (n=41). Data expressed as mean and SEM. No differences between groups means exist. (Mann Whitney U test).

4.3.4 Relationships amongst circulating inflammatory cytokine concentrations

A Spearman's rho correlation performed on circulating concentrations of cytokines of all participants (n=75) revealed a significant positive correlation between IL-6 and TNF α circulating concentrations ($r=0.325$, $p=0.004$) [Figure 4.2]. This indicates that concentrations of inflammatory cytokines IL-6 and TNF- α may increase in tandem. There were no other significant relationships between concentrations of any other cytokines with one another.

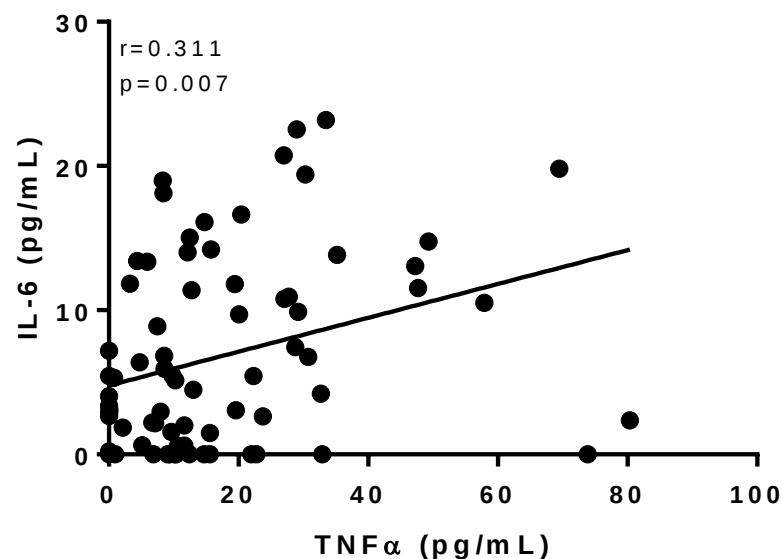


Figure 4.2 Correlation between IL-6 and TNF α concentrations. A significant positive correlation exists between IL-6 and TNF α concentrations according to a Pearson's correlational analysis.

4.3.5 Inflammatory cytokine whole blood mRNA expression in depressed patients compared with healthy controls

Demographic and clinical data relating to the subset of participants that had whole blood mRNA expression measured can be found in Appendix 4a. There were no differences between depressed patients and healthy controls in regard to age, gender, or BMI. Consistent with a diagnosis of MDD, depressed patients exhibited significantly higher depressive symptoms, sleep disturbance, and early life adversity.

Gene expression of IFN- γ , IL-1 β , and TNF α was measured in whole blood from depressed patients relative to control subjects.

Basal IL-1 β mRNA expression was observed in all blood samples studied and an average Ct for controls was determined to be 28.5. The housekeeping gene GAPDH had an average Ct of 25.0, therefore the Δ Ct of IL-1 β was 3.5.

Basal IFN- γ mRNA expression (a Ct of less than 45) was observed in 35 of the 38 control samples and 51 of the 55 depressed patient samples. The average Ct for IFN- γ in the control group was 34.5, while the average Ct for the housekeeping gene GAPDH was 25.3, resulting in an average Δ Ct of 9.2 for IFN- γ .

Basal TNF α mRNA expression was observed in all control samples and 54 of the 55 depressed patient samples. The average Ct of TNF α in the control group was 30.3, while the average Ct for housekeeping gene GAPDH was 25.1, resulting in an average Δ Ct of 5.2 for TNF α .

A Mann-Whitney U test revealed significantly higher expression of IL-1 β mRNA in depressed patients compared to healthy controls ($Z=-2.236$, $p=0.025$) [Figure 4.3a]. There was no significant difference in IFN γ mRNA expression ($Z=-0.762$, $p=0.446$) or TNF α mRNA expression ($Z=-1.071$, $p=0.284$) between depressed patients and healthy controls [Figure 4.3b,c].

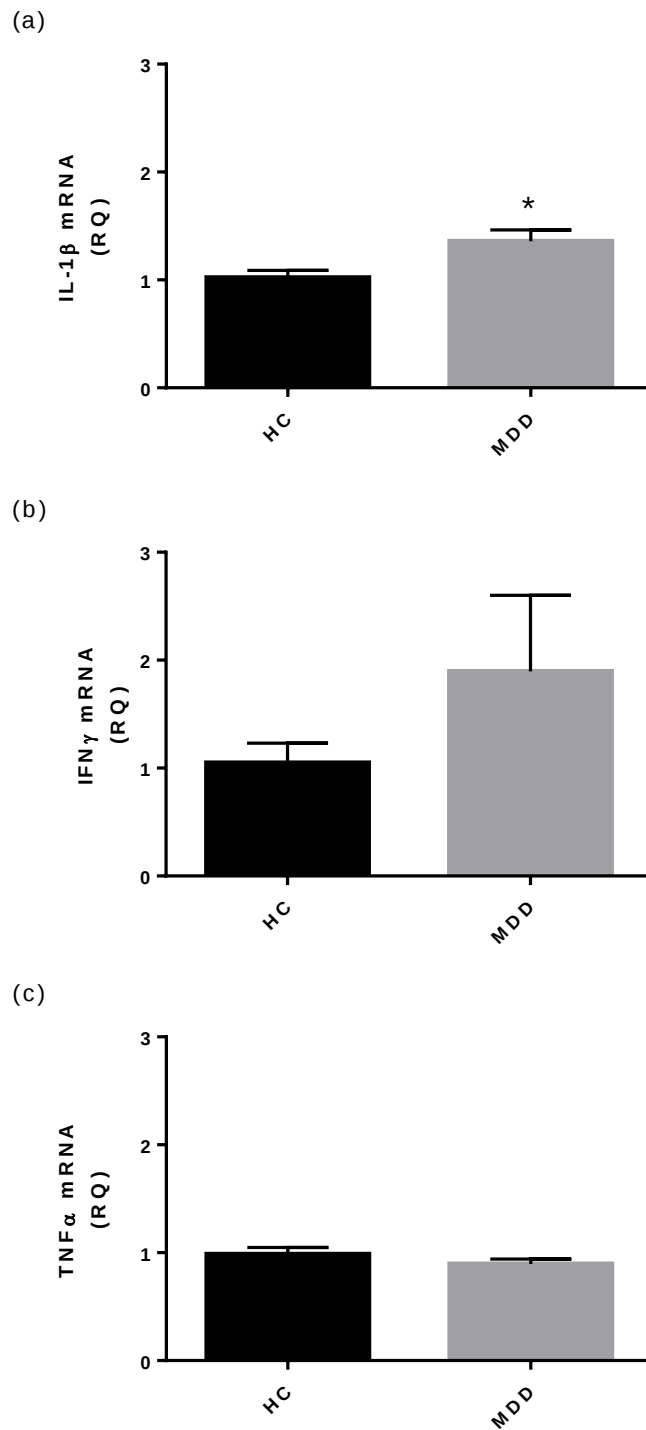


Figure 4.3 Whole blood mRNA expression of inflammatory cytokines in depressed patients and healthy controls. Relative quantification of mRNA expression for (a) IL-1 β , (b) IFN- γ , and (c) TNF- α in depressed patients (a: n=55, b: n=52, c: n=54) compared with healthy controls (a: n=38, b: n=35, c: n=38). Data expressed as mean with SEM. Statistical analysis was conducted with a Mann Whitney U test. p* $\leq$ 0.05 vs. controls.

4.3.6 Relationships between cytokine mRNA expression and psychiatric rating scales

Within the depressed group, no significant relationships exist between mRNA expression of cytokines and depressive symptom severity as measured by HAM-D or CES-D.

A Spearman's rho correlation analysis between IL-1 β mRNA relative quantification and PSQI score for the entire cohort revealed a significant positive relationship ($r=0.250$, $p=0.018$). However, within group analysis revealed no relationships within the depressed and control groups individually (MDD: $r=0.24$, $p=0.087$; HC: $r=-0.012$, $p=0.944$) [Figure 4.4a]. No significant relationships exist between sleep quality and transcriptional expression of IFN- γ or TNF- α .

A Spearman's rho correlation analysis revealed that within the depressed group there was medium effect size significant positive correlation between IL-1 β gene expression and CTQ global score with a ($r=0.332$, $p=0.015$) [Figure 4.4b]. No significant relationships exist between global CTQ scores and transcriptional expression of IFN- γ or TNF- α .

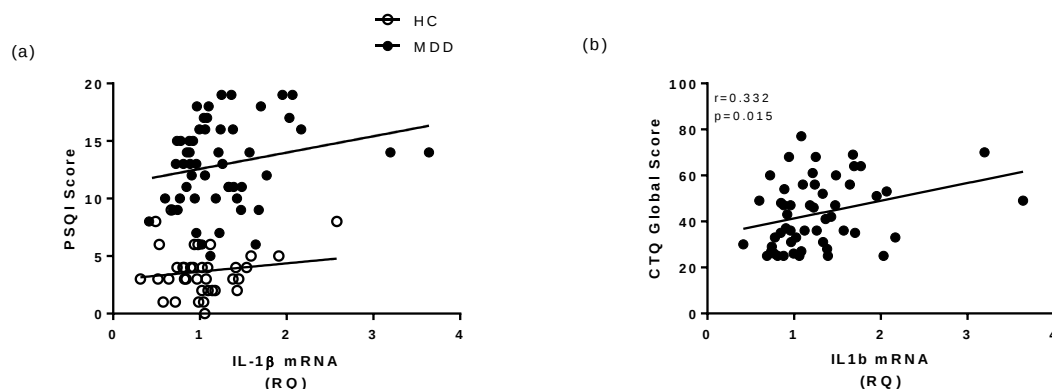


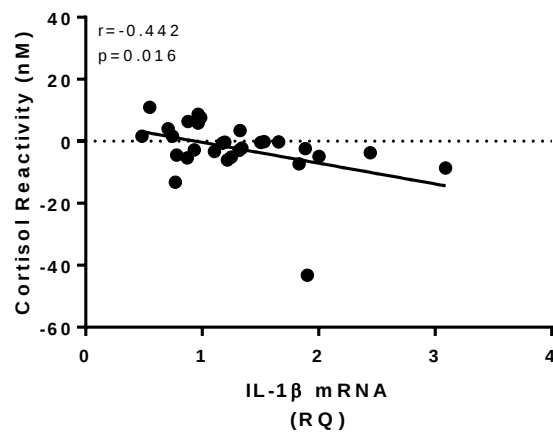
Figure 4.4 Correlations between mRNA expression of inflammatory cytokines and psychiatric rating scales. A Spearman's rho correlation revealed a significant positive correlation between (A) IL-1 β mRNA expression and PSQI in the entire cohort but not within groups, and (B) a significant positive correlation between IL-1 β mRNA expression and CTQ Global score in the MDD group.

4.3.7 Relationship between immune system measures and HPA axis measures

There were no significant correlations between IFN- γ , TNF α , IL-6, or CRP and morning cortisol concentrations or CAR parameters within the depressed patient group. However, a Spearman's rho analysis of immune system measures and log transformed HPA axis measures revealed some significant relationships within the entire cohort. Circulating concentration of IL-1 β were positively correlated with cortisol concentration at T720 (n=37, $r=0.533$, $p=0.001$), as was the PM average cortisol concentration (n=37, $r=0.392$, $p=0.016$). Circulating IL-6 concentration was negatively correlated to wakening cortisone concentration among the entire cohort (n=55, $r=-0.276$, $p=0.041$). In contrast, there were no significant correlations between IFN- γ , TNF α , IL-6, or CRP and morning cortisol concentrations or CAR parameters.

Within the depressed group, there was a significant negative correlation between whole blood IL-1 β mRNA expression and cortisol reactivity ($r=-0.442$, $p=0.016$) [Figure 4.5a]. There was also a significant positive correlation between whole blood IFN- γ mRNA expression and cortisol concentration at T30 ($r=0.387$, $p=0.020$) within the depressed group [Figure 4.5b]. There were no significant relationships found between mRNA expression of TNF α with any HPA axis measures within the depressed cohort.

(a)



(b)

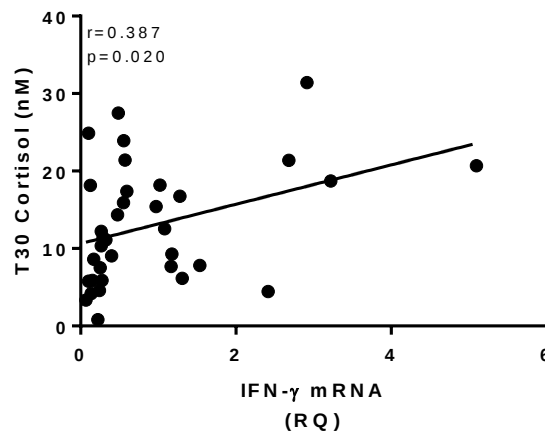


Figure 4.5 Correlational analysis between mRNA expression of cytokines and HPA axis measures in depressed patients. A Spearman's rho correlation revealed (a) a significant negative correlation between mRNA expression of IL-1 β and morning cortisol reactivity within the depressed group and (b) a significant positive correlation between mRNA expression of IFN- γ and T30 cortisol concentration within the depressed group.

4.3.8 Voxel based morphometry hippocampal and amygdalar volumes in depressed patients and healthy controls

Demographic and clinical data relating to the subset of participants that completed structural MRI scans for the study can be found in Appendix 4b. There were no differences in age, gender, or BMI between depressed patients and healthy controls. Consistent with a diagnosis of MDD, depressed patients exhibited significantly higher depressive symptoms, sleep disturbance, and early life adversity.

Voxel based morphometry (VBM) analysis was used to calculate volumes of whole left and right hippocampus and left and right amygdala.

A univariate general linear model analysis of variance was performed using age, gender and total intracranial volume (TIV) as covariates [Table 4.4]. This analysis revealed no significant difference in whole hippocampal volumes between depressed patients and healthy controls [Figure 4.6a, b]. VBM analysis also revealed no significant difference in left amygdala volume between depressed patients and healthy controls [Figure 4.7a]. However, a trend towards higher right amygdalar volume in the depressed group was detected [Figure 4.7b].

Table 4.4 Grey Matter Volume data for patients with MDD and healthy controls who completed structural brain scans.

Brain Region	Patients (n=47)	Controls (n=35)	Statistics (p-value)
Left Hippocampus (mm ³)	3944.97 (56.41)	4086.69 (66.83)	F=0.936, p=0.336
Right Hippocampus (mm ³)	4011.59 (51.62)	4094.81 (62.54)	F=0.065, p=0.779
Left Amygdala (mm ³)	1575.34 (28.29)	1576.96 (33.76)	F=0.331, p=0.567
Right Amygdala (mm ³)	1694.84 (29.52)	1647.33 (36.99)	F=3.421, p=0.068

Data expressed as mean Grey Matter volume in mm³ with SEM in parentheses. Statistical analysis was performed using a Univariate General Linear Model analysis with Age, Gender, Smoking and Total Intracranial Volume (TIV) as covariates.

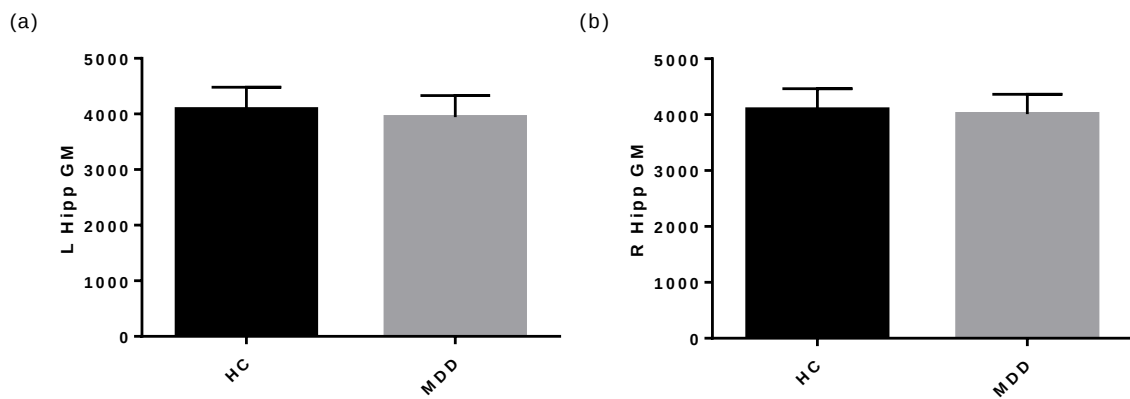


Figure 4.6 Hippocampal grey matter volumes in depressed patients and healthy controls. Grey matter volumes expressed as means and SEM. No difference in (a) left hippocampal volume or (b) right hippocampal volume between depressed patients and healthy controls. (Univariate analysis of variance, co-varying for age, gender, and TIV).

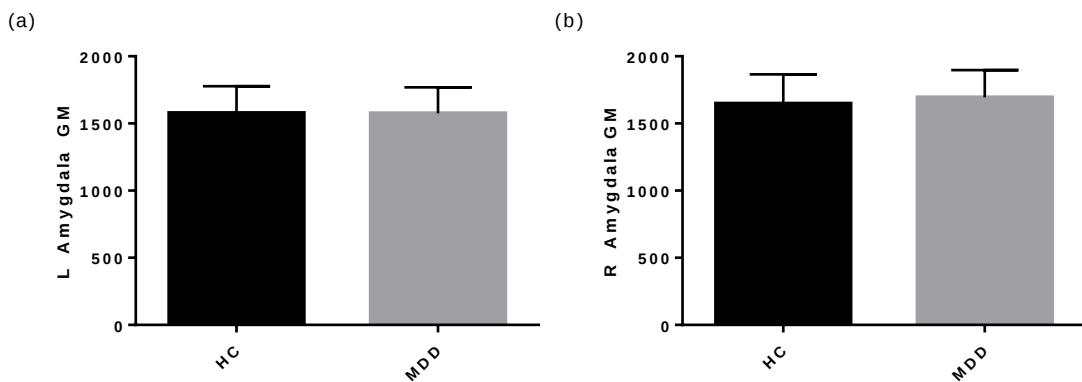


Figure 4.7 Amygdalar grey matter volumes in depressed patients and healthy controls. Grey matter volumes expressed as means and SEM. No difference in (a) left amygdalar volume or (b) right amygdalar volume between depressed patients and healthy controls. (Univariate analysis of variance, co-varying for age, gender, and TIV).

4.3.9 Hippocampal subfield volumes in depressed patients and healthy controls

Further volumetric analysis was carried out to compare the left and right CA3, CA4, subiculum, and hippocampal fissure in depressed patients and healthy controls. The anatomy of the subfields of interest that were investigated in this study is depicted in Figure 4.8.

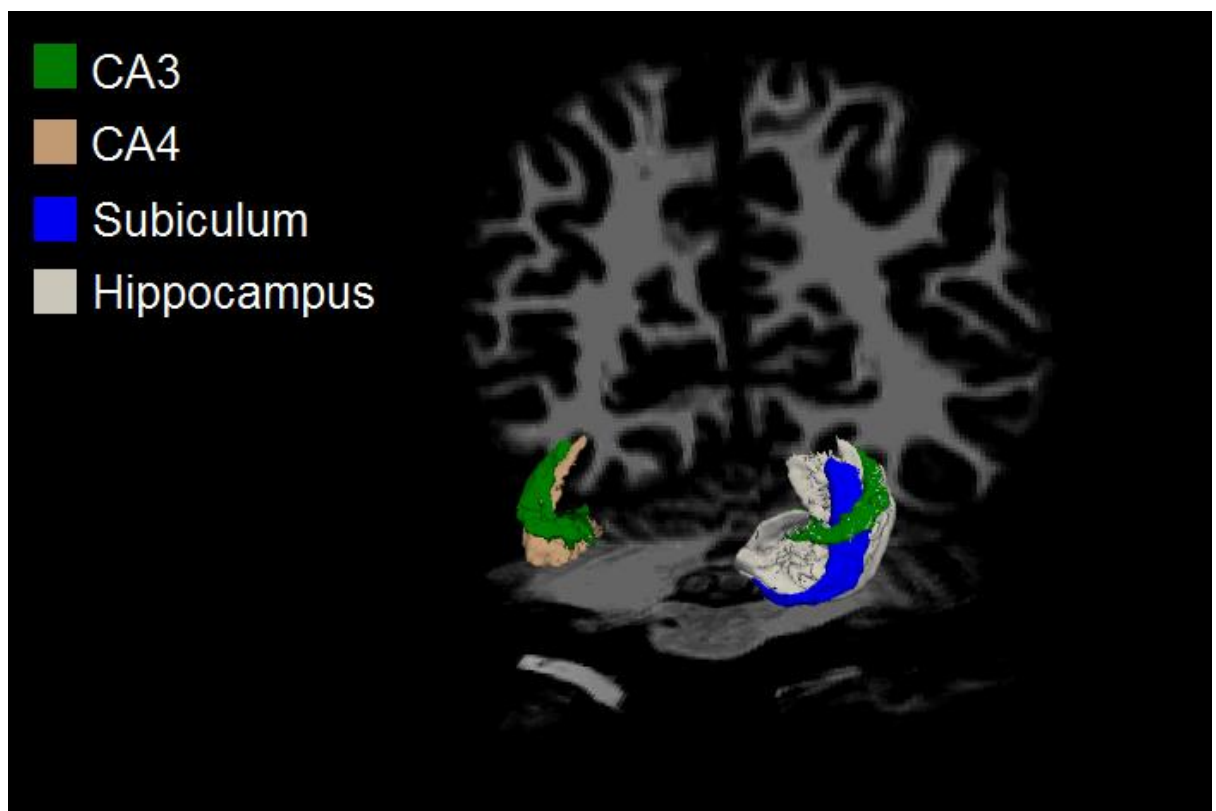


Figure 4.8 Hippocampal subfields implicated in depression. Structural brain image displaying hippocampal subfield areas of interest. The blue area highlights the left subiculum. The grey region encompasses the remainder of the whole left hippocampus. Green areas indicate left and right CA3. The brown colour indicates the CA4 subfield. Figure constructed using Free Surfer.

4.3.9.1 Hippocampal CA3 and CA4 subfield volumes in depressed patients and healthy controls

A univariate analysis of variance using age, gender, and TIV as covariates was performed to determine if differences in left subfield volumes existed between depressed patients and healthy controls.

The analysis revealed that depressed patients had significantly lower left CA3 volume than healthy controls [Table 4.5] [Figure 4.9a]. Analysis also showed several significant differences between subfield volumes of the right hippocampus [Table 4.6]. The right CA3 and CA 4 were smaller in volume in the depressed group compared to controls [Figure 4.9b, 4.10b]. Finally, the right hippocampal fissure was significantly larger in depressed patients than healthy controls.

Table 4.5 Left hippocampal subfield volumes for patients with MDD and healthy controls who completed structural brain scans.

Left Brain Region	Patients (n=47)	Controls (n=35)	Statistics (p-value)
CA3	211.54 (3.97)	225.93 (4.77)	F=4.089, df=1, p=0.047*
CA4	250.34 (4.06)	264.55 (4.89)	F=3.501, df=1, p=0.065
Subiculum	397.16 (5.35)	406.20 (4.87)	F=0.915, df=1, p=0.342
Hippocampal-fissure	160.24 (4.07)	155.05 (4.46)	F=1.125, df=1, p=0.292
Whole left hippocampus	3352.02 (43.17)	3487.44 (54.01)	F=2.967, df=1, p=0.089

Data are expressed as mean volume and SEM in parenthesis. Statistical analysis was performed using a Univariate General Linear Model analysis with Age, Gender, and Total Intracranial Volume as covariates.

Table 4.6 Right hippocampal subfield volumes for patients with MDD and healthy controls who completed structural brain scans.

Right Brain Region	Patients (n=47)	Controls (n=35)	Statistics (p-value)
CA3	227.60 (3.38)	244.19 (4.44)	F=5.884, df=1, p=0.018*
CA4	258.80 (3.62)	274.31 (4.44)	F=4.198, df=1, p=0.044*
Subiculum	382.94 (4.93)	396.34 (7.13)	F=0.674, df=1, p=0.414
Hippocampal-fissure	151.64 (3.47)	137.82 (2.97)	F=7.562, df=1, p=0.008**
Whole right hippocampus	3354.11 (39.85)	3456.09 (49.96)	F=0.567, df=1, p=0.454

Data are expressed as mean volume and SEM in parenthesis. Statistical analysis was performed using a Univariate General Linear Model analysis with Age, Gender, and Total Intracranial Volume as covariates.

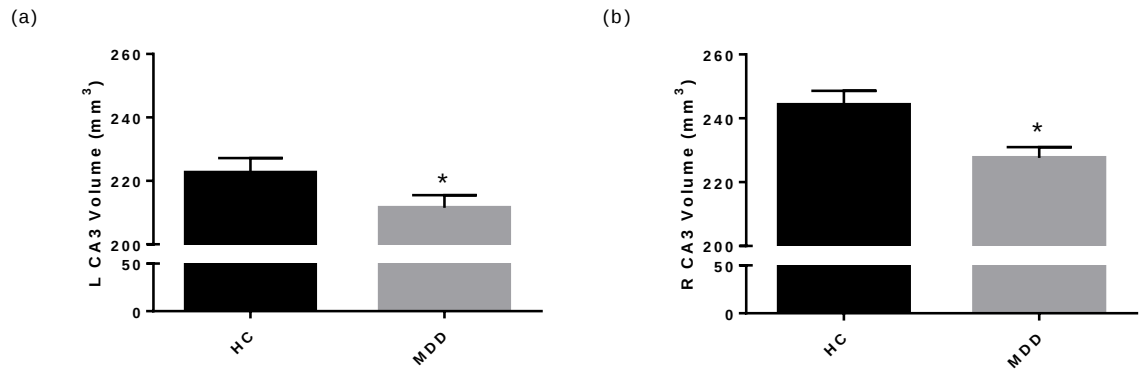


Figure 4.9 Left and right hippocampal CA3 volumes in depressed patients and healthy controls. Hippocampal subfield volumes for (a) left CA3 and (b) right CA3 in depressed patients (n=47) and healthy controls (n=35). Data expressed as mean and SEM. * $p \leq 0.05$ vs. control (Univariate General Linear Model analysis of variance with age, gender, and TIV Volume as covariates).

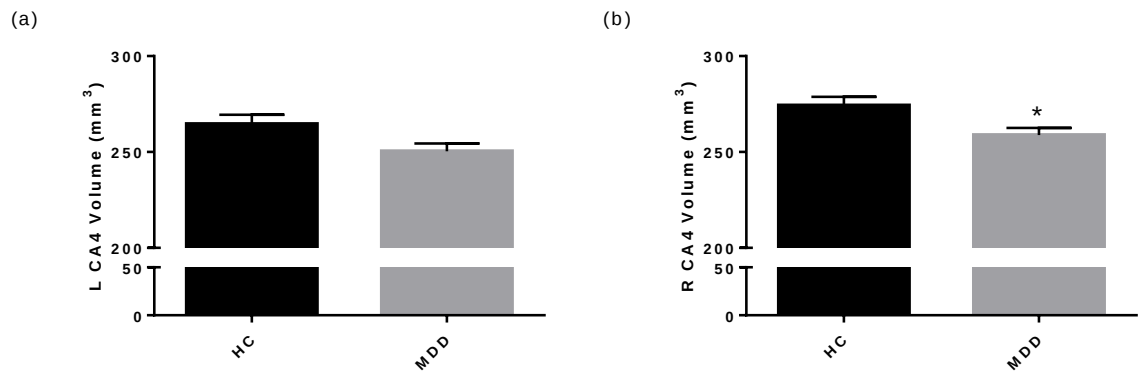
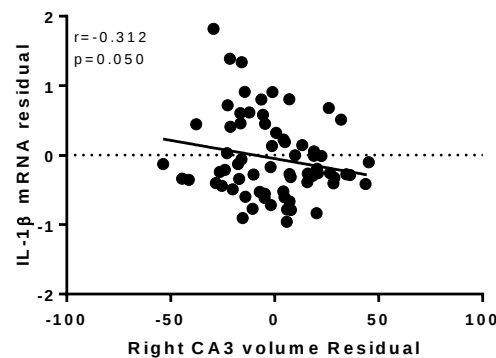


Figure 4.10 Left and right hippocampal CA4 volumes in depressed patients and healthy controls. Hippocampal subfield volumes for (a) left CA4 and (b) right CA4 in depressed patients (n=47) and healthy controls (n=35). Data expressed as mean and SEM. * $p \leq 0.05$ vs. control (Univariate General Linear Model analysis of variance with age, gender, and TIV as covariates).

4.3.9.2 Relationship between immune activity and CA3 and CA4 volumes

A partial correlation correcting for age, gender, and TIV revealed significant relationships between right CA3 and CA4 volumes with transcriptional expression of IL-1 β among the entire cohort. There was a significant negative correlations between IL-1 β gene expression and CA3 ($r=-0.370$, $p=0.019$) [Figure 4.11a] and between IL-1 β gene expression and CA4 ($r=-0.312$, $p=0.050$) [Figure 4.11b]. However, within group analysis revealed that no significant correlation existed between these variables within the depressed group alone. No correlations exist between hippocampal subfields and the other circulating of whole blood mRNA inflammatory markers measured.

(a)



(b)

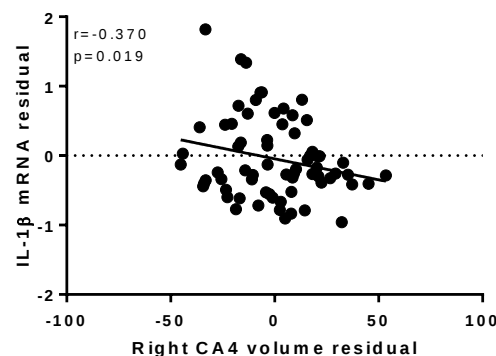


Figure 4.11 Relationship between Right CA3 and CA4 volumes and whole blood mRNA expression of IL-1 β . A significant negative relationship exists between IL1b and (a) right CA3 volume and (b) CA4 volume among the entire cohort. (Partial correlation correcting for age, gender, and TIV).

4.3.9.3 Relationship between subiculum and depressive symptoms

A univariate general linear model analysis using age, gender, and TIV as covariates did not reveal a difference in left or right subiculum volume between depressed patients and healthy controls [Figure 4.12a, b]. However, a partial correlation between subiculum volumes and psychiatric rating scales correcting for age, gender, and TIV revealed a significant negative relationship between left subiculum volume and CES-D score ($r=-0.406$, $p=0.010$) [Figure 4.13]. The relationship between left subiculum volume was further investigated within groups, and a negative trend was revealed between subiculum volume and CES-D score ($r=-0.237$, $p=0.136$).

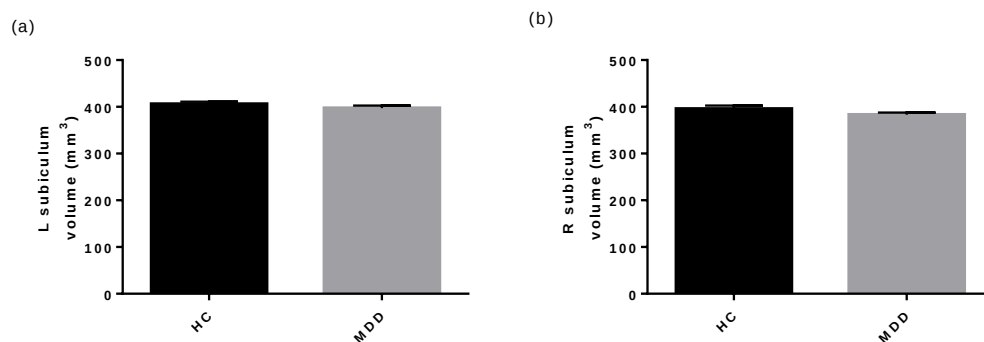


Figure 4.12 Left and right hippocampal subiculum volumes in depressed patients and healthy controls. Hippocampal subfield volumes for (a) left subiculum and (b) right subiculum in depressed patients ($n=47$) and healthy controls ($n=35$). Data expressed as mean and SEM. (Univariate General Linear Model analysis of variance with age, gender, and TIV as covariates).

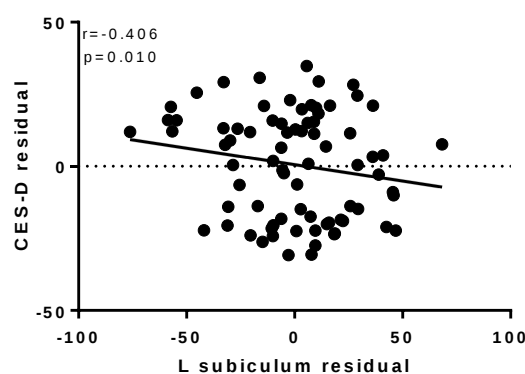


Figure 4.13 Relationship between left subiculum volume and CES-D score. A significant negative relationship exists between CES-D scores and left subiculum volumes among the entire cohort according to a partial correlation controlling for age, gender, and TIV of the entire cohort.

4.2.9.4 Hippocampal fissure volumes in depressed patients and healthy controls

A univariate general linear model analysis of variance using age, gender, and TIV as covariates revealed a significantly larger right hippocampal fissure volume in depressed patients compared to healthy controls [Figure 4.14]. No difference between left hippocampal fissure volumes was detected.

4.2.9.5 Relationship between hippocampal fissure volumes and HPA axis activity within MDD patients

A partial correlation controlling for age, gender, and TIV revealed significant correlations between right hippocampal volumes and HPA axis measurements within the depressed group. Right hippocampal fissure was positively correlated to peak morning cortisol ($r=0.404$, $p=0.022$) [Figure 4.15a]. Additionally, right hippocampal fissure was positively correlated to wakening cortisol concentration ($r=0.018$, $p=0.435$) and average morning cortisol ($r=0.409$, $p=0.020$) [Figure 4.15b, c], reinforcing the existence of a relationship between the volume of this hippocampal subfield and morning cortisol activity within MDD patients.

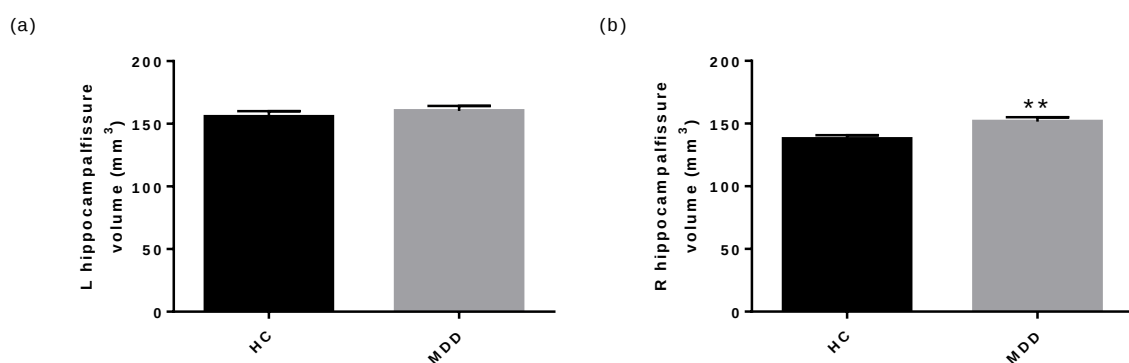
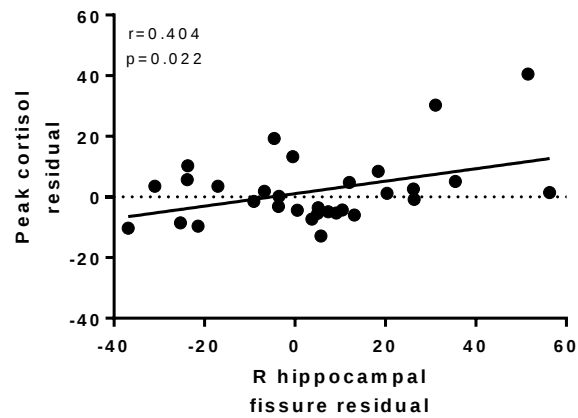
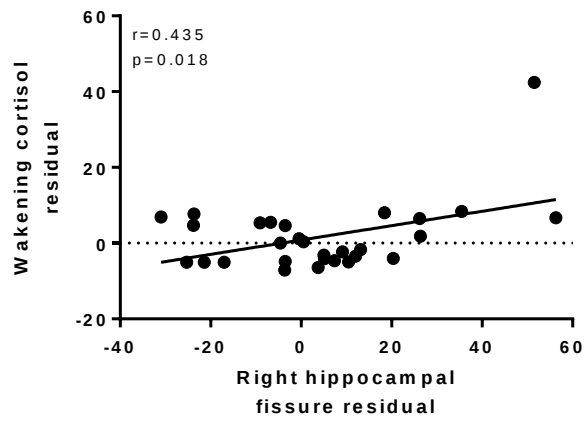


Figure 4.14 Left and right hippocampal fissure volumes in depressed patients and healthy controls. Subfield volumes for (a) left hippocampal fissure and (b) right hippocampal fissure in depressed patients ($n=47$) and healthy controls ($n=35$). Data expressed as mean and SEM. ** $p \leq 0.010$ vs. healthy controls (Univariate General Linear Model analysis of variance with age, gender, and TIV as covariates).

(a)



(b)



(c)

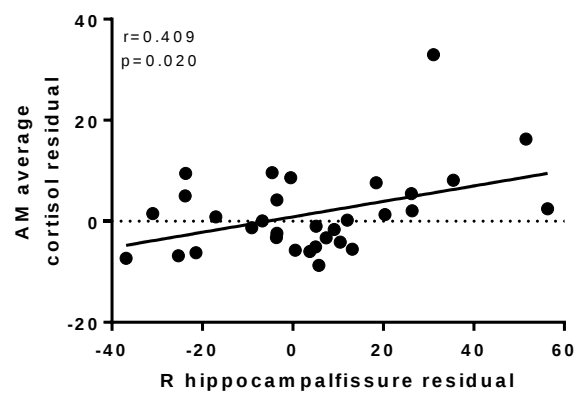


Figure 4.15 Relationship between right hippocampal fissure and HPA axis measures. Within the depressed group, a significant positive relationship exists between right hippocampal fissure volume and (a) peak cortisol, (b) wakening cortisol, and (c) morning average cortisol concentrations. (Partial correlation controlling for age, gender, and TIV).

4.4 Discussion

The present study sought to explore the involvement of the inflammatory response system in the pathophysiology of depression. To evaluate immune activity, several circulating inflammatory cytokines were measured in plasma and the relative gene expression of some of those cytokines was quantified in whole blood of depressed patients and healthy controls. The data resulting from cytokine immunoassays was inconclusive in regard to an inflammatory profile in the depressed cohort, as no significant differences were found between groups. However, gene expression of IL-1 β was significantly higher in the depressed cohort, suggesting that the differences in immune activation do exist but are subtle. It has been suggested that inflammation mediates brain tissue damage (Calabrese et al., 2015), therefore this study explored regional brain volume differences between depressed patients and healthy controls. A subset of participants completed structural MRI scans which were used to measure grey matter volumes of the hippocampus, an area believed to be damaged in depression, and the amygdala, a region suggested to be hyper-activated in depression (Drevets, 2001; Adolphs and Tranel, 2004; Lange and Irle, 2004). Analysis using VBM revealed a trend toward increased right amygdalar volume in the depressed group, but there was no difference in whole hippocampal grey matter volumes in depressed patients according to this initial assessment. To elucidate more discrete volume data from the hippocampus, another method was implemented to assess volumetric differences in specific hippocampal subfields associated with HPA axis activity. This subfield analysis revealed a significantly smaller left and right hippocampal CA3 and a significantly smaller right CA4 in the depressed group compared to controls, suggesting damage had occurred in these specific areas of the hippocampus. In summation, the data resulting from these comparisons support the theory of a mildly increased inflammatory profile in depression and specific yet significant differences in regional brain matter volumes. Furthermore, significant associations between both inflammation and HPA axis activity, and hippocampal volumes and HPA axis activity were revealed by correlational analyses. This finding suggests that the differences in cytokine expression and brain volume assessed in this chapter are related to HPA axis dysregulation reported in the previous chapter (Chapter 3), giving further endorsement to a biological explanation of MDD that takes account of HPA axis and immune dysregulation.

4.4.1 Circulating inflammatory profile in depressed patients

The cytokines assessed in circulating plasma by immunoassay in this study were IL-6, IL-1 β , IFN- γ , and TNF- α , and C-reactive protein was also measured. The data resulting from these immunoassays did not reveal a difference in circulating inflammation between depressed patients and healthy controls. There were also no correlations between cytokine concentration and severity of depression symptoms. This is in contrast to many previous studies that have found a significant increase in expression of circulating cytokines in depression (Tiemeier et al., 2003; Alesci et al., 2005; Raison et al., 2006). This discrepancy may be due to the fact that many samples assayed had cytokine concentrations falling below the detectable level measured by ELISA. Cytokines, including the ones measured in this study, are considered small proteins and exist in low concentrations typically well below 100 pg/ml in plasma in healthy people. Concentrations of cytokines increase 1,000-fold in response to infection (Cohen, 2010), but in psychiatric disorders they may only increase marginally (Maes, 1994), resulting in subtle differences in inflammatory profile that are difficult to detect. This could account for the lack of significant differences in circulating cytokine concentrations between depressed patients and healthy controls. Analysing samples for a wider range of inflammatory cytokines, with the addition of analysis of chemokines or macrophages associated with inflammation, may give a more accurate indication of overall immune activity in depressed patients compared to controls. Using more sensitive assay kits with a lower limit of detection would also be beneficial in future studies.

4.4.2 Transcriptional expression of inflammatory cytokines, and the significance of IL-1 β in depression pathophysiology

Whole blood transcriptional expression of IL-1 β , IFN- γ , and TNF- α was measured by qPCR. The resultant relative quantification data indicated a significantly higher level of IL-1 β expression in depressed patients compared to healthy controls, but no difference in mRNA expression of IFN- γ , and TNF- α [Figure 4.3]. While the present study did not replicate previous findings that have indicated increased gene expression or involvement of IFN- γ or TNF- α in depression (Mihailova et al., 2016; Fabbri et al., 2016), the increase of IL-1 β gene expression demonstrates altered immune activation in the MDD group.

Circulating IL-1 β is produced by active macrophages in the periphery as well as by glial cells and neurons in the central nervous system (Dinarello, 1996). This interleukin is known as a driver of immune responses (van de Veerdonk and Netea, 2013) and promotes sickness behaviour (Dantzer et al., 2008). Knowledge of the role of IL-1 β in mood disorders has been established through several means including epidemiological data (Owen et al., 2001), studies showing the modification of IL-1 β expression following treatment of mood disorders (Maes and Rief, 2012), and the behavioural alterations documented following administration of IL-1 β in pre-clinical studies (Anisman and Hymie, 2009). The finding that IL-1 β was elevated in whole blood mRNA of depressed patients but not in circulating plasma could be due to mRNA expression being a potentially more stable marker while circulating measures can be acutely altered.

The finding that whole blood IL-1 β gene expression was significantly positively correlated to severity of psychiatric symptoms is consistent with previous findings in the literature that have shown a relationship between this gene and experiences associated with depression (Levine et al., 1999). Specifically, the present study found a significant positive correlation between gene expression of IL-1 β and PSQI score among the entire cohort [Figure 4.4a] which has been observed in previous research (Krueger, 2008). Also, one of the most critical findings of this set of analysis was that whole blood mRNA expression of IL-1 β was positively correlated to global CTQ scores within the depressed group [Figure 4.4b]. In some studies, childhood trauma has been shown to be significantly associated with increased IL-1 β (Levine et al., 2015). This study showed immune activation was related to higher sleep disturbance and childhood trauma which are both associated with depression, further implicating the involvement of IL-1 β mRNA expression in depression pathophysiology.

4.4.3 Grey matter differences in MDD

Structural MRI scans analysed by VBM for measurement of hippocampal and amygdala volumes revealed a trend toward a larger right amygdala in depressed patients compared to healthy controls. The amygdala is part of the limbic system and is believed to play a role in memory processing and emotional reactivity (Adolphs et al., 1997). Previous studies have found increased amygdalar activity and increased amygdalar volumes in depressed patients, therefore these results are supported by the literature (Hamilton et al., 2008). There were no differences in hippocampal grey matter volume detected by VBM analysis despite

previous reports of this finding in depressed patients (Bremner et al., 2000; Lange and Irle, 2004). Accordingly, we chose to probe more nuanced differences in the substructures within the hippocampus and subsequently conducted a volumetric analysis of hippocampal subfields, regardless of grey or white matter composition. While VBM has the ability to accurately calculate grey matter, white matter, and cerebrospinal fluid volumes, a hippocampal subfield analysis implementing Free Surfer ROI analysis gives a different, equally valid, specific and accurate calculation of volume for hippocampal subfields (Giuliani et al., 2005; Iglesía et al., 2015).

4.4.4 Hippocampal subfield volumes in MDD patients compared to healthy controls

The present study found significant differences in CA3 and CA4 hippocampal subfield volumes in patients with MDD compared to healthy controls. A diagram of these subfields can be found on the following page [Figure 4.16]. The left and right CA3 were significantly smaller in depressed patients compared to controls [Figure 4.9]. Only the right CA4 was shown to be decreased in depressed patients, while the left CA4 showed no difference between groups [Figure 4.10]. It has been suggested that the CA3 plays a role in associative memory recall and memory sequence (Jensen and Lisman, 1996; Nakazawa et al., 2002). The exact function of the CA4 region has not yet been established, but neurogenesis in the CA region in general has been shown to be suppressed during stress (McEwen and Magarinos, 2001), and another recent study showed lower CA1-3 volumes in medicated MDD patients compared to healthy controls (Huang et al. 2013), implying that the CA region as a whole is altered in depression. The decreases in hippocampal subfield volumes in depressed patients support the theory that the hippocampus is damaged in depression (MacQueen and Frodl, 2011).

4.4.5 Possible mechanisms of immune activation mediating grey matter damage

Analysis revealed that as gene expression of IL-1 β increased, right hippocampal CA3 and CA4 volumes decreased in a negative correlation of the entire cohort [Figure 4.11]. This demonstrates a relationship between increased inflammation and decreased brain volume, suggesting that dysregulation of one could lead to damage in the other. Considering the

mounting scientific evidence pointing to a relationship between immune activation and hippocampal volume reduction in human studies and animal models, this data supports the hypothesis that there is a link between these two biological alterations (Goshen et al., 2008; Kuzumaki et al., 2010; Zunszain et al., 2012).

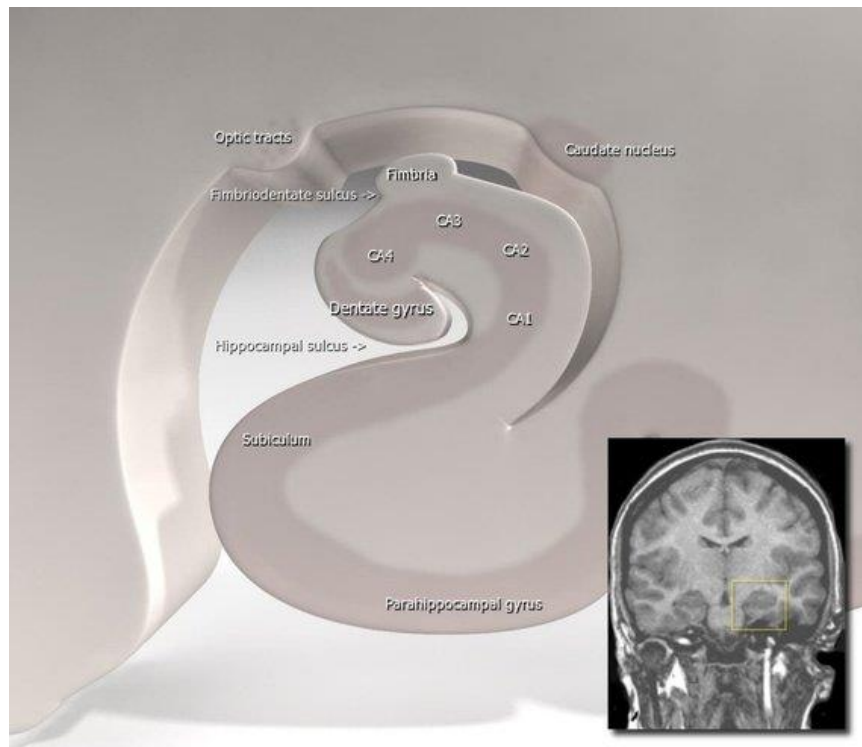


Figure 4.16 Hippocampal subfield illustration. Illustration showing a representation of hippocampal anatomy including CA3, CA4, and subiculum subfields. (Gaillard, 2006).

4.4.6 Relationship between HPA axis activity and immune activation

Inflammation is known to be a biological consequence of stress and HPA axis alterations (Miura, 2008). The current study revealed several significant relationships between measures that indicate HPA axis activity and immune activity. For example, within the depressed group, whole blood mRNA expression of IL-1 β was significantly negatively correlated to morning cortisol reactivity [Figure 4.5a]. Decreased morning cortisol reactivity was a measure found to be associated with depression diagnosis in the previous chapter of

this study (Chapter 3), indicating that decreased CAR reactivity could be a marker of depression. Its relationship within the depressed group to whole blood mRNA expression of IL-1 β is indicative of a link between HPA and immune dysregulation in depression. Also, increased gene expression of inflammatory cytokine IFN- γ was associated with increased cortisol at T30 within the depressed group, giving more support to the theory that increased dysfunction of the HPA axis is related to increased inflammation [Figure 4.5b]. These findings are all in accordance with previous studies that have found links between HPA axis and immune system activation in tandem, and in depression (Pace, 2007; Bellavance et al., 2014; Wolkow et al., 2015; Otte et al., 2016).

4.4.7 Relationship between HPA axis activity and hippocampal volumes

It has been established that the hippocampus plays a role in regulation of the HPA axis as this brain region's activity has been shown to alter levels of circulating glucocorticoids (Herman et al., 2003). Furthermore, HPA dysregulation and hippocampal volume reduction have been observed simultaneously in depressed patients (Vythilingam et al., 2002). In this study, a significant positive correlation was revealed between the hippocampal fissure subfield, which was significantly increased in volume in depressed patients, and waking cortisol, another hallmark of depression pathophysiology [Figure 4.15a]. The hippocampal fissure, also known as the hippocampal sulcus, is the groove or space between the bends of the CA and subiculum subfields of the hippocampus. As the other subfields decrease in volume, the fissure subsequently increases in volume as the space between regions becomes larger. This increase in hippocampal fissure volume and its relationship to increased waking cortisol concentration contribute further evidence of the relationship between HPA axis and hippocampal alterations in depression.

There have been findings in other studies that indicate HPA axis alterations in depression are associated with hippocampal CA subfield changes specifically. One study found that smaller volumes in the CA2/3-dentate gyrus subfield of the hippocampus were associated with depressive symptoms and hyper-reactivity of cortisol secretion during the day in multiple sclerosis patients (Gold et al., 2010). Another recent study found that depressed patients with reduced mRNA expression of glucocorticoid inducible genes also showed blunted cortisol responses and reduced hippocampal CA1 volumes (Frodl and Amico, 2014). While no relationships between CA subfield volumes were correlated to measures of the HPA axis in

the present study, there is ample evidence that hippocampal subfield volumes should continue to be assessed in relation to HPA axis dysregulation and depression pathophysiology.

4.4.8 Limitations and future directions

Future studies of this kind should look at a wider range of cytokines, as well as chemokines and macrophages, to get a broader indication of immune activity. It has previously been shown that macrophages and chemokines are related to depression (Smith, 1991; Wohleb et al., 2015), and if they had been included as targets in this study, a more distinct immune profile may have been detected. The relationship between presence of macrophages and regional brain volume could also reveal further relationships between inflammation and neurodegeneration that have been missed by the limited number of markers observed by the present study.

More reliable or sensitive ELISA methods should be sought in future investigations of inflammation in depression to ensure that low levels of these small proteins are able to be detected at a higher rate. For example, sensitivity may be improved by increasing incubation times of samples, and by increasing the coating concentration of the detection antibody (Cox et al., 2012). Also, the use of zeros for values that fell below the limit of detection (LOD) may be considered a limitation. Other methods such as replace undetectable values with the $LOD/2$ or $LOD/\sqrt{2}$ to have been used in previous cytokine research (Ashwood et al., 2011), and may more accurately describe the plasma concentrations of these proteins.

Mechanisms linking inflammation and brain tissue dysfunction or loss are poorly understood and seem like a promising route to gaining understanding of many diseases that involve neurodegeneration and inflammation. Few studies have been conducted showing the relationship between inflammation and specific hippocampal subfields such as the CA region which was shown to be significantly altered in this study. Gaining a better understanding of the relationship between these two dysfunctions in a depressed population is essential to furthering knowledge of the underlying pathophysiology of the disorder.

4.4.9 Conclusion

The results of these analyses give evidence of increased inflammation and altered brain volumes in depressed patients compared to healthy controls. Increased whole blood mRNA expression of IL-1 β and decreased hippocampal subfields in depressed patients are indicative of involvement of the immune system in the pathophysiology of depression. Future research into the mechanisms by which inflammation may induce loss of brain volume should be addressed. Significant associations between immune alterations, brain volume differences, and HPA axis dysregulation provide a rational basis to further explore a theory of depression aetiology that encompasses all of these pathways.

Chapter 5

Tryptophan depletion and kynurenine pathway induction in Major Depressive Disorder

5.1 Introduction

The monoamine hypothesis of depression has been the most widely supported biological explanation of depression for the past several decades, particularly because it takes account of the mechanism by which serotonin reuptake inhibitors (SSRIs) act upon by enhancing serotonergic transmission (Albert and Benkelfat, 2013). This theory suggests that the cause of depression is depletion of neurotransmitters, particularly serotonin, which has been shown to be reduced in limbic regions through both in vivo imaging and post mortem human studies of depression (Kambeitz et al., 2015). However, about 40% of patients with MDD do not achieve remission within two adequate trials of antidepressant treatment (Sinyor et al., 2010), and recurrence of the disorder is extremely common (Burcusa and Iacono, 2007). For these reasons, it is essential to further investigate other biological systems that might play a role in depression in conjunction with serotonin depletion. Tryptophan is the precursor of serotonin (5-HT). Since serotonin cannot cross the blood brain barrier, its levels are primarily influenced by tryptophan which is supplied through the diet and competes with other neutral amino acids to cross the blood brain barrier (Pardridge, 1977; Fernstrom, 1983). The ratio of total tryptophan to neutral amino acids has been shown to be decreased in depressed patients, suggesting that tryptophan availability to the brain is reduced in MDD (Cowen et al., 1989). Many other studies have shown tryptophan depletion in plasma of patients with MDD (DeMyer et al., 1981; Joseph et al., 1984; Møller, 1985; Cowen et al., 1989; Maes et al., 1991). Tryptophan is also the precursor to kynurenine and its products, which form the kynurenine pathway of tryptophan metabolism, another pathway implicated in the aetiology of depression.

The rate limiting enzymes of kynurenine formation are indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). Pro-inflammatory cytokines such as TNF- α and IFN- γ activate IDO, and stress hormones such as cortisol activate TDO (Christmas et al., 2011; Miura et al., 2008). Stress and immune stimuli promote kynurenine metabolism and are theorised to deprive the 5-HT pathway of tryptophan, ultimately reducing 5-HT synthesis (Maes, 2011). Furthermore, metabolites of the KP have neurotoxic and neuroprotective functions in the brain. For example, 3-hydroxykynurenine (3HK) has been shown to produce free radicals and quinolinic acid is potentially excitotoxic as it agonizes the NMDA receptor (Saito et al., 1992; Ghafourifar and Cadenas, 2005; Pierozan et al., 2012; Pierozan et al., 2015). Meanwhile, kynurenic acid has been shown to have the opposite effects on the NMDA receptor as its endogenous antagonist, and has protective effects within the CNS

(Andine et al., 1988; Hilmas et al., 2001; Urenjak and Obrenovitch, 2000). An increased kynurenine/tryptophan ratio has been shown in depressed adolescents, suggesting upregulation of the kynurenine pathway. Moreover, the same study reported a positive correlation between depressive symptoms and kynurenine levels (Gabbay et al., 2010). Kynurenine pathway induction has also been interpreted through the finding of increased TDO2 positive white matter in brains of depressed patient in post mortem studies (Miller et al., 2006).

The kynurenine pathway is functionally related to both the hypothalamic pituitary adrenal (HPA) axis via glucocorticoid induced TDO and the immune system via cytokine induced IDO. These systems have been shown to influence each other, and contribute to the severity of depression. For example, elevated quinolinic acid has been reported in association with increased inflammation (Steiner et al., 2011), strengthening the evidence of a link between these two proposed mechanisms that each appear to be involved in depression. Furthermore, studies have proposed that the hippocampal atrophy that appears in chronic depression may be associated with imbalances in neurotoxic and neuroprotective activities of the kynurenine pathway (Miura et al., 2008), suggesting a link between the many proposed biological pathways of depression. Alterations of the HPA axis and immune system in a depressed cohort have been shown in the present study (Chapter 3 & 4), and the relationship between those systems and the kynurenine pathway of tryptophan metabolism are of interest. The interplay of the alterations of these three pathways should be further investigated in MDD in order to enhance our understanding of their relationship to each other during a depressive episode, and to develop a biological model of depression that encompasses each.

Study Aims:

1. To evaluate tryptophan metabolism and kynurenine pathway activation in depressed patients compared to healthy controls.
2. To assess ratios of potentially neurotoxic and neuroprotective products of the kynurenine pathway.
3. To investigate the relationship between expression of HPA axis variables, inflammatory markers, and KP metabolites.

5.2 Methods

In this study, kynurenine pathway metabolism was assessed in 60 depressed patients and 34 healthy controls. Then, kynurenine pathway variables were correlated with HPA axis and immune system variables measured in Chapter 2 and Chapter 3 respectively. All biological data was used in regression analysis to determine relationships between dysfunction of these three networks in MDD. Depressed patients were recruited from the psychiatric outpatient clinic at Sheaf House in Tallaght Hospital and at the Mary Mercer Healthy Centre in Jobstown, Dublin 24. Recruitment of depressed patients was based on criteria for a Major Depressive Episode (DSM-5, American Psychiatric Association, 2013). Additional inclusion and exclusion criteria for depressed patients and healthy controls were as stated in Chapter 2. Psychiatric rating scale data was collected for participants with measures of depressive symptoms, sleep disturbance, and childhood trauma being quantified with the use of the HAM-D, CES-D, PSQI, and CTQ standardised scales.

Two 9 ml EDTA tubes of blood were collected from all participants at the time of recruitment or within one week for use in measurement of concentrations of circulating kynurenine pathway metabolites including tryptophan, kynurenine, 3-HK, quinolinic acid, and kynurenic acid. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to measure concentrations of these metabolites which was carried out in the laboratory of Dr. Andre Liesener at Boehringer Ingelheim and is described at length in Chapter 2.

Gene expression of genes encoding for kynurenine pathway enzymes IDO1, IDO2, KMO, Kynureninase and KAT1 were also assessed. One 2.5 ml PAXgene DNA tube of blood was collected and stored as described in Chapter 2. Total RNA was isolated from the whole blood and equalized before it was used for cDNA synthesis. Analysis of gene expression of the target genes was conducted using Real-Time PCR methods and Taqman® Gene Expression Assays (Applied Biosystems, UK). Relative quantification data was calculated using the $\Delta\Delta C_t$ method and GAPDH as an endogenous control, as described in greater detail in Chapter 2.

Linear regression analysis of biological variables was performed as a predictor of CES-D score in depressed patients and healthy controls in order to determine the relationship between key variables and depression severity. Binary logistic regression analysis to predict diagnosis status was performed to determine an equation that best predicted a diagnosis of MDD using biological variables. Other data are presented as mean with standard error of the mean (SEM) or standard deviation (SD) where appropriate. All data were tested for

normality using the Shapiro-Wilk test. Non-parametric data were analysed using the Mann-Whitney U test for independent sample comparisons. Correlational analysis was carried out using Spearman's rho correlation statistics or partial correlation, or partial correlations correcting for age, gender, and TIV when correlating with MRI data. All statistical analyses were considered significant when $p \leq 0.05$. All data were analysed using SPSS (Version 16). Graphs were generated using GraphPad Prism Software Version 5.00 (GraphPad software, Inc).

5.3 Results

5.3.1 Demographic data for depressed patients and controls whose circulating KP metabolites were measured

This study included 60 depressed patients and 35 healthy controls. Analysis revealed that there were no differences in age, gender, or BMI between depressed patients and healthy controls in this cohort [Table 5.1]. However, the depressed group had a significantly higher rate of smokers ($p=0.001$) compared to healthy controls (MDD: 42.4% smokers vs. HC: 9.1% smokers), and healthy controls were significantly more educated on the ISCED scale ($p<0.001$) compared to depressed patients [Table 5.1].

Table 5.1 Demographic data for patients with MDD and healthy controls whose circulating KP metabolite concentrations were measured.

	Patients (n=60)	Controls (n=35)	Statistics (p-value)
Age (years)	27.79 (8.13)	28.53 (7.47)	$t=0.736$, $df=90$, $p=0.667$
Gender (female/male)	42/16	18/16	$\chi^2=3.583$, $p=0.072$
BMI	24.60 (5.65)	23.87 (3.89)	$t=0.537$, $df=69$, $p=0.537$
Smoking (Yes/No)	24/34	31/3	$\chi^2=10.957$, $p=0.001^{**}$
ISCED Education	3.47 (1.50)	6.03 (1.92)	$t=6.888$, $df=83$, $p<0.001^{***}$

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Student's t-test (Age, BMI, Education), and Chi squared (χ^2) test (Gender, Smoking).

5.3.2 Clinical data for depressed patients and healthy controls

Consistent with a diagnosis of MDD, patients scored significantly higher compared to healthy controls on the HAM-D and CES-D [Table 5.2], signifying elevated depressive symptoms. Patients also exhibited significantly increased sleep disturbance through elevated PSQI scores [Table 5.2]. In the depressed group, a significantly larger number of participants had experienced early life adversity (MDD: 66.1% vs. HC: 18.2%), with significantly higher Global CTQ scores than healthy controls [Table 5.2]. Of the depressed cohort, 22 were medication free, 22 were prescribed SSRIs, 8 were prescribed SNRIs, and 7 were on other medications including benzodiazepines, melatonergics, anti-epileptics or a combination of medications. Healthy controls were not on medication at the time of the study.

Table 5.2 Clinical data for patients with MDD and healthy controls whose circulating KP metabolite concentrations were measured.

	Patients (n=58)	Controls (n=34)	Statistics (p-value)
HAM-D	22.75 (5.24)	2.15 (2.31)	Z=-7.949, p<0.001***
CES-D	38.98 (9.77)	5.94 (5.79)	Z=-7.845, p<0.001***
PSQI	13.35 (3.61)	3.61 (1.78)	Z=-7.697, p<0.001***
CTQ Global Score	43.94 (15.10)	29.75 (8.00)	Z=-4.576, p<0.001***
Early Life Adversity (Yes/No)	37/17	6/26	$\chi^2=19.907$, p<0.001***
Antidepressant (Yes/No)	35/23		

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).

5.3.3 Circulating tryptophan pathway metabolites in depressed patients and healthy controls

5.3.3.1 Tryptophan pathway concentration data

Tryptophan, kynurenine, quinolinic acid, kynurenic acid, and 3-hydroxykynurenine concentrations were measured in plasma by LC-MS [Table 5.3]. A Mann-Whitney U test revealed significantly lower tryptophan concentrations in depressed patients compared to healthy controls ($Z=-3.258$, $p=0.001$) [Figure 5.1a]. Kynurenine concentrations were lower in depressed patients compared to healthy controls, though statistical significance of this difference was narrowly missed ($Z=-1.913$, $p=0.056$) [Figure 5.1b].

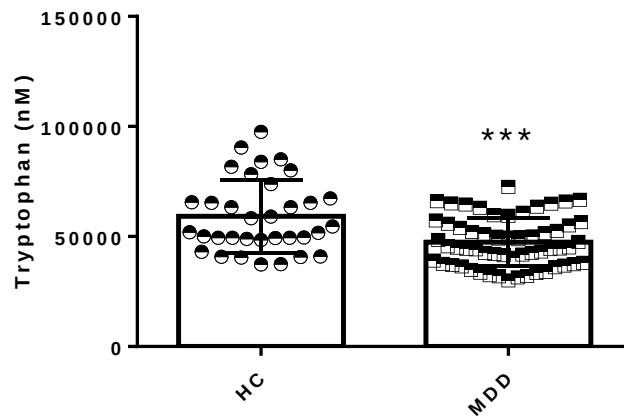
Concentrations of downstream kynurenine pathway metabolites were measured with LC-MS. Analysis revealed no differences in 3-hydroxykynurenine concentration between depressed patients and healthy controls [Figure 5.2a]. There were also no differences in quinolinic acid concentration between groups [Figure 5.2b]. However, kynurenic acid concentration was found to be significantly lower in depressed patients compared to healthy controls [Figure 5.3].

Table 5.3 Circulating tryptophan pathway concentration data for patients with MDD and healthy controls.

	Patients (n=58)	Controls (n=34)	Statistics (p-value)
Tryptophan (nM)	47496.67 (1418.2)	59148 (2826.2)	$Z=-3.258$, $p=0.001$ ***
Kynurenine (nM)	2467.45 (76.3)	2819.85 (149.3)	$Z=-1.913$, $p=0.056$
3HK (nM)	34.45 (1.7)	37.44 (2.5)	$Z=-1.123$, $p=0.262$
Quinolinic Acid (nM)	289.77 (12.1)	278.8 (79.1)	$Z=-0.342$, $p=0.732$
Kynurenic Acid (nM)	33.04 (1.5)	39.14 (2.1)	$Z=-2.105$, $p=0.035$ *

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

(a)



(b)

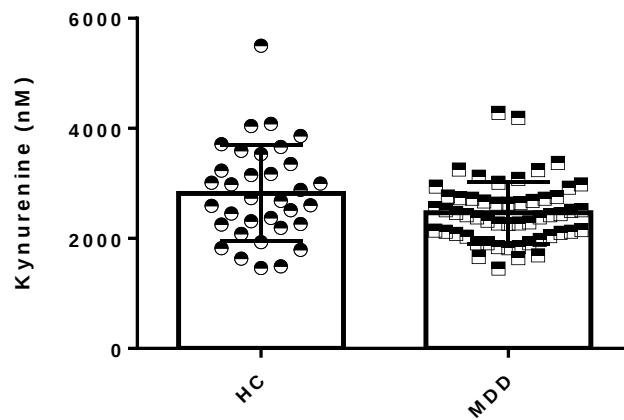
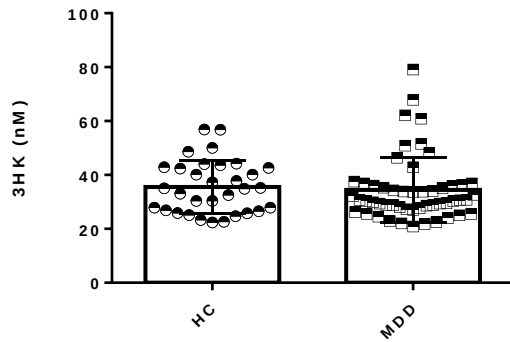


Figure 5.1 Tryptophan and kynurenine concentrations in depressed patients and healthy controls. Plasma concentrations of (a) tryptophan, and (b) kynurenine in depressed patients (n=58) and healthy controls (n=34). Data expressed as mean and SEM. (Mann-Whitney U test). *** $p \leq 0.001$ vs healthy controls.

(a)



(b)

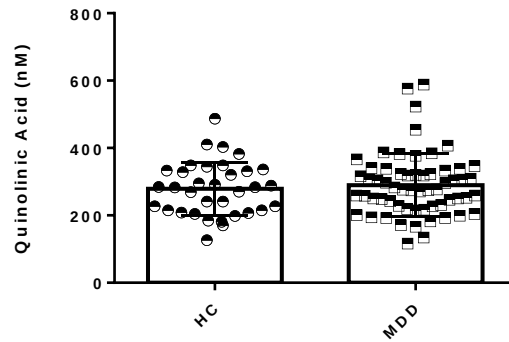


Figure 5.2 3-Hydroxykynurenine and Quinolinic acid concentrations in depressed patients and healthy controls. Plasma concentrations of (a) 3-hydroxykynurenine, and (b) quinolinic acid in depressed patients (n=58) and healthy controls (n=34). Data expressed as mean and SEM. (Mann-Whitney U test).

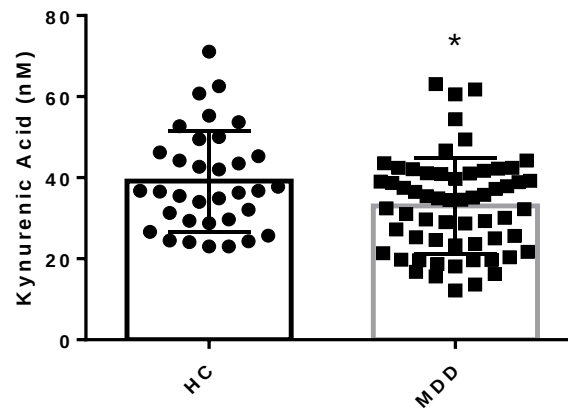


Figure 5.3 Kynurenic acid concentrations in depressed patients and healthy controls. Plasma concentrations of kynurenic acid in depressed patients (n=58) and healthy controls (n=34). Data expressed as mean and SEM. (Mann-Whitney U test). * $p \leq 0.05$ vs healthy controls.

5.3.3.2 Kynurenine pathway metabolite ratios as an indicator of KP activity

Analysis carried out by a Mann-Whitney U test revealed some differences between ratios of kynurenine pathway metabolites. There were no differences in kynurenine/tryptophan ratio between the depressed group and controls ($Z=-1.275$, $p=0.202$) [Figure 5.4].

Quinolinic acid/kynurenine ratios were significantly higher in the depressed group compared to the controls ($Z=-2.018$, $p=0.044$) [Figure 5.5a]. There was no difference between kynurenic acid/kynurenine ratios between groups ($Z=-0.659$, $p=0.510$) [Figure 5.5b].

Finally, the kynurenic acid/quinolinic acid ratio was calculated for participants and a Mann-Whitney U test revealed a significantly lower ratio in depressed patients compared to healthy controls ($Z=-2.416$, $p=0.016$) [Figure 5.6].

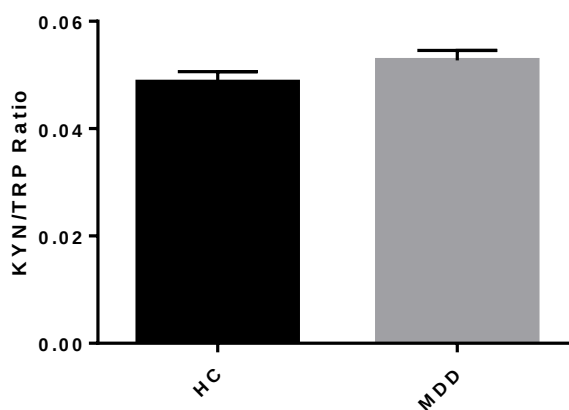


Figure 5.4 KYN/TRP ratios in depressed patients and healthy controls. Ratios of kynurenine concentration to tryptophan concentration in depressed patients ($n=38$) and healthy controls ($n=58$). Data expressed as mean and SEM. (Mann-Whitney U test).

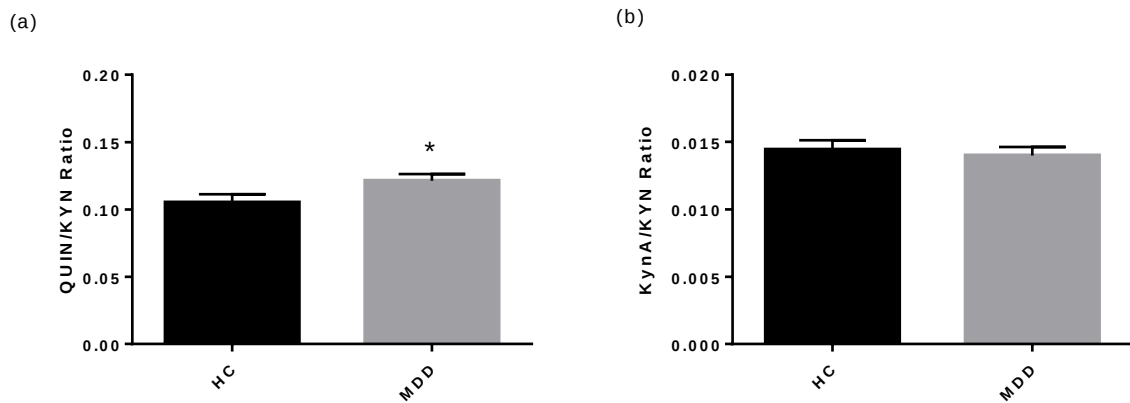


Figure 5.5 QUIN/KYN ratios and KynA/KYN ratios in depressed patients and healthy controls. Ratios of (a) quinolinic acid to kynurenine concentration and (b) kynurenic acid to kynurenine concentration in depressed patients (n=58) and healthy controls (n=34) are depicted as mean and SEM. (Mann-Whitney U test). * $p \leq 0.05$ vs healthy controls.

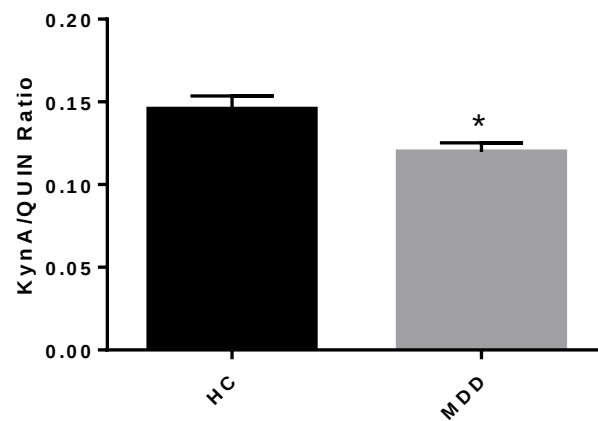


Figure 5.6 KynA/QUIN ratios in depressed patients and healthy controls. Ratios of kynurenic acid concentration to quinolinic acid concentration in depressed patients (n=58) and healthy controls (n=34). Data expressed as mean and SEM. (Mann-Whitney U test). * $p \leq 0.05$ vs healthy controls.

5.3.4 Associations between circulating concentrations of kynurenine pathway metabolites and depression symptoms

Several significant relationships were revealed by conducting a Spearman's rho correlation between circulating kynurenine pathway concentrations and ratios with psychiatric rating scales. Within the depressed group alone, HAM-D score was positively correlated to kynurenine concentration ($r=0.342$, $p=0.011$) [Figure 5.7]. In the entire cohort, tryptophan concentrations were negatively correlated to CES-D ($r=-0.213$, $p=0.042$). The quinolinic acid/kynurenine ratio was significantly positively correlated to CES-D score within the entire cohort ($r=0.212$, $p=0.050$), and the kynurenic acid/quinolinic acid ratio was negatively correlated to CES-D ($r=-0.259$, $p=0.013$), PSQI ($r=-0.308$, $p=0.004$), and CTQ global score ($r=-0.277$, $p=0.010$) within the entire cohort. However, within group analysis revealed no relationships between KP ratios and severity of these experiences within the depressed group alone.

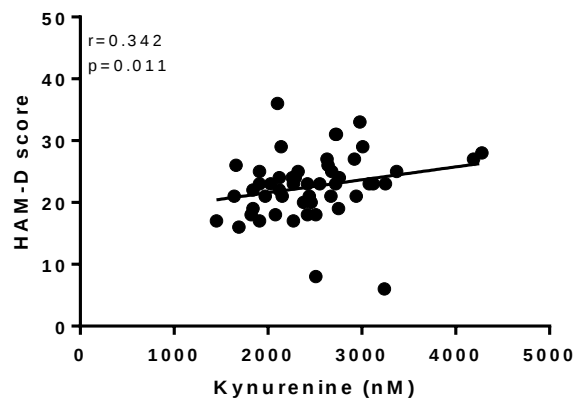


Figure 5.7 Relationship between circulating kynurenine concentration and HAM-D scores of MDD patients. A significant positive relationship exists between circulating kynurenine concentration and HAM-D scores of depressed patients. (Spearman's rho correlation).

5.3.5 Relative whole blood mRNA expression of tryptophan pathway metabolites in depressed patients and healthy controls

Gene expression of several tryptophan pathway metabolites was measured in whole blood from depressed patients relative to control subjects. The demographic and clinic data relating to this subset of participants can be found in Appendix 4a. The relative quantification of each tryptophan pathway encoding gene measured in depressed patients compared to healthy controls are reported in Table 5.4.

Table 5.4 Whole blood mRNA expression data for patients with MDD and healthy controls.

	Patients (n=55)	Controls (n=38)	Statistics (p-value)
SLC6A4 RQ	1.10 (0.17)	0.98 (0.12)	Z=-0.685, p=0.494
IDO1 RQ	0.76 (0.11)	1.08 (0.27)	Z=-1.642, p=0.101
IDO2 RQ	1.29 (0.22)	1.07 (0.26)	Z=-0.896, p=0.370
KMO RQ	1.21 (0.13)	1.01 (0.09)	Z=-0.602, p=0.547
Kynureninase RQ	1.21 (0.19)	1.02 (0.10)	Z=-0.032, p=0.975
KAT1 RQ	0.91 (0.20)	1.01 (0.04)	Z=-1.548, p=0.122

Data expressed as mean RQ with SEM in parentheses. Statistical analysis was performed using a Mann Whitney U test.

5.3.5.1 Relative whole blood mRNA expression of whole blood IDO enzymes in depressed patients and healthy controls

Transcriptional expression of IDO1, IDO2, and TDO2 were measured by qPCR. Expression of TDO2 fell below the limits of detection in most samples therefore cannot be reliably reported.

Basal IDO1 mRNA expression was observed in all blood samples studied and an average Ct for controls was determined to be 32.7. The housekeeping gene GAPDH had an average Ct of 25.0, therefore the Δ Ct of IDO1 was 7.7. Basal IDO2 mRNA expression was observed in 26 of the 38 control samples and 34 of the 55 depressed patient samples. The average Ct for IDO2 in the control group was 35.5, while the average Ct for the housekeeping gene GAPDH was 25.0, resulting in an average Δ Ct of 10.5 for IDO2.

A Mann-Whitney U test indicated no difference in whole blood transcriptional expression of IDO1 or IDO2 between depressed patients and healthy controls in this study [Table 5.4].

5.3.5.2 Relative whole blood mRNA expression of KYN-QUIN branch of KP enzymes in depressed patients and healthy controls

Basal KMO mRNA expression was observed in all blood samples studied and an average Ct for controls was determined to be 33.8. The housekeeping gene GAPDH had an average Ct of 25.1, therefore the Δ Ct of KMO was 8.7. Basal kynureninase mRNA expression was observed in all control samples and 54 of the 55 depressed patient samples. The average Ct for kynureninase in the control group was 31.5, while the average Ct for the housekeeping gene GAPDH was 25.5, resulting in an average Δ Ct of 6.0 for kynureninase.

A Mann-Whitney U test revealed no significant differences in the whole blood transcriptional expression of KMO and kynureninase between depressed patients and healthy controls in this study [Table 5.4].

5.3.5.3 Relative whole blood mRNA expression of KYN-KynA branch of KP enzymes in depressed patients and healthy controls

Basal KAT1 mRNA expression was observed in all whole blood samples and an average Ct for controls was determined to be 31.8. The housekeeping gene GAPDH had an average Ct of 25.4, therefore the Δ Ct of KMO was 6.4. The transcriptional expression of KAT2 was also measured but was below the limits of detection in most samples.

A Mann-Whitney U test between the whole blood transcriptional expression of KAT1 showed a trend towards lower expression of KAT1 mRNA in depressed patients compared to healthy controls but no significant difference was detected [Table 5.4].

5.3.5.4 Relative whole blood mRNA expression of SLC6A4 in depressed patients and healthy controls

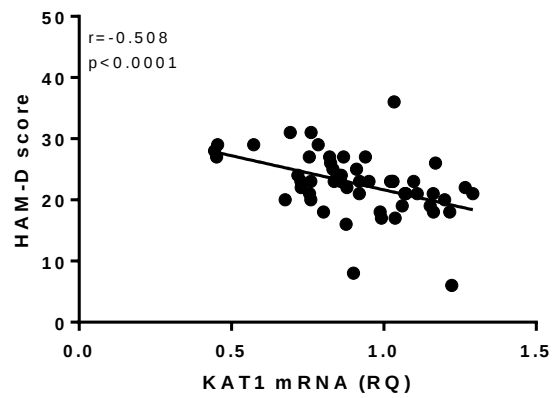
Gene expression of SLC6A4 was measured in whole blood from depressed patients relative to healthy control subjects. Basal expression of SLC6A4 was measured in 36 of the 38 control samples and 45 of the 55 depressed patient samples. The average Ct for the control group was 34.5 and the average Ct for the housekeeping gene GAPDH was 25.6. This results in an average Δ Ct of 8.9 for SLC6A4.

A Mann-Whitney U test revealed no significant difference in whole blood transcriptional expression of SLC6A4 between depressed patients and healthy controls [Table 5.4].

5.3.5.5 Relationship between whole blood kynurenine pathway enzyme gene expression and psychiatric rating scales

A Spearman's rho correlational analysis between whole blood transcriptional expression of KAT1 and psychiatric rating scale scores revealed several significant negative associations within the depressed group. There was a highly significant negative correlation between transcriptional expression of KAT1 and HAM-D score ($r=-0.508$, $p<0.0001$) [Figure 5.8a]. Also, whole blood KAT1 mRNA expression was negatively correlated to childhood trauma questionnaire scores ($r=-0.322$, $p=0.019$) [Figure 5.8b].

(a)



(b)

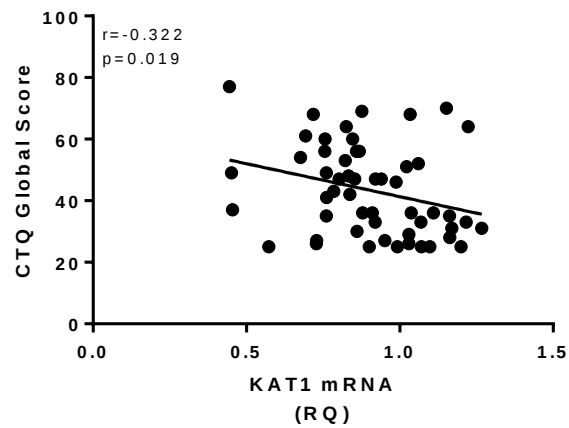


Figure 5.8 Relationship between whole blood KAT1 mRNA expression and psychiatric rating scales in MDD patients. Significant negative correlations exist between relative quantification mRNA expression of KAT1 in whole blood and (a) HAM-D scores of depressed patients and (b) CTQ global scores of MDD patients according to a Spearman's rho analysis.

5.3.6 Evaluation of relationships between kynurenine pathway measurements

A Spearman's rho analysis revealed several relationships between kynurenine pathway variables within the entire cohort where there were no significant differences between group means. First, IDO1 mRNA expression was significantly positively correlated to quinolinic acid concentration ($r=0.236$, $p=0.036$) [Figure 5.9].

Spearman's rho analysis revealed a significant negative correlation between mRNA expression of IDO1 and mRNA expression of SLC6A4 ($r=-0.275$, $p=0.013$) [Figure 5.10]. In contrast, there was no significant negative association between SLC6A4 and other components of the KP such as IDO2, KMO, or kynureninase.

Whole blood mRNA expression of IDO1 was also positively correlated to transcriptional expression of KMO ($r=0.313$, $p=0.002$), as well as positively correlated to transcriptional expression of kynureninase ($r=0.352$, $p=0.001$) [Figure 5.11a, b]. Additionally, IDO2 was significantly positively correlated to kynureninase ($r=0.288$, $p=0.027$), but with a weaker relationship than IDO1.

Within the depressed group, which had significantly lower circulating kynurenic acid concentrations, correlational analysis revealed a significant negative relationship between kynurenic acid concentration and whole blood transcriptional expression of KMO ($r=-0.396$, $p=0.005$) [Figure 5.12a]. Also, KMO mRNA expression and kynureninase mRNA expression were significantly positively correlated within depressed patients ($r=0.709$, $p<0.001$) [Figure 5.12b], which is representative of their relationship to each other in the chain of tryptophan metabolism.

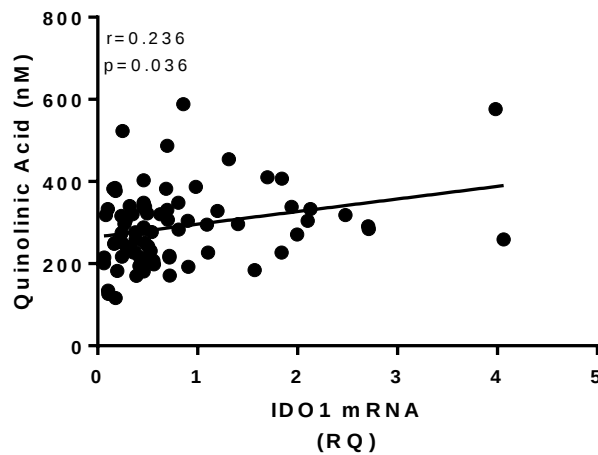


Figure 5.9 Relationships between whole blood mRNA expression of whole blood IDO1 mRNA and circulating QUIN. A significant positive correlation exists between whole blood mRNA expression IDO1 and circulating quinolinic acid in the whole participant group. (Spearman's rho correlation).

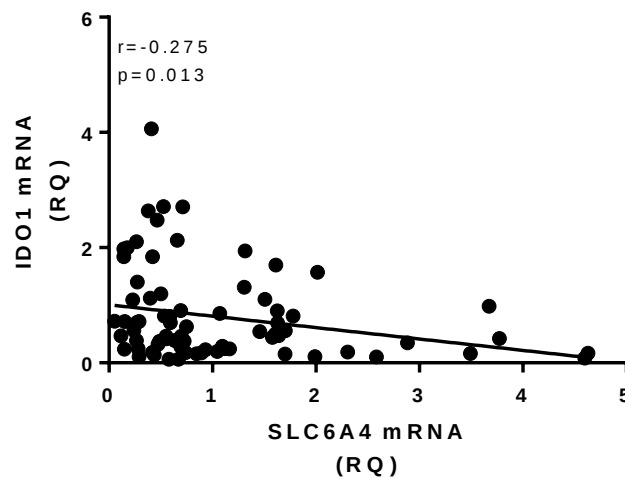


Figure 5.10 Correlational analysis between whole blood IDO1 mRNA expression and SLC6A4 mRNA expression. A significant negative relationship exists between normalised relative quantification mRNA expression of IDO1 and SLC6A4 in whole blood. Analysis was performed by Spearman's rho correlation on all participants.

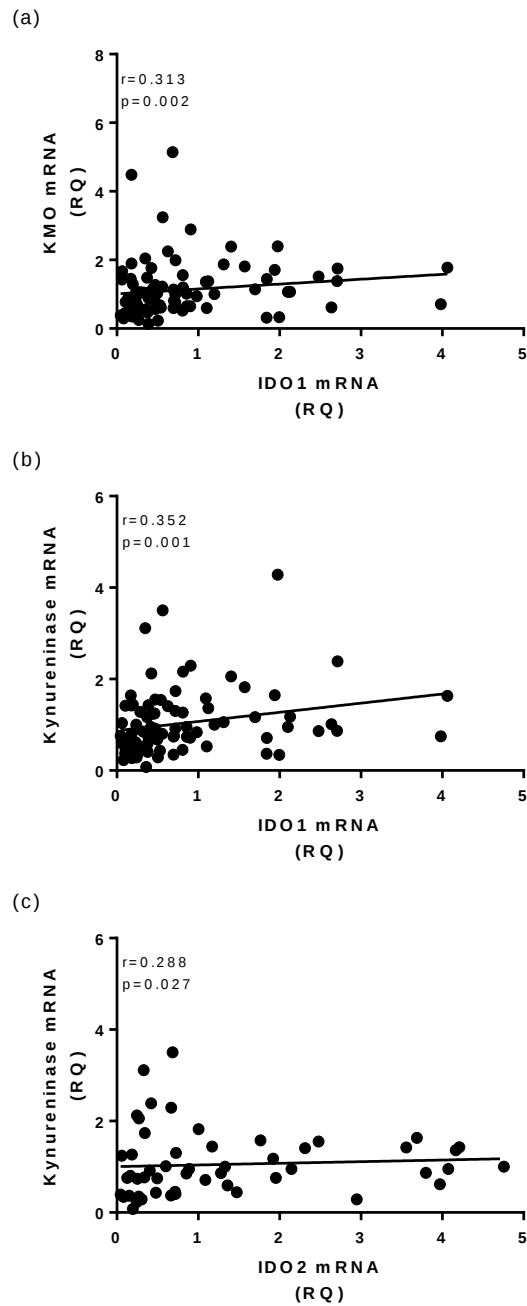
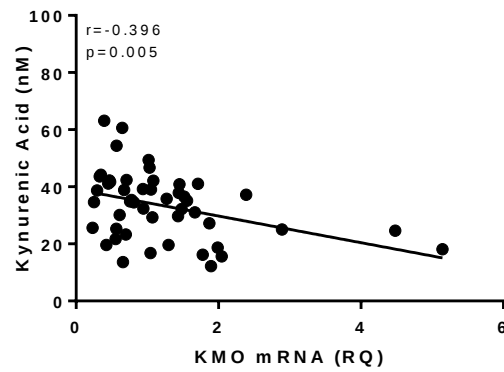


Figure 5.11 Significant correlations between mRNA expressions of multiple kynurenine pathway metabolites. A Spearman's rho correlation conducted on mRNA expression data revealed several significant relationships between metabolites of the Kynurenine Pathway. Significant positive correlations exist between (a) KMO and IDO1 mRNA expression, (b) kynureninase and IDO 1 mRNA expression, and (c) kynureninase and IDO2 mRNA expression of all participants.

(a)



(b)

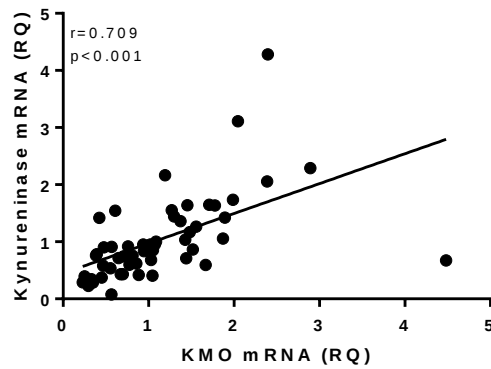


Figure 5.12 Associations between mRNA expression of KMO and other KP markers within MDD. Within the MDD group, (a) a significant negative relationship exists between circulating kynurenic acid concentrations and whole blood mRNA expression of KMO and (b) a significant positive relationship exists between whole blood mRNA expression of kynureninase and mRNA expression of KMO. Analysis was performed by Spearman's rho correlation.

5.3.7 Relationships between KP metabolites and HPA axis measures in MDD

A Spearman's rho correlation revealed many significant relationships between cortisol data and kynurenine pathway markers within the depressed group which are displayed in Table 5.5. Most significantly, the kynurenine/tryptophan ratio was strongly correlated to waking cortisol concentration ($p < 0.001$) [Figure 5.13]. Kynurenine/tryptophan ratio was also negatively correlated to morning average cortisol concentration, peak cortisol concentration, and intercept of the regression line fitted through morning cortisol data. Kynurenine concentration in depressed patients was negatively correlated to waking cortisol and positively correlated to AUCi, reactivity, and slope of the regression line fitted through morning cortisol data. 3-HK was negatively correlated to average morning cortisol concentration, peak cortisol concentration, and intercept of the regression line fitted through morning cortisol data [Table 5.5].

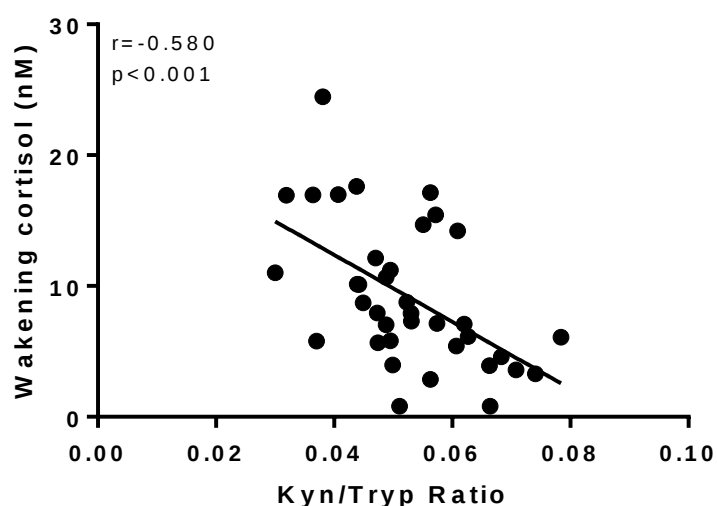


Figure 5.13 Correlation between waking cortisol and kynurenine/tryptophan ratio in MDD patients. A significant negative relationship exists between waking cortisol concentration and kynurenine/tryptophan ratio within MDD patients. (Spearman's rho analysis).

Table 5.5 Correlational analysis of kynurenine pathway measurements and HPA axis variables within MDD patients.

Biological parameter	Wakening Cortisol	Morning Average Cortisol	AUCi	Peak	Reactivity	Slope	Intercept
Tryptophan	r=0.122 p=0.448	r=0.138 p=0.360	r=0.098 p=0.571	r=0.157 p=0.298	r=0.052 p=0.754	r=-0.024 p=0.881	r=0.129 p=0.414
Kynurenine	r=-0.417 p=0.009	r=-0.220 p=0.156	r=0.344 p=0.050	r=-0.196 p=0.208	r=0.451 p=0.006	r=0.321 p=0.046	r=-0.315 p=0.051
3-HK	r=-0.307 p=0.078	r=-0.406 p=0.010	r=-0.196 p=0.307	r=-0.412 P=0.009	r=-0.055 p=0.765	r=-0.096 p=0.584	r=-0.365 p=0.031
Quinolinic acid	r=-0.463 p=0.002	r=-0.245 p=0.101	r=0.324 p=0.054	r=-0.176 p=0.242	r=0.247 p=0.130	r=0.224 p=0.154	r=-0.284 p=0.068
Kynurenic acid	r=-0.186 p=0.244	r=-0.154 p=0.308	r=0.070 p=0.687	r=-0.143 p=0.344	r=0.127 p=0.442	r=0.092 p=0.561	r=-0.180 p=0.253
Kyn/Tryp	r=-0.580 p<0.001*	r=-0.437 p=0.003	r=0.176 p=0.328	r=-0.424 p=0.005	r=0.261 p=0.124	r=0.213 p=0.193	r=-0.467 p=0.003

Statistical analysis was performed using the Spearman rho correlation coefficient. $p < 0.05$ was considered significant ($n=46$). Bold type face represents significant findings. Asterisks indicate significant findings following correction for multiple testing, adjusted p -value $p \leq 0.001$.

5.3.8 Relationship between KP metabolites and inflammatory markers

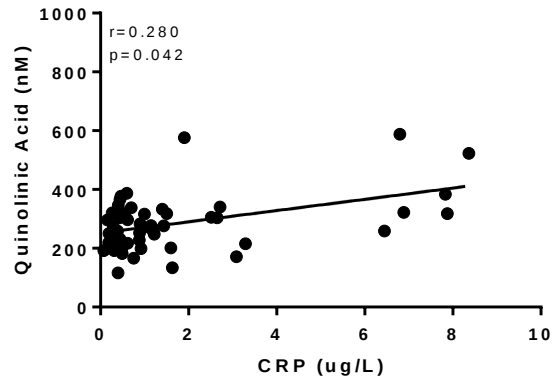
Correlational analysis revealed several relationships between inflammatory markers and kynurenine pathway metabolites within depressed patients. A positive correlation exists between circulating quinolinic acid and CRP concentrations ($r=0.280$, $p=0.042$) [Figure 5.14a]. Quinolinic acid/kynurenine ratio was positively correlated to circulating IFN- γ concentration ($r=0.400$, $p=0.035$) [Figure 5.14b]. Finally, kynurenic acid/quinolinic acid ratio was significantly negatively associated with whole blood transcriptional expression of IL-1 β ($r=-0.307$, $p=0.032$) [Figure 5.14c].

When assessing variables with no mean differences between groups, a Spearman's rho test performed on all participants revealed a significant positive relationship between circulating concentration of CRP and circulating concentration of quinolinic acid ($r=0.298$, $p=0.006$) and quinolinic acid/kynurenine ratio ($r=0.322$, $p=0.004$).

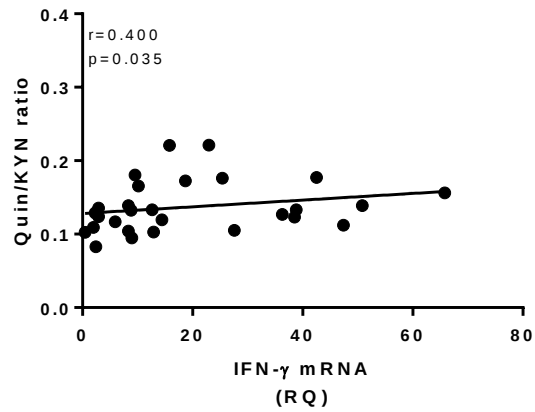
5.3.9 Relationship between kynurenine pathway activity and hippocampal subfield volumes

A partial correlation analysis controlling for age, gender, and total intracranial volume revealed several relationships between KP measures and hippocampal volumes among the entire cohort. A significant negative correlation was determined to exist between IDO2 mRNA expression and some hippocampal subfields. Whole blood transcriptional expression of IDO2 was significantly negatively correlated with right subiculum volume ($r=-0.381$ $p=0.011$), left subiculum volume ($r=-0.515$ $p=0.005$), and right whole hippocampal volume ($r=-0.457$ $p=0.014$) [Figure 5.15]. Additionally, analysis revealed significant positive correlations between KAT1 mRNA expression and left subiculum volume ($r=0.273$ $p=0.030$), and KAT1 mRNA expression with left whole hippocampal volume ($r=0.386$ $p=0.043$) [Figure 5.16a, b].

(a)



(b)



(c)

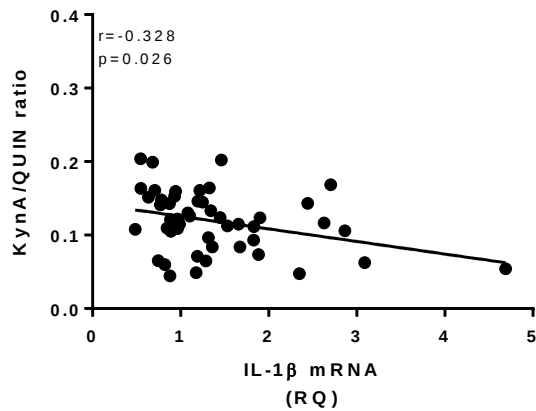


Figure 5.14 Relationships between inflammatory markers and indices of KP activity within MDD. A significant positive relationship exists between (a) circulating quinolinic acid and circulating CRP concentrations, and (b) quinolinic acid/kynurenine ratio and circulating concentration of IFN- γ within the MDD group. A negative relationship exists between kynurenic acid/quinolinic acid ratio and whole blood mRNA expression of IL-1 β in MDD patients. (Spearman's rho analysis).

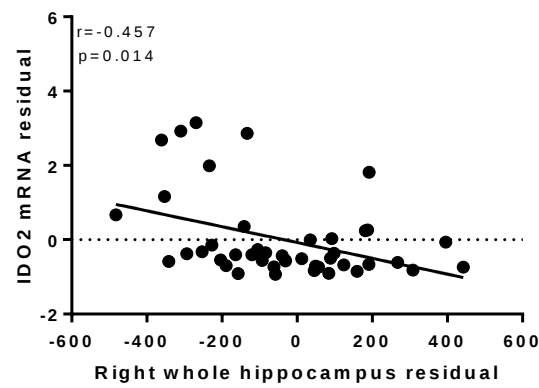
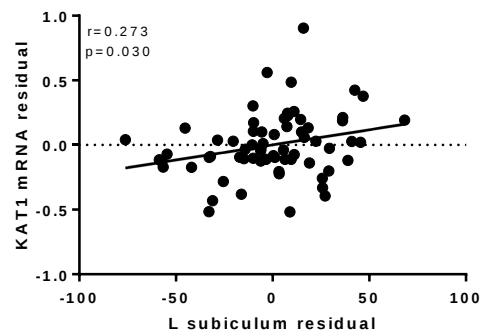


Figure 5.15 Relationship between whole blood IDO2 mRNA expression and hippocampal subfields. A significant negative correlation exists between IDO2 mRNA expression and right whole hippocampal volume according to a partial correlation corrected for age, gender, and TIV.

(a)



(b)

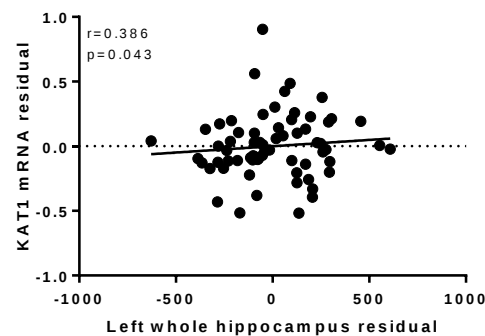


Figure 5.16 Relationships between whole blood KAT1 mRNA expression and hippocampal subfields. A significant positive correlation exists between KAT1 mRNA expression and (a) left subiculum volume and (b) left whole hippocampal volume according to a partial correlation corrected for age, gender, and TIV.

5.3.10 Linear regression analysis of biological variables as a predictor of CES-D score in depressed patients and healthy controls

A linear regression analysis was calculated to predict CES-D scores based on average morning cortisol concentration, cortisol reactivity, transcriptional expression of IL-1 β , and kynurenic acid/quinolinic acid ratio as independent variables. Analysis included 47 participants in total, with 29 depressed patients and 18 healthy controls that had measures for each of the five variables in the regression equation. A significant regression equation was found ($F(1,41)=3.015$, $p=0.029$), with an R^2 of 0.227. Further detail regarding the significance of each independent variable in predicting CES-D score is detailed in Table 5.6. Participant's CES-D score can be predicted based on the following equation:

$$y = 24.848 + 0.476a + 0.294b + 6.492c - 88.385d$$

Where

y=CES-D score

a=AM average Cortisol (nM)

b=Cortisol RT

c=IL-1 β mRNA RQ

d=KynA/QUIN ratio

Table 5.6 Linear regression analysis for prediction of CES-D score.

	Beta	Sig. (p-value)	Collinearity VIF
Constant		0.022	
AM Cortisol (nM)	.198	0.192	1.185
Cortisol RT	.150	0.343	1.297
IL-1 β mRNA RQ	.287	0.111	1.638
KynA/QUIN ratio	-.230	0.132	1.190

Data includes key variables from linear regression analysis and their corresponding B values and p-values for significance. VIF=Variance inflation factor.

5.3.11 Binary logistic regression analysis for predicting a diagnosis of MDD

A binary logistic regression analysis was calculated to predict whether or not a participant had a diagnosis of MDD based on average morning cortisol concentration, cortisol reactivity, transcriptional expression of IL-1 β , and kynurenic acid/quinolinic acid ratio as covariates. Analysis was performed on 47 participants, including 30 depressed patients and 17 healthy controls. A significant regression equation was found ($X^2=17.048$, $df=4$, $p=0.002$), with a resultant Nagelkerke R^2 value of 0.417. Prediction of a participant's diagnosis was based on the following equation:

$$y = -0.587 + 0.090a - 0.019b + 2.304c - 15.349d$$

Where

y=Diagnosis (0=Healthy Control; 1=MDD)

a=AM average Cortisol (nM)

b=Cortisol RT

c=IL-1 β mRNA RQ

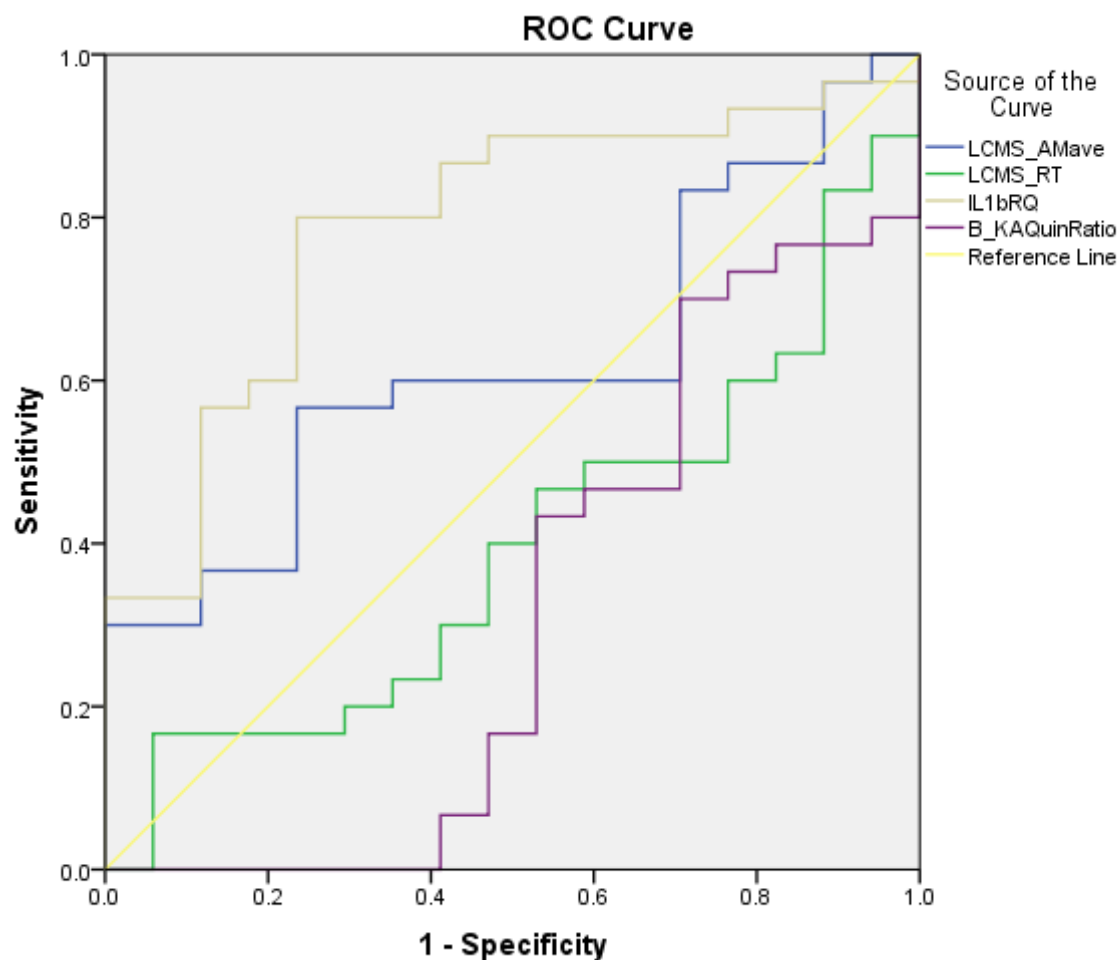
d=KynA/QUIN ratio

This model correctly predicted 72.3% of participant's diagnosis status. It correctly predicted 90.0% of depressed patients to have an MDD diagnosis, but only accurately predicted 47.1% of healthy controls as not have an MDD diagnosis. It appears that this model may overestimate the cases of depression despite its high accuracy within the patients group. Further information regarding individual significance of each independent variable in this regression model is detailed in Table 5.7. Receiver operating characteristic (ROC) curve is displayed in Figure 5.17 to illustrate the sensitivity and specificity of the variables in this model as a predictor of diagnosis.

Table 5.7 Binary logistic regression analysis for prediction of depression diagnosis.

	B Value	Sig. (p-value)
Constant	-.587	0.783
AM Cortisol (nM)	.090	0.265
Cortisol RT	-.019	0.766
IL-1 β mRNA RQ	-15.349	0.113
KynA/QUIN ratio	2.304	0.063

Data includes key variables from binary logistic regression analysis and their corresponding B values and p-values for significance.



Area Under the Curve

Test Result Variable(s)	Area	Std. Error ^a	Asymptotic Sig. ^b	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
LCMS_AMave	.624	.082	.163	.463	.784
LCMS_RT	.396	.084	.241	.231	.561
IL1bRQ	.788	.069	.001	.654	.923
B_KAQuinRatio	.316	.088	.037	.144	.488

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Figure 5.17 ROC Analysis using variables from binary logistic regression equation. The figure is a plot of the sensitivity and specificity of each variable as a binary classifier for depression diagnosis where Healthy Control=0 and MDD=1. The corresponding data table describes the parameters of each variable's predictive sensitivity and specificity.

5.3.12 Tree analysis revealing strongest biological profiles in depressed patients and healthy controls

Classification analysis was carried out using the Chi Square Automatic Interaction Detection (CHAID) method of Tree analysis. All biological variables were entered as independent variables, and diagnosis (depressed patient or healthy control) was selected as the dependent variable. A minimum parent node of 10 and minimum child node of 5 were specified for the output of this analysis. A decision tree was generated with 5 terminal nodes and was 3 levels deep. Thresholds for grouping were determined by automatically by the CHAID programme. The resultant primary grouping variable, the most decisive variable, was kynurenine concentration. Kynurenine concentration was divided into 2 levels, and grouped the participants into Node 1, a concentration of ≤ 890.5 pg/ml, or Node 2, a concentration of > 890.5 pg/ml. Fifty-four of 72 depressed patients fell into Node 1 classification, while 20 of 60 total healthy controls were also grouped into Node 1. Node 2 accounted for 18 depressed patients and 40 healthy controls and was a terminating node.

Next, Node 1 was further classified based on transcriptional expression of IL-1 β into Nodes 3 and 4. Node 3 represented a low transcriptional expression of IL-1 β , with RQ values ≤ 0.78 , while Node 4 represented a higher category of transcriptional expression of IL-1 β , with RQ values > 0.78 . The resultant Node 3 was inclusive of 11 healthy controls and 8 MDD patients, while Node 4 was inclusive of 9 healthy controls and 46 MDD patients.

To further classify these biological groupings, Node 3 was divided into two additional groupings defined by low (≤ 0.86) and high (> 0.86) relative quantification of IDO2, which were named Node 5 and 6 respectively. Node 6 was inclusive of 7 controls and 0 depressed patients, while Node 7 was made up of 4 controls and 8 depressed patients. Node 4 was subsequently broken down into two further nodes of low (≤ 3596 mm³) and high (> 3596 mm³) right whole hippocampal volumes, which defined Nodes 7 and 8 respectively. Node 7 was composed of 4 healthy controls and 42 depressed patients. Node 8 was comprised of 5 healthy controls and 4 depressed patients. Results are depicted in Figure 5.18.

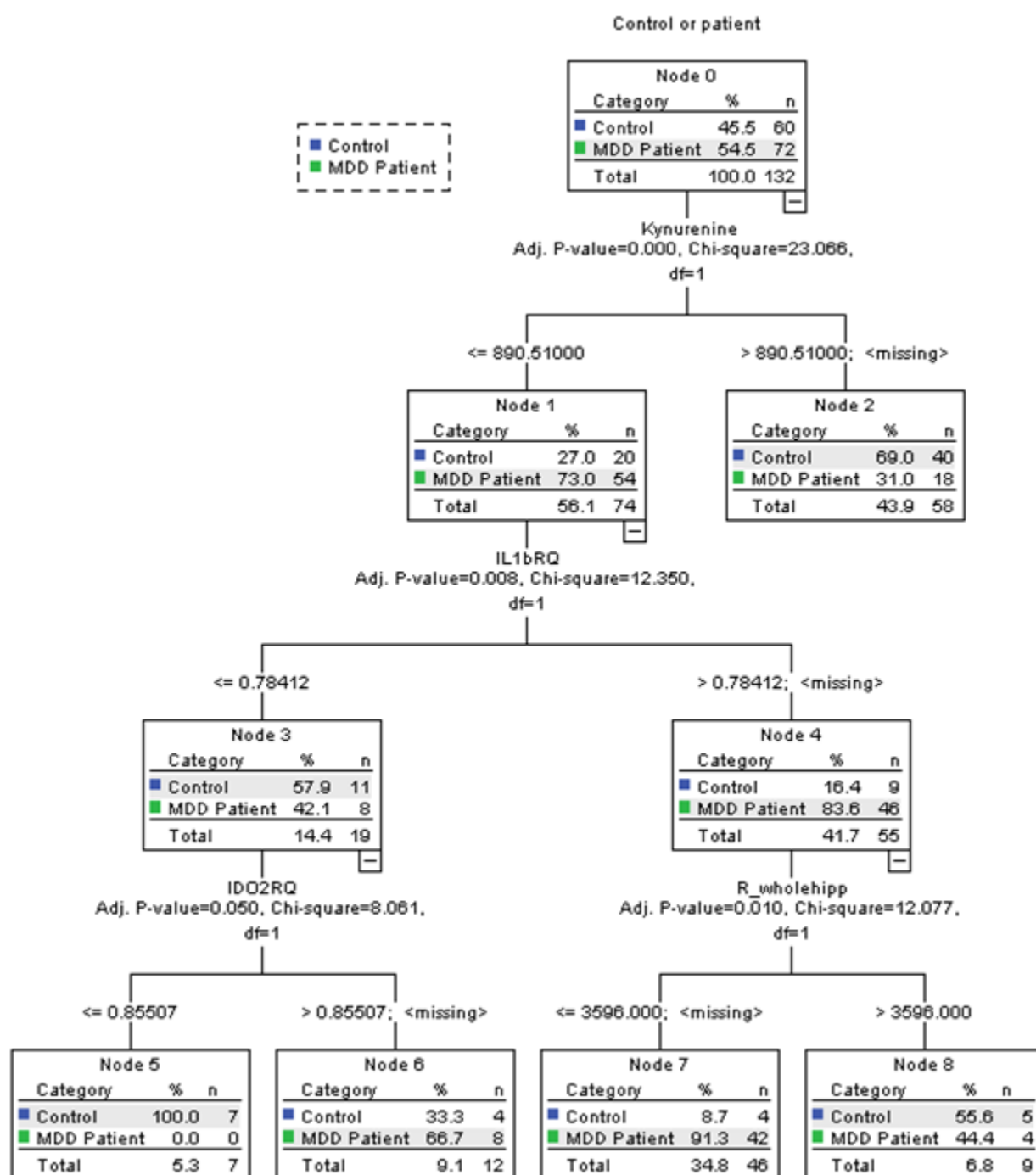


Figure 5.18 CHAID tree classification analyses of biological variables for depressed patients and healthy controls. Tree created by the CHAID model (Chi-squared Automatic Interaction Detection) for biological pathways measured in depressed patients and healthy controls.

5.4 Discussion

The results of this study provide evidence of kynurenine pathway disruption in major depressive disorder. Tryptophan and several of its catabolites were measured in circulating plasma, and genes encoding for enzymes involved in kynurenine pathway activation were assessed in PAXgene whole blood mRNA. In this study, depressed patients showed significant tryptophan depletion, a trend toward lower kynurenine, and significantly reduced kynurenic acid with no change in quinolinic acid concentration. This profile of kynurenine pathway activation can also be described by the differing ratios of KP catabolites between depressed patients and healthy controls. For example, a significantly higher quinolinic acid/kynurenine ratio in depressed patients indicated an increase in neurotoxic substances in relation to its precursor. A significant decrease in kynurenic acid/quinolinic acid ratio reiterated the deficiency in the potentially protective products of the KP relative to potentially excitotoxic products of the KP present in circulating plasma of depressed patients compared to healthy controls. Correlational analysis established that increased kynurenine was related to severity of depression symptoms within the MDD group, providing evidence that induction of the kynurenine pathway is exacerbated in tandem with symptomatology. Furthermore, several measures of kynurenine pathway activity were found to be significantly correlated to measures of HPA axis activation within the depressed group, for example, kynurenine/tryptophan ratio was negatively associated with wakening cortisol concentration. Some measures of kynurenine pathway activity were associated with measures of inflammation within depression, such as the negative relationship between kynurenic acid/quinolinic acid ratio and whole blood mRNA expression of IL-1 β . It is known that HPA axis activation and immune activity can induce metabolism of tryptophan, yet the relationship between these pathways and expression of downstream kynurenine pathway metabolites has rarely been reported in depression research. Altogether, these findings indicate disruption of the kynurenine pathway in MDD through decreased metabolites and enzymes of the kynurenic acid branch of the KP, and increased quinolinic acid branch products. This dysregulation exists in conjunction with alterations of the HPA axis and immune system, suggesting that the biological causes and consequences of depression may be explained by a network that encompasses all of these pathways.

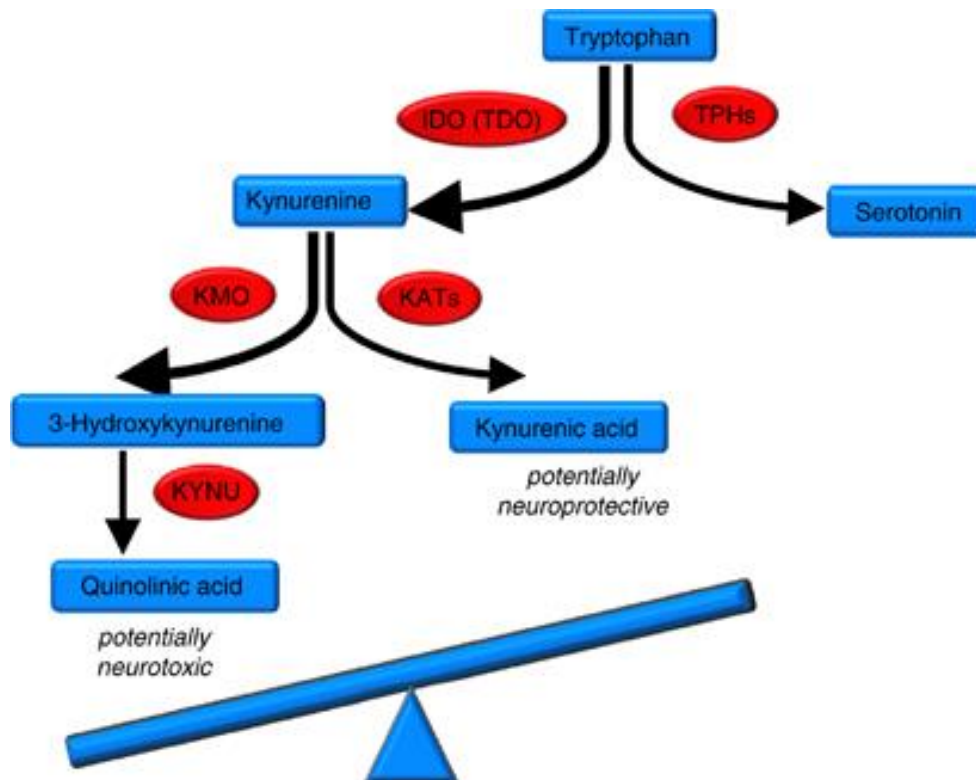


Figure 5.19 Kynurenine pathway of tryptophan metabolism schematic. Tryptophan is the precursor to serotonin and kynurenine. IDO and TDO represent the first and rate limiting enzymes of tryptophan metabolism into kynurenine. Kynurenine is degraded into 3-HK via the enzyme KMO. 3-HK is eventually catabolised by kynureninase into quinolinic acid, a neurotoxic product of the KP. Kynurenine is differentially metabolised via KATs into kynurenic acid, a neuroprotective product of the KP (Zunszain et al., 2012).

5.4.1 Altered tryptophan and kynurenine concentrations in depression

Circulating tryptophan concentrations were found to be significantly lower in depressed patients than in healthy controls in this study [Figure 5.1a]. Additionally, tryptophan concentrations were inversely correlated to CES-D scores of depression symptom severity across depressed and controls patients. Tryptophan depletion is a well-known biological alteration associated with depression (DeMyer et al., 1981; Cowen, 1989; Maes et al., 1991). Tryptophan is the precursor to serotonin, and its depletion is thought to be responsible for the reduction of serotonin levels in the brains of depressed patients (Maes et al., 2011). Tryptophan is alternatively catabolised into kynurenine, which also showed a trend toward lower concentrations in depressed patients than healthy controls [Figure 5.1b]. Concentrations of kynurenine in depressed patients have been previously been reported to

be both increased (Myint et al., 2007) and decreased (Maes et al., 2011) in depression, and the kynurenine to tryptophan ratio has also shown to be increased in some studies (Maes and Rief, 2012; Bryleva and Brundin, 2016). In this study, we did not find a difference in kynurenine/tryptophan ratio between patients and controls [Figure 5.4]. This could be due to further kynurenine catabolism into downstream metabolites which is not represented by the kynurenine/tryptophan ratio. Tryptophan in the blood that is not converted to either serotonin or kynurenine is used as a building block for protein biosynthesis.

5.4.2 Kynurenine pathway induction by indoleamine-2,3-dioxygenase

Assessment of relative mRNA expression of IDO1 and IDO2 as indicators of indoleamine-2,3-dioxygenase activity revealed no significant differences between depressed patients and healthy controls in this study [Table 5.4]. This contradicted the hypothesis that kynurenine pathway activation would occur via IDO increases induced by immune activity in depressed patients (Dantzer et al., 2008). However, correlational analysis on the whole study population did reveal that tryptophan concentration was negatively correlated to IDO2 mRNA expression, indicating that as tryptophan was depleted, IDO2 was more prevalent. Additionally, quinolinic acid was positively correlated to IDO1 in the whole cohort [Figure 5.9]. These findings suggest that as IDO expression increases, kynurenines (including potentially neurotoxic catabolites) also increase. Finally, it was determined that whole blood IDO1 mRNA expression was negatively correlated to SLC6A4 mRNA expression [Figure 5.10], suggesting that reduced availability of the serotonin transporter may be related to increased IDO activity. This evidence supports the hypothesis that IDO induces tryptophan and serotonin depletion, shunting tryptophan catabolism down the kynurenine pathway.

5.4.3 Breakdown of kynurenine into potentially neurotoxic catabolites

The pathway of kynurenine metabolism contains a potentially neurotoxic branch. Kynurenine is broken down into 3-HK, which is converted into 3-hydroxyanthranilic acid (3-HAA). Next, 3-HAA is metabolised into 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD) which is finally catabolised into quinolinic acid [Figure 5.19]. Quinolinic acid is known to have toxic effects on the central nervous system (Lugo-Huitrón, 2013), as is its precursor 3-HK (Reyes-Ocampo et al., 2015). 3-HK is neurotoxic via generation of oxidative stress and the release of free hydroxyl radicals (Eastman and Guilarte, 1989). In

rat brains, it has been shown to potentiate excitotoxic neuronal loss in the striatum and apoptotic neuronal death in the cortex (Schwarcz et al., 2012). Quinolinic acid was first shown to have neurotoxic effects by Lapin (1996) who observed convulsions in mice after quinolinic acid injections. It is an agonist of the N-methyl-D-aspartate (NMDA) receptor and induces oxidative stress (Stone, 1993; Cabrera et al., 2000). Moreover, quinolinic acid was shown to be increased in post-mortem subgenual anterior cingulate cortices of patients with severe depression (Steiner et al., 2011).

While this study found no differences in circulating concentrations of 3-HK and quinolinic acid [Figure 5.2a,b], the ratio of quinolinic acid/kynurenine was significantly higher in depressed patients compared to healthy controls [Figure 5.5a]. This finding has been reported among both current and remitted depressed patients (Savits et al., 2015), showing that these data are in accordance with the most recent findings in kynurenine pathway assessment in MDD. Additionally, the difference in quinolinic acid to kynurenine ratio suggests that the relative levels rather than the absolute concentrations of quinolinic acid and kynurenine is the more prominent abnormality in depression.

The transcriptional expression of several kynurenine pathway enzymes was assessed. Kynurenine 3-monooxygenase (KMO) is the enzyme that catalyses the conversion of kynurenine to 3-HK. There was no difference in mRNA expression of KMO between depressed patients and controls [Table 5.4], but KMO was positively correlated to IDO1 mRNA expression [Figure 5.11a]. There was also no difference in whole blood mRNA expression of kynureninase, the enzyme that catalyses the conversion of 3-HK to 3-HAA, however kynureninase expression was positively correlated to IDO1 and IDO2 mRNA expression [Figure 5.11b and c]. Moreover, expression of KMO and kynureninase were positively associated with each other [Figure 5.12b]. Finally, whole blood mRNA expression of KMO was negatively associated with whole blood mRNA expression of kynurenic acid [Figure 5.12a], showing that the quinolinic acid arm of the kynurenine pathway is favoured over the kynurenic acid arm with increased KMO. These relationships give evidence of kynurenine pathway functioning, as each of these enzymes assists in further degradation of each component of the quinolinic acid arm of the kynurenine pathway.

5.4.4 Breakdown of kynurenine into potentially neuroprotective catabolites

One of the most crucial findings in this study was the significantly lower kynurenic acid levels in plasma of depressed patients compared to healthy controls [Figure 5.3]. This supported the hypothesis of reduced potentially neuroprotective products of the KP in depressed patients. Kynurenic acid is known to be a NMDA antagonist with anti-excitotoxic and anticonvulsant properties (Birch et al., 1988; Stone et al., 2013). Its reduction has been shown in depression (Myint, 2007), though not all studies have had findings that agree with this (Hughes et al., 2012). Reduction of the kynurenic acid to kynurenine ratio has also been shown in some studies of depression (Myint et al., 2007), though the findings have not been consistent across studies according to a review by Lopresti et al. (2014). The present research found no difference between groups [Figure 5.5b], though this does not necessarily prove a lack of dysfunction in this branch of the kynurenine pathway.

Whole blood transcriptional expression of kynurenine amino transferase (KAT) 1, one of the KATs enzymes that catalyse conversion of kynurenine to kynurenic acid, was measured. No difference was found between its expression in depressed patients and controls, but it was found to be significantly negatively correlated to HAM-D scores within the depressed group [Figure 5.8a]. Moreover, expression of KAT1 was also negatively associated with CTQ scores among depressed patients, suggesting that depression and trauma are associated with a deficiency in this branch of kynurenine metabolism [Figure 5.8b].

5.4.5 Decrease in ratio of kynurenic acid to quinolinic acid ratio in depression

One of the major findings of this study was the significantly lower kynurenic acid to quinolinic acid ratio detected in depressed patients [Figure 5.6]. This figure represents the proportionality of the final products of two branches of the kynurenine pathway. Quinolinic acid and kynurenic acid have opposing functions on NMDA receptors, therefore a difference in this ratio is indicative of possible dysregulation of NMDA receptor activation (Savitz et al., 2015). The finding of a significant difference in kynurenic acid to quinolinic acid ratio between groups, as opposed to finding differences in both of the individual metabolites between groups, suggests that the relative levels of these metabolites to each other rather than their absolute concentrations is the most pertinent dysfunction of the KP in depression (Gupta et al., 2012).

5.4.6 Relationship between KP induction and HPA axis activation

Previous studies have found a relationship between HPA axis activation and KP dysregulation in depression, as expression of TDO, one of the enzymes that catalyses the conversion of tryptophan to kynurenine is induced by glucocorticoid activity observed in depression (O'Farrell and Harkin, 2015). While this study found several significant relationships between kynurenine pathway and hypothalamic pituitary adrenal axis activity within the depressed group, the directionality of these relationships was not as anticipated. For example, wakening cortisol concentration was significantly negatively correlated to the kynurenine/tryptophan ratio [Figure 5.13]. In the same vein, peak cortisol concentration was negatively associated with circulating 3-HK concentration [Table 5.5]. These relationships suggest overall kynurenine pathway depletion in relation with increased HPA axis activation, rather than kynurenine pathway induction. While this study found greater expression of HPA markers of stress in the previous chapter, increased cortisol levels were not associated with greater levels of kynurenine production.

5.4.7 Relationship between KP induction and immune activation

Another key finding in this study was the significant positive correlation between circulating CRP concentration and quinolinic acid concentration within the depressed group [Figure 5.14a]. Also, whole blood mRNA expression of IFN- γ was positively correlated to quinolinic acid/kynurenine ratio among depressed patients [Figure 5.14b], and whole blood mRNA expression of IL-1 β was significantly negatively associated with kynurenic acid to quinolinic acid ratio within the depressed group [Figure 5.14c]. This gives additional indication that increased inflammatory markers are associated with a kynurenine pathway metabolism profile that favours the quinolinic acid branch over the kynurenic acid branch of metabolism. These associations add ample evidence to the growing literature suggesting immune activation is largely responsible for kynurenine pathway induction in depression (Capuron et al., 2003).

5.4.8 Relationship between KP induction and brain volumes

Analysis revealed that mRNA expression of IDO2 was negatively correlated to left subiculum volume, right subiculum volume, and right whole hippocampal volume [Figure 5.15]. These

results indicate that specific hippocampal subfields are reduced in volume as the kynurenine pathway is induced by IDO. Furthermore, mRNA expression of KAT1 was positively correlated to left subiculum volume and left whole hippocampal volume [Figure 5.16a,b], suggesting that when the neuroprotective branch of the kynurenine pathway is more active, hippocampal volume integrity is increased. An imbalance of neuroprotective to neurotoxic products of the kynurenine pathway has been found to be associated with hippocampal grey matter volume loss in psychiatric disorders (Savits et al., 2015), though the present study is among one of the first to explore the relationship between kynurenine pathway activity and volume of limbic structures such as the hippocampus. These findings give strong evidence that differences in kynurenine pathway activation in depression are driven by the immune system, as hippocampal volume is known to be reduced as a consequence of inflammation (Kesler et al., 2013). Moreover, the region found to be associated with KP dysfunction, the subiculum, is thought to play a role in inhibitory control of the HPA axis (O'Mara, 2005). This reasserts the need to consider the kynurenine pathway in conjunction with HPA and immune alterations when describing the pathophysiology of MDD.

5.4.9 Linear regression analysis of biological variables in predicting depression diagnosis

As part of this study's analysis, a binary logistic regression analysis for prediction of depression diagnosis was calculated, and resulted in an equation that included average morning cortisol concentration, cortisol reactivity, whole blood mRNA expression of IL-1 β , and kynurenic acid/quinolinic acid ratio as variables. This analysis indicated that, overall, the model applied can statistically significantly predict the dependent variable, which in this case is diagnosis of MDD. Finding a model that could significantly predict MDD diagnosis would be extremely helpful clinically, and this is a preliminary attempt to establish such a model.

5.4.10 Logistic regression analysis of biological variables to predict CES-D score

A linear regression analysis was calculated to predict scores of the self-rated CES-D scale of depression symptoms in patients and controls. This resulted in an equation that included average morning cortisol concentration, cortisol reactivity, transcriptional expression of IL-1 β , and kynurenic acid/quinolinic acid ratio as variables. Overall, analysis indicated that the model applied can statistically significantly predict the dependent variable of CES-D score

among participants. Discovery of a model that significantly predicts depression symptom severity could be extremely useful. Increased depression symptom severity is associated with increased benefit of anti-depressant treatment (Fournier et al., 2010). Therefore, development of a model such as this could be helpful in determining the most effective treatment plan for a patient. Since depressed patients who do achieve remission must often complete several trials of anti-depressants (STAR-D), optimisation of a model such as this could be extremely beneficial in choosing a treatment through this objective measure.

5.4.11 Limitations and future directions

Studies that hope to investigate alterations of the kynurenine pathway in depression may benefit from assessing markers that were not quantified in this study. For example, measurement of TDO in depressed patients and healthy controls in conjunction with the variables collected in the present study would greatly improve the understanding of the extent that the HPA axis initiates kynurenine pathway activity. In this study, we measured mRNA expression of KAT1, but not KAT2, 3, or 4. A more complete assessment of the neuroprotective branch of the kynurenine pathway would include measurement of each of the KAT enzymes. Furthermore, picolinic acid is another neurotoxic product of the kynurenine pathway that was not assessed here and would be valuable in acquiring a more complete understanding of the kynurenine pathway dysfunction that occurs in MDD.

One limitation in regard to the regression modelling in this study was that not all participants provided a complete set of biological samples to the study [Figure 5.20]. This resulted in subsets of variables being unmeasured in many patients. For example, some patients gave saliva samples for cortisol analysis and completed an MRI scan, but were unable to donate a blood sample therefore mRNA and circulating proteins could not be measured. In some cases, patients were not able to undergo an MRI scan due to metal in their body. This resulted in exclusion of a large number of individuals from regression modelling when including MRI measures as a variable, thus brain volume variables were impractical to include in the regression modelling. Future studies should aim to ensure that all participants are able to give the full range of samples in order to avoid having to exclude any subject or variable from regression analysis.

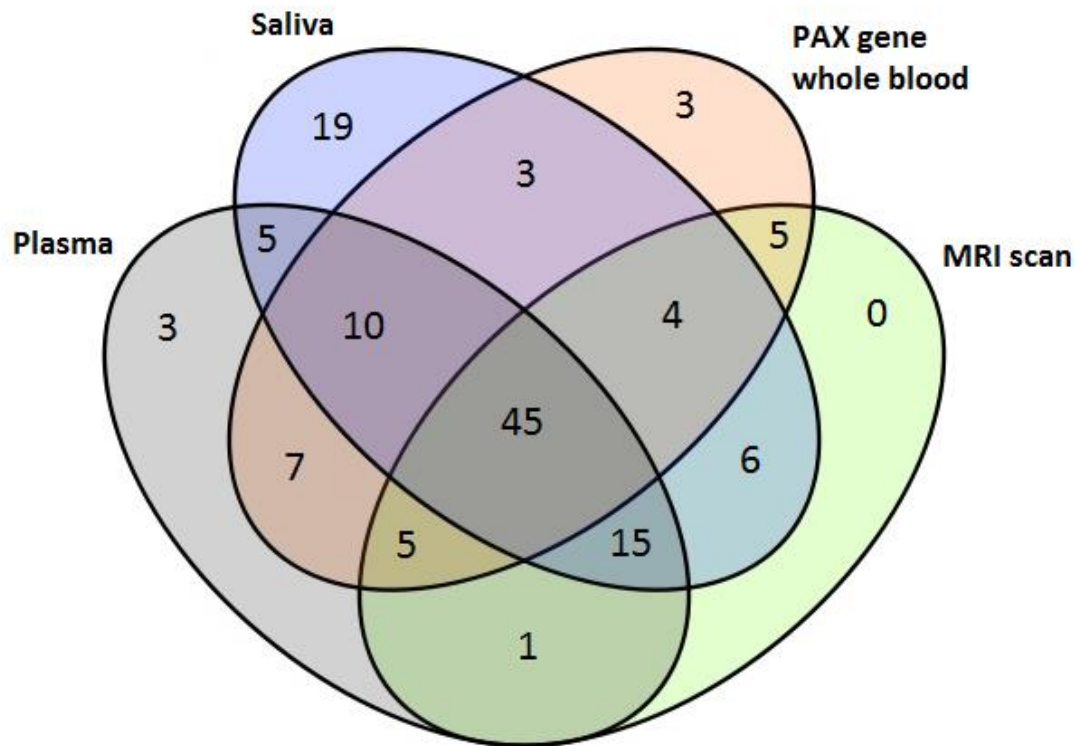


Figure 5.20 Biological sample collection summary. Venn diagram depicting the total number of each sample type collected. Forty-five participants donated the full range of samples including saliva, PAXgene whole blood, plasma, and an MRI scan.

5.4.12 Conclusions

This study has shown significant alterations in the kynurenine pathway of tryptophan metabolism in MDD. Tryptophan depletion and the decrease of kynurenic acid were significant in the patient group, strongly supporting the hypothesis that depression may occur due to dysfunction of this pathway. Relationships were detected between several markers of immune activation and kynurenine pathway dysfunction, furthering the evidence that inflammation plays a large role in upsetting the balance of kynurenine pathway metabolites that is witnessed in depressed patients. Moreover, this study gives some of the first evidence of a relationship between circulating kynurenine pathway metabolites and reduced hippocampal subiculum volume, a region associated with inhibitory control of the HPA axis. Overall, this study provides much support for a model of depression that encompasses kynurenine pathway alterations in conjunction with HPA axis and immune system dysregulation.

Chapter 6

**Differing biological profiles for depressive subtypes and
symptom clusters**

6.1 Introduction

Major Depressive Disorder (MDD) is a serious illness affecting about 350 million people worldwide and is the number one cause of disability (World Health Organisation, 2016). Despite the prevalence of this disorder, the exact aetiology of MDD is unknown. Treatment response is extremely variable between subsets of patients, and only about half of patients achieve remission with two adequate trials of anti-depressant treatment (Huynh and McIntyre, 2008). Moreover, depression is a very heterogeneous disorder, and patients' experiences often present as opposing extremes such as insomnia or hypersomnia, anorexia or hyperphagia, and motor agitation or leaden paralysis. Many phenotypes of depression exist, but distinct biological pathways have not yet been identified for these varying subtypes which include atypical and melancholic depression (American Psychiatric Association, 2000). Early life adversity, specific depressive symptom profiles, atypical subtype, recurrence, and antidepressant medication use are all factors that should be accounted for in the search to establish a biological mechanism that explains depression pathophysiology.

Within depression, severity of individual symptoms may vary greatly between patients. The Hamilton Depression Rating Scale (HAM-D) was developed in 1960 with its latest revision being completed in 1980, with a purpose of measuring severity of depression in an individual by assessing mood, feelings of guilt, suicidal ideation, insomnia, agitation, anxiety, weight loss, and somatic symptoms (Hamilton, 1980). However, when looking at specific subsets of HAM-D items, for example anxiety or insomnia cluster scores, it is possible to more accurately quantify the individual's depressive phenotype. Furthermore, because of the heterogeneous nature of depression, it is essential to determine the relationship between biological variables and specific symptoms of the disorder, rather than depression severity in general (Fried and Nesse, 2015). Previous studies have shown how increased anxiety symptoms can modulate inflammation (Salim et al., 2012), while the HPA axis is highly influenced by sleep disruption (Buckley and Schatzberg, 2005). It is critical to investigate the biological differences associated with specific depressive symptoms to gain insight into the diverse presentations of the illness.

Atypical depression is characterised by the presence of reverse vegetative symptoms of hypersomnia or hyperphagia as well as mood reactivity, and has been shown to have a distinct or even opposite pathophysiology to that of non-atypical or melancholic subtypes of depression (Blanco et al., 2012). Only a few studies of MDD have stratified patients by these

clinical subtypes despite their distinctiveness and relative stability (Lamers et al., 2013). Pure melancholic subtype comprises between 25-30% of all patients with MDD, while pure atypical subtype accounts for 15-30% of patients (Gold and Chrousos, 2002). Studies that have assessed biological differences between depressive subtypes have honed in on the differential HPA axis activity between atypical and non-atypical depressives. For example, atypical depressives have been shown to have reduced activity of the HPA axis and corticotropin releasing hormone (CRH) compared to healthy controls, rather than the hyperactivity of the HPA axis seen in non-atypical or melancholic subtypes when compared to healthy controls (Chrousos and Gold, 1992; Asnis et al., 1995; Levitan et al., 2009). However, no research to date has explored the activation of the kynurenine pathway with regard to atypical and non-atypical subtypes of MDD.

Additionally, research has made it clear that a history of childhood trauma increases the risk for depression and suicide in adulthood (Felitti et al., 1998; Edwards et al., 2003; Chapman et al., 2004). Evidence exists to support dysregulation of the HPA axis and immune system as a response to childhood trauma or early life adversity. For example, Heim et al. reported that women with a history of childhood sexual or physical abuse exhibited increased pituitary-adrenal and autonomic responses to stress compared with controls. Women who were depressed and had been abused exhibited more than 6-fold greater ACTH responses to stress than controls (Heim et al., 2008). Another study found elevated circulating IL-6 in male depressed patients who had experienced early life adversity (Pace et al., 2006). Moreover, early life stress exposure in depressed patients has been associated with smaller hippocampal volumes, and hippocampus is involved in HPA axis regulation (Frodl et al., 2002; Saleh et al., 2016). These findings indicate that early life adversity has lasting damaging effects on the HPA axis and immune systems that are evident within a non-depressed population and exacerbated within a depressed population. It is essential to determine how biological systems are affected differently between depressed patients with and without ELA in order to better understand the pathophysiology underlying trauma in a depressed population.

Since depression is recurrent in about 45% of patients (Rush et al., 2009), determining the biological differences between patients presenting with their first episode of depression compared to patients who have had multiple episodes is of interest. Patients presenting with their first episode of depression may not have developed the degree of biological dysregulation that recurrently depressed patients exhibit, as recurrent depressives tend to

have more severe symptoms and increased comorbidities (Gili et al., 2010; Roca et al., 2011). While greater inflammation has been reported in recurrent depressed patients (Valkanova et al., 2013), no studies have been published concerning the differences in kynurenine pathway activation between recurrent and first presentation patients. Disruptions of the kynurenine pathway are gaining credibility as a prominent explanation for depression aetiology and exploration of the effects of chronicity of disease are necessary for validation of this hypothesis.

About half of depressed patients achieve remission with two trials of antidepressant medication (Huynh and McIntyre, 2008). The lack of improvement of depressive symptoms in treatment resistant patients who are medicated with antidepressants is not well understood. However, it has been shown that treatment with antidepressants can increase circulation of inflammatory cytokines (Vogelzangs et al., 2014). Additionally, it has been reported that despite increased circulating tryptophan after antidepressant treatment, no differences in the balance of metabolites of the kynurenine pathway have been detected in previous research (Hannestad et al., 2011; Vogelzangs et al., 2014). The current study aims to contribute to the limited literature that exists concerning kynurenine pathway markers during the use of antidepressant medication.

While the previous chapters of this thesis have investigated alterations of the HPA axis (Chapter 3), immune system (Chapter 4), and kynurenine pathway (Chapter 5) between healthy controls and depressed patients as a whole, the assessment of biological variables of these systems will now focus on depression subtypes in order to further probe the differences in activation of these pathways in varying presentations of the disorder. A greater understanding of the biological profiles and varying forms of biological dysfunction associated with specific depression subtypes has the possibility to better inform treatment targets for patients with particular symptom profiles or clinical features. Early life adversity, HAM-D subscale scores, atypical subtype, recurrence, and antidepressant medication use have been assessed to gain further insight into the complexities of depression aetiology and will be presented here.

Study Aims:

1. To investigate the differences in activation of the kynurenine pathway and immune activity in depressed patients with and without early life adversity.
2. To assess how severity of specific symptom clusters of depression are related to expression of biomarkers.
3. To determine differences in HPA axis activity between depressed patients with the atypical subtype compared to those without.
4. To investigate effects of recurrent depression on the immune system and kynurenine pathway compared to depressed patients who have presented with only one episode.
5. To determine difference in expression of biological markers in patients who are being treated with antidepressants compared to those that are treatment naïve.

6.2 Methods

6.2.1 Participants

In total, 72 patients with MDD and 60 healthy controls were recruited for participation in this study. Depressed participants were recruited from the psychiatric outpatient clinic at Sheaf House in Tallaght Hospital and at the Mary Mercer Health Centre in Jobstown, Dublin 24. Patients who presented to the clinic with depression were considered for the study. They were evaluated for study eligibility based on DSM-5 criteria for a depressive episode and a score of 17 or above on the Hamilton Depression Rating Scale (Hamilton, 1960). Healthy control subjects with no history of depression were recruited from the local community. Further inclusion and exclusion criteria for this study are as detailed in Chapter 2.

6.2.2 Childhood Trauma Questionnaire and Early Life Adversity

The Childhood Trauma Questionnaire (CTQ) is a standardised, 28-item self-report instrument which assesses five categories of childhood maltreatment including emotional, physical, and sexual abuse, and emotional and physical neglect. The questionnaire is comprised of five questions for each subscale of childhood maltreatment, in addition to 3 items meant to evaluate minimization and denial to identify participants who might be under reporting traumatic events. Each abuse subscale breaks down into classifications of no adversity, low, moderate, or severe adversity based on specific cut-off points [Table 6.1]. Participants scoring in the moderate to severe range in at least one of the items were classified as having a positive history of early life adversity (ELA). Reliability and validity of the CTQ has been established for validity from structured interviews, stability over time, and corroboration (Bernstein et al., 2003).

Table 6.1 Classification range for early life adversity scores.

	No adversity	Low	Moderate	Severe
Emotional abuse	5-8	9-12	13-15	16-25
Physical abuse	5-7	8-9	10-12	13-25
Sexual abuse	5	6-7	8-12	13-25
Emotional neglect	5-9	10-14	15-17	18-25
Physical neglect	5-7	8-9	10-12	13-25

Cut-offs provided by Heim et al. (2009).

6.2.3 Hamilton Rating Scale for Depression and its subscales

Severity of depression symptoms was assessed in patients using the HAM-D 21 scale. The HAM-D total score is based on the sum of the first consecutive 17 items in the scale, and the subsequent 4 items provide supplemental clinical information regarding diurnal variation, derealisation and depersonalisation, delusional and obsessional symptoms. Higher HAM-D scores are indicative of increased severity of depression. The HAM-D 21 question scale can be viewed in full in Appendix 3b. Depressive symptoms were assessed using several symptom clusters based on specific subsets of HAM-D items, including the following:

- (1) The core depression subscale was defined by depressed mood, feelings of guilt, work activities, psychomotor retardation, anxiety (psychological) and somatic symptoms (general) items of the HAM-D (Bech et al., 1975). This subscale covers the affective core symptoms of depression severity.
- (2) Insomnia or sleep difficulty is a 3 item cluster defined by insomnia early, middle, and late (Manber et al., 2005).
- (3) The anxiety subscale was a five item cluster calculated by the sum of agitation, anxiety (psychological), anxiety (somatic), hypochondriasis and insight scores.
- (4) The somatic or vegetative symptom cluster was defined by somatic (gastrointestinal), somatic (general), genital symptoms and weight loss items (Shafer, 2006).
- (5) Anxiety/somatisation factor scores were based on 6 items used to define anxious depression (Farabaugh et al., 2010; Fava et al., 2008). The sum of anxiety (psychological), anxiety (somatic), somatic (gastrointestinal), somatic (general), hypochondriasis and insight was taken to calculate a score for the anxiety/somatisation factor.

6.2.4 Atypical Depression

Each patient was assessed for criteria that would further classify their depression as the atypical subtype or not. In order to be defined as atypical subtype, the participant would have to have shown mood reactivity, or brightening of mood in response to potential or actual positive events, and at least two of the following: weight gain or increase in appetite, hypersomnia, leaden paralysis, and longstanding interpersonal rejection sensitivity. These symptoms were assessed at the time of recruitment.

6.2.5 Laboratory methods

Biological data was generated as previously described (Chapter 2) from blood samples, saliva samples, and MRI scans collected from participants at the time of recruitment. LC-MS was used to quantify salivary cortisol measurements. ELISA was used for circulating cytokine quantification. LC-MS/MS was used to determine circulating kynurenine pathway metabolite concentrations. RT-PCR was used to determine relative quantification of inflammatory markers and KP enzymes.

6.2.6 Statistical analysis

Data are presented as mean with standard error of the mean (SEM) or standard deviation (SD) where appropriate. All data were tested for normality using the Shapiro-Wilk test. Normally distributed data were analysed using a student's t-test. Non-parametric data were analysed using a Mann-Whitney U test for independent sample comparisons and Kruskal-Wallis one-way ANOVA for more than two independent samples. Correlational analysis between biological markers and HAM-D subscale scores was carried out using Spearman's rho correlation statistics, or partial correlations correcting for age, gender, and total intracranial volume when correlating hippocampal volumes to subscale scores. Considering exploratory nature of the correlational analyses, unadjusted p-values are reported throughout (Rothman, 1990; Perneger, 1998). All statistical analyses were considered significant when $p \leq 0.05$. All data were analysed using SPSS (Version 16). Graphs were generated using GraphPad Prism Software Version 5.00 (GraphPad software, Inc).

6.3 Results

6.3.1 Early life adversity

6.3.1.1 Childhood trauma subscale scores and ELA in depressed patients and healthy controls

Participants in the depressed cohort reported significantly more childhood traumatic events than healthy control participants according to global CTQ scores, and depressed patients had much higher rates of early life adversity (ELA) (MDD: 66.19% vs HC: 19.30%) ($\chi^2=27.555$, $p<0.001$). Of the depressed patients in this study, 24 reported moderate to severe emotional abuse, while 2 healthy controls had a history of emotional abuse. Emotional abuse scores were significantly higher in patients than controls [Table 6.2]. Of the depressed patients, 13 reported moderate to severe physical abuse, while 1 control had a childhood history of physical abuse, and physical abuse subscale scores were significantly higher in depressed patients accordingly [Table 6.2]. There was no significant difference in sexual abuse scores between depressed patients and healthy controls [Table 6.2], as seven depressed patients and 3 healthy controls were sexually abused as children. Moderate to severe emotional neglect was experienced by 21 depressed patients and no healthy controls, resulting in significantly higher emotional neglect subscale scores in depressed patients. Moderate to severe physical neglect was reported in 11 depressed patients and 2 healthy controls, again resulting in higher physical neglect subscale scores in depressed patients. Interestingly, healthy controls scored significantly higher on the minimization and denial scale compared to depressed patients [Table 6.2].

Table 6.2 Individual childhood trauma sub-type scores in depressed patients and healthy controls.

CTQ subscales	Controls (n=57)	MDD (n=66)	Statistics
Emotional abuse	5.39 (2.16)	10.98 (5.30)	Z=-5.345, $p<0.001$ ***
Physical abuse	5.75 (1.55)	7.71 (3.96)	Z=-2.829, $p=0.005$ **
Sexual abuse	5.63 (2.78)	7.00 (5.83)	Z=-1.192, $p=0.223$
Emotional neglect	6.82 (2.21)	11.86 (4.84)	Z=-5.506, $p<0.001$ ***
Physical neglect	5.54 (1.45)	7.14 (2.53)	Z=-4.500, $p<0.001$ ***
Minimization/denial	0.68 (0.99)	0.13 (0.34)	Z=-2.843, $p=0.004$ **
CTQ Global Score	30.14 (7.49)	44.67 (16.44)	Z=-5.418, $p<0.001$ ***

Data expressed as mean with SD in parentheses. Scores measured using the 28-item CTQ. Statistical analysis was performed using a Mann-Whitney U test. ** $p\leq 0.01$ vs healthy controls; *** $p\leq 0.001$ vs healthy controls.

6.3.1.2 Demographic and clinical data for depressed patients with ELA and without ELA

Of the depressed patients recruited to the study that completed the childhood trauma questionnaire, scores indicated that 45 had experienced early life adversity (ELA) and 23 had not. Meanwhile, only 11 healthy controls had experienced early life adversity while 46 had not. Patients with MDD who had experienced ELA and those who had not experienced ELA exhibited no differences between age, gender, BMI, smoking, or education [Table 6.3]. Analysis also revealed no differences in HAM-D scores or CES-D scores [Table 6.3], indicating no difference in depression symptomatology between depressed patients with and without ELA [Table 6.3]. These results indicate that the demographics of depressed patients with and without ELA are comparable. Additionally, the data suggests that patients who have experienced ELA did not have greater severity of depression symptoms or sleep disruption. However, HAM-D scores were significantly correlated to CTQ global scores within the depressed patient group ($r=0.278$, $p=0.028$), indicating that a relationship between trauma and depression severity exists.

Table 6.3 Demographic and clinical data for depressed patients with and without ELA.

	MDD with ELA (n=45)	MDD without ELA (n=23)	Statistics (p-value)
Age (years)	28.11 (8.93)	27.61 (7.98)	$Z=-0.169$, $p=0.866$
Gender (female/male)	29/16	16/7	$\chi^2=0.178$, $p=0.673$
BMI	24.22 (6.20)	25.01 (5.24)	$Z=-1.018$, $p=0.308$
Smoking (Yes/No)	21/24	6/17	$\chi^2=2.693$, $p=0.101$
ISCED Education	3.45 (1.43)	3.40 (1.57)	$Z=-0.452$, $p=0.651$
HAM-D	23.02 (5.72)	22.26 (3.03)	$Z=-0.899$, $p=0.369$
CES-D	40.30 (9.69)	38.27 (8.80)	$Z=-0.919$, $p=0.358$
PSQI	13.58 (3.35)	12.02 (3.82)	$Z=-1.663$, $p=0.096$
Antidepressant (Yes/No)	28/17	11/12	$\chi^2=1.290$, $p=0.305$

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

6.3.1.3 Inflammation differences between MDD patients with and without ELA

Analysis revealed significant differences in circulating IL-1 β and whole blood transcriptional expression of IL-1 β between patients with and without early life adversity. Circulating plasma IL-1 β was significantly higher in patients who had experienced early life adversity than those who had not ($t=-2.282$, $p=0.026$) [Figure 6.1a]. Similarly, mRNA expression of IL-1 β was significantly higher in patients who had experienced early life adversity than those who had not ($t=-2.204$, $p=0.032$) [Figure 6.1b]. In contrast, there were no significant differences detected between expression of other inflammatory markers such as CRP, IL-6, IFN- γ , or TNF- α between ELA and no ELA depressed patients.

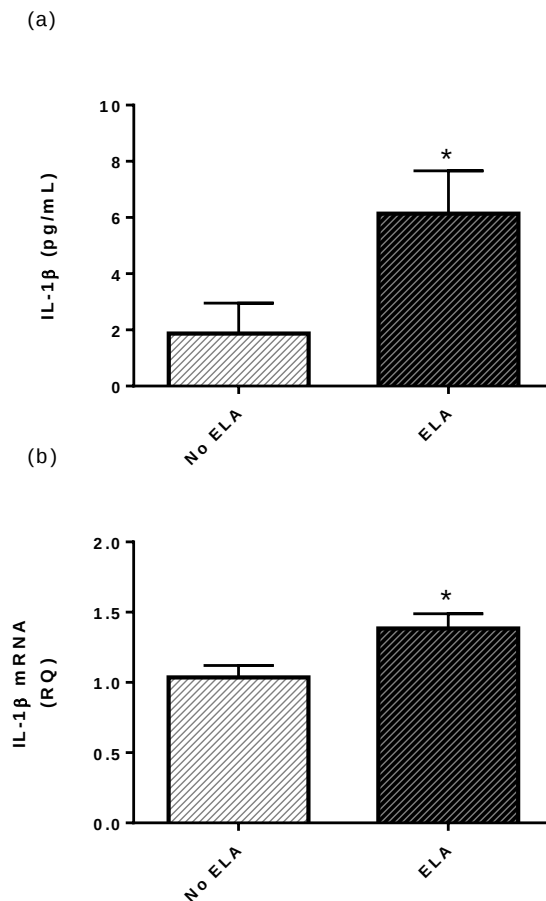


Figure 6.1 Circulating concentration and mRNA expression of IL-1 β in MDD patients with and without ELA. Data expressed as mean and SEM. (a) Plasma concentrations of IL-1 β in depressed patients with ELA ($n=39$) compared to patients without ELA ($n=17$) and (b) normalised mRNA expression of IL-1 β in depressed patients with ELA ($n=36$) compared to patients without ELA ($n=18$). (Independent samples t-test). * $p\leq 0.05$ vs no ELA.

6.3.1.4 Hippocampal volume differences between MDD patients with and without ELA

A univariate analysis of variance general linear model (GLM) using age, gender, and total intracranial volume (TIV) as covariates revealed differences in hippocampal subfield volumes between depressed patients with and without ELA. There was no significant difference in right CA3 volume between groups ($F=0.986$, $p=0.327$) [Figure 6.2a], but the left CA3 volume was significantly higher in patients who had experienced ELA ($F=8.244$, $p=0.007$) [Figure 6.2b].

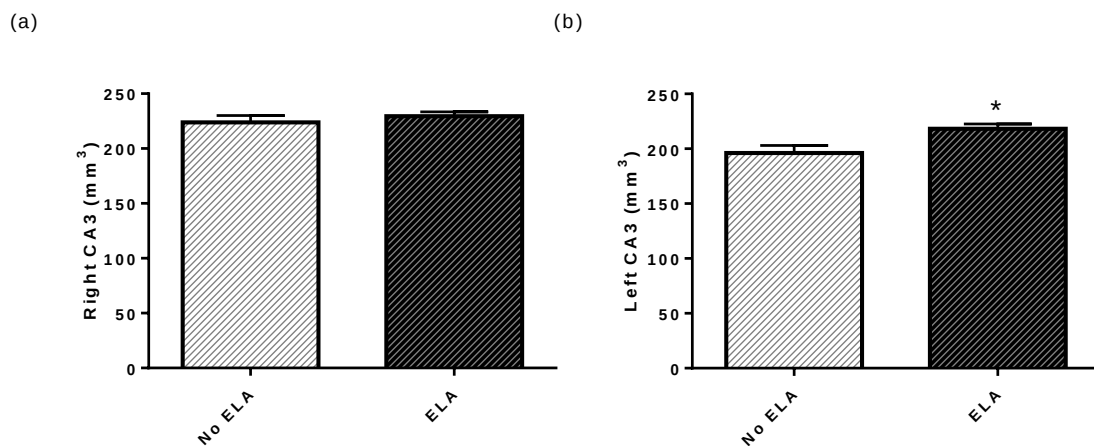


Figure 6.2 Left and right hippocampal CA3 volumes in MDD patients with and without ELA.

Data displayed as means and SEM. Hippocampal CA3 volumes in patients with ELA ($n=30$) compared to patients without ELA ($n=15$). (Univariate GLM with age, gender, and TIV as covariates). * $p < 0.05$ compared to MDD patients without ELA.

6.3.2 Relationship between HAM-D symptom cluster subscales and biological measures in MDD patients

6.3.2.1 HAM-D symptom cluster subscale scores in depressed patients

Symptom cluster subscale scores were calculated for MDD patients from the 21 question HAM-D scale. The distribution of subscale scores amongst depressed patients is depicted in Table 6.4.

Table 6.4 Symptom cluster subscales in depressed patients.

Symptom cluster (range of possible scores)	Maximum	Minimum	Mean	Standard deviation
Core depression (0-12)	8.00	1.00	4.78	1.71
Sleep difficulty (0-6)	6.00	0.00	3.66	1.91
Anxiety symptoms (0-16)	11.00	2.00	6.87	1.97
Somatic symptoms (0-8)	6.00	0.00	3.14	1.55
Anxiety/Somatisation factor (0-18)	12.00	2.00	7.81	1.97
HAM-D Suicidality (0-4)	4.00	0.00	1.66	1.45
MINI Suicidality (0-52)	52.00	0.00	12.11	13.91

Data derived from HAM-D scores of depressed patients (n=70) and MINI module C (n=55).

6.3.2.2 Relationship between core depression scores and biological measures in MDD patients

A Spearman's rho correlational analysis revealed several significant relationships between core depression scores and biological markers in patients with MDD. Core depression scores were significantly negatively correlated to whole blood mRNA expression of SLC6A4 ($r=-0.386$, $p=0.012$) [Figure 6.3a], indicated that decreased serotonin transporter expression is related to an increase in core depression symptoms. Core depression symptoms were also positively correlated to whole blood transcriptional expression of IFN- γ ($r=0.315$, $p=0.029$) and IDO1 ($r=0.336$, $p=0.015$), demonstrating increased inflammation and IDO1 activity as core depression symptoms increase [Figure 6.3b, c]. Additionally, core depression symptoms were negatively correlated to kynurenic acid/kynurenine ratio values ($r=-0.309$, $p=0.023$) [Figure 6.3d]. In contrast, other inflammatory markers were not found to be significantly positively correlated to core depression symptoms.

6.3.2.3 Relationship between insomnia and sleep disturbance symptom cluster and biological markers in MDD patients

A Spearman's rho correlational analysis revealed several significant relationships between insomnia and sleep disturbance symptom cluster scores calculated from the 21 question HAM-D in depressed patients. First, insomnia and sleep disturbance symptom cluster scores were significantly positively correlated to whole blood mRNA expression of SLC6A4 ($r=0.380$, $p=0.013$) [Figure 6.4]. Also, a partial correlation correcting for age, gender, and TIV revealed that sleep disturbance symptom cluster scores were significantly positively correlated to left ($r=0.323$, $p=0.045$) and right ($r=0.328$, $p=0.041$) hippocampal subiculum subfields, and significantly positively correlated to the whole right hippocampal volume ($r=0.377$, $p=0.018$) [Figure 6.5a-c].

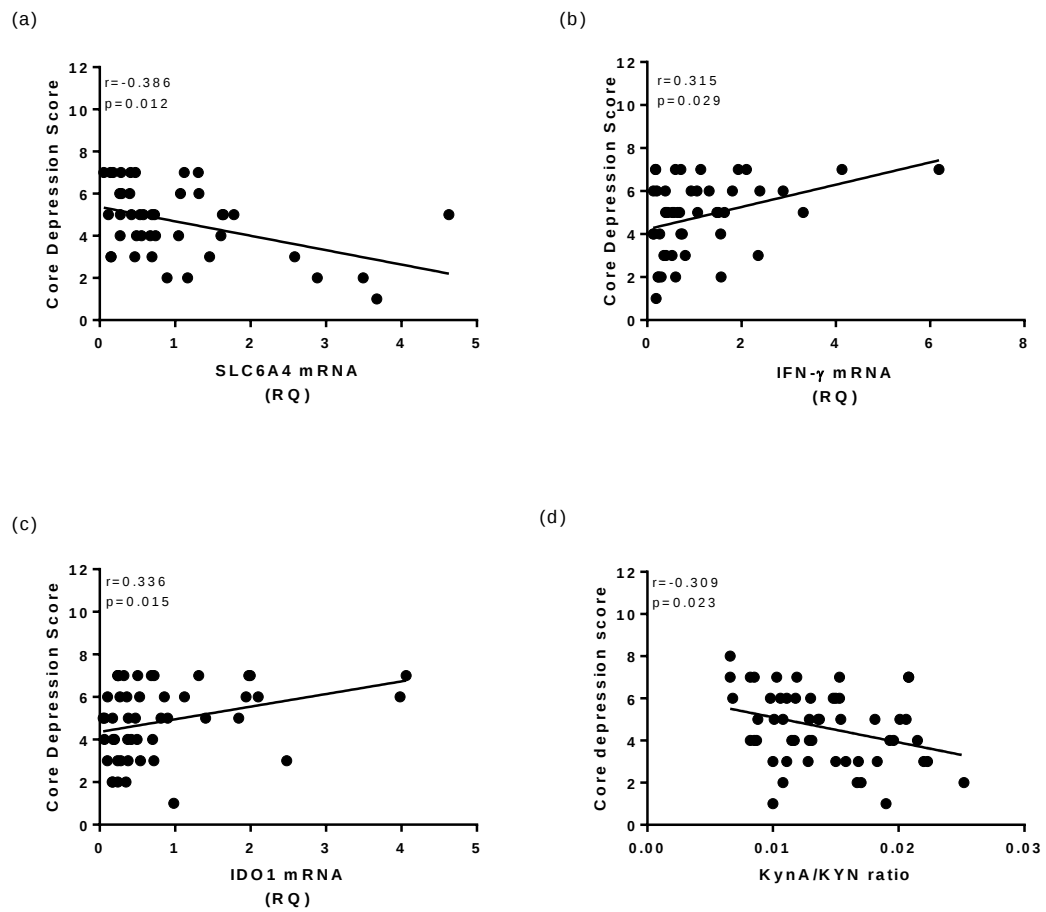


Figure 6.3 Whole blood mRNA expression associated with core depression subscale in MDD patients. Core depression subscale scores were (a) significantly negatively correlated to whole blood mRNA expression of SLC6A4, (b) significantly positively correlated to whole blood mRNA expression of IFN- γ , and (c) significantly positively correlated to whole blood mRNA expression of IDO1 in whole blood. Core depression score was (d) significantly negatively correlated to kynurenic acid/kynurenine ratio. Correlational analysis was conducted with a Spearman's rho analysis.

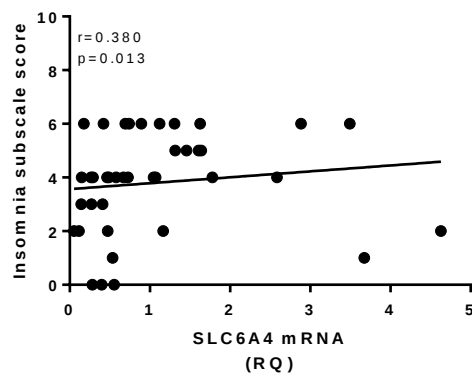
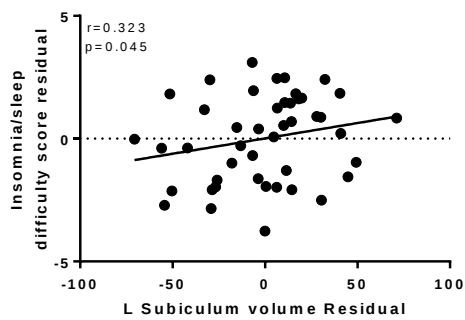
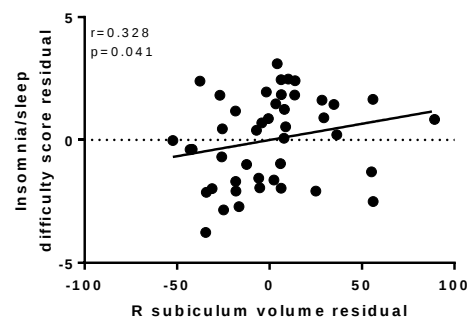


Figure 6.4 Relationship between insomnia subscale and whole blood mRNA expression of SLC6A4. A significant positive relationship exists between HAM-D insomnia subscale scores and transcriptional expression of SLC6A4. (Spearman's rho correlation).

(a)



(b)



(c)

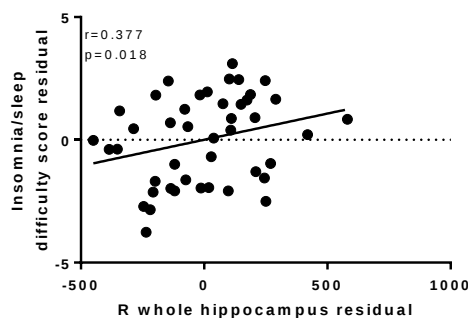


Figure 6.5 Relationships between insomnia subscale scores and hippocampal subfield volumes. A significant positive correlation exists between insomnia subscales scores and (a) left subiculum volume and (b) right subiculum volume. A significant positive correlation exists between (c) insomnia subscale scores and whole right hippocampal volume. (Partial correlation using age, gender, and TIV as covariates).

6.3.2.4 Relationship between anxiety symptom cluster and biological markers in MDD patients

Correlational analysis revealed a significant positive relationship between anxiety symptom cluster scores and circulating TNF- α concentrations ($r=0.274$, $p=0.041$) [Figure 6.6a] in depressed participants. A significant negative relationship between anxiety subscale score and transcriptional expression of KMO was also determined ($r=-0.299$, $p=0.032$) [Figure 6.6b]. Spearman's rho analysis also revealed a significant positive correlation between kynurenic acid/quinolinic acid ratio and anxiety symptom cluster score ($r=0.305$, $p=0.019$) [Figure 6.6c].

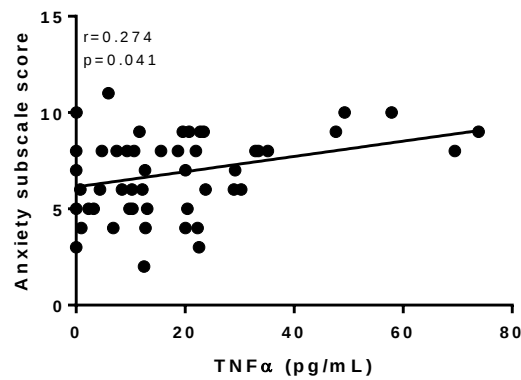
6.3.2.5 Relationship between somatic or vegetative symptom cluster and biological markers in MDD patients

A partial correlation correcting for age, gender, and TIV indicated a trend towards a significant negative relationship between somatic/vegetative HAM-D subscale scores and left hippocampal CA3 volume of depressed patients ($r=-0.309$, $p=0.055$). No significant relationships exist between somatic/vegetative subscale scores and any of the circulating cytokine concentrations, whole blood mRNA expression, or kynurenine pathway measurements.

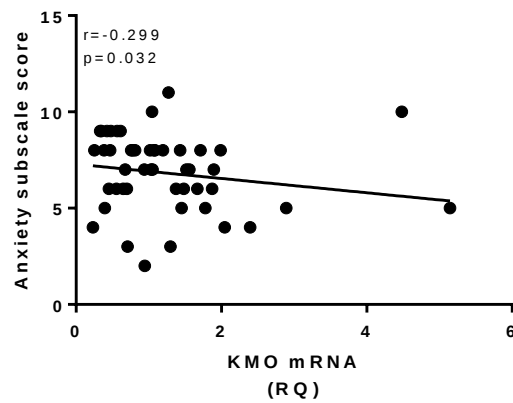
6.3.2.6 Relationship between anxiety/somatisation factor and biological markers in MDD patients

A Spearman's rho correlation revealed a significant negative relationship between anxiety/somatisation factor score and whole blood transcriptional expression of KMO ($r=-0.299$, $p=0.032$) [Figure 6.8a]. Anxiety/somatisation factor score was also positively correlated to kynurenic acid/quinolinic acid ratio ($r=0.279$, $p=0.032$) [Figure 6.8b].

(a)



(b)



(c)

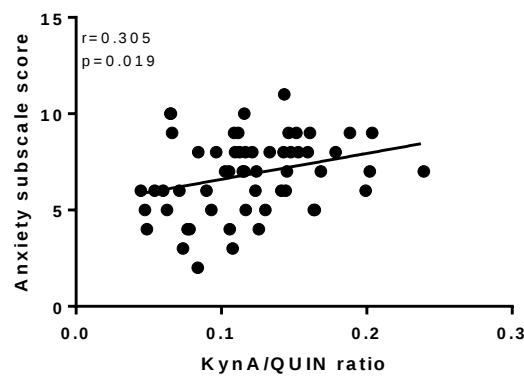


Figure 6.6 Significant relationships between anxiety subscale scores and biological measures. (a) The anxiety subscale of the HAM-D was significantly positively correlated to circulating concentration of TNF- α (pg/ml). (b) A significant negative correlation exists between anxiety subscale score and transcriptional expression of KMO mRNA, and (c) a significant positive correlation exists between anxiety subscale score and kynurenic acid/quinolinic acid ratio. (Spearman's rho correlation).

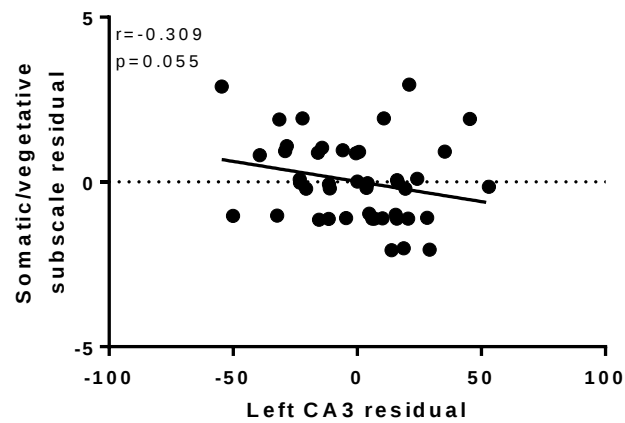
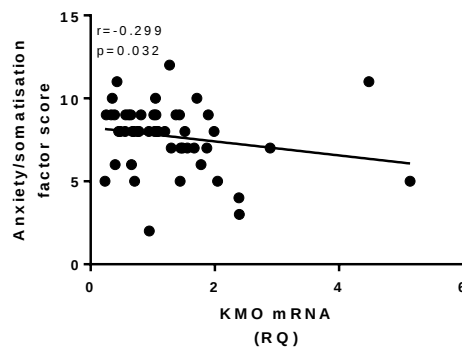


Figure 6.7 Relationship between somatic/vegetative symptom cluster score and left CA3 volume. A non-significant negative correlation exists between HAM-D somatic/vegetative subscale scores and left hippocampal CA3 volume (mm³). (Partial correlation correcting for age, gender, and TIV).

(a)



(b)

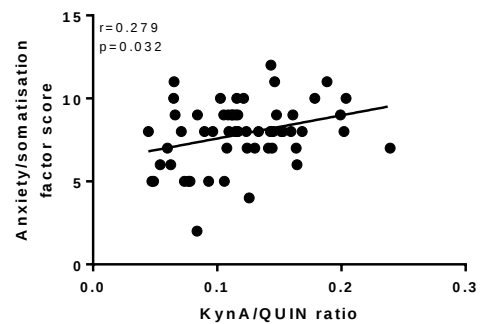


Figure 6.8 Significant relationships between anxiety/somatisation factor and biological markers. (a) A significant negative relationship exists between HAM-D anxiety/somatisation factor scores and whole blood transcriptional expression of KMO. (b) A significant positive relationship exists between HAM-D anxiety/somatisation factor scores and kynurenic acid/quinolinic acid ratio. (Spearman's rho correlation).

6.3.2.7 Relationship between HAM-D suicidality scores and biological measures in MDD patients

Several significant relationships were revealed when a Spearman's rho correlational analysis was carried out between HAM-D suicidality scores and biological measures in depressed patients. Ordinal data such as suicidality scores can be assessed with a Spearman's rho correlation since this test is valid in correlating ordinal data. A significant positive relationship exists between suicidality scores and cortisol reactivity ($r=0.311$, $p=0.040$) [Figure 6.9a], indicating an association between increased suicidality and increased reactivity of the HPA axis. Suicidality scores were shown to be significantly negatively correlated to whole blood transcriptional expression of IL-1 β ($r=0.377$, $p=0.006$) [Figure 6.9b]. Transcriptional expression of KAT1 was found to be significantly negatively correlated to suicidality score ($r=0.454$, $p=0.001$) [Figure 6.9c]. Finally, correlational analysis revealed a significant positive relationship between kynurenine concentration and suicidality scores ($r=0.334$, $p=0.014$) [Figure 6.9d]. These results may indicate specific alterations in the HPA axis, inflammatory markers, and kynurenine pathway in association with suicidality within a diagnosis of MDD.

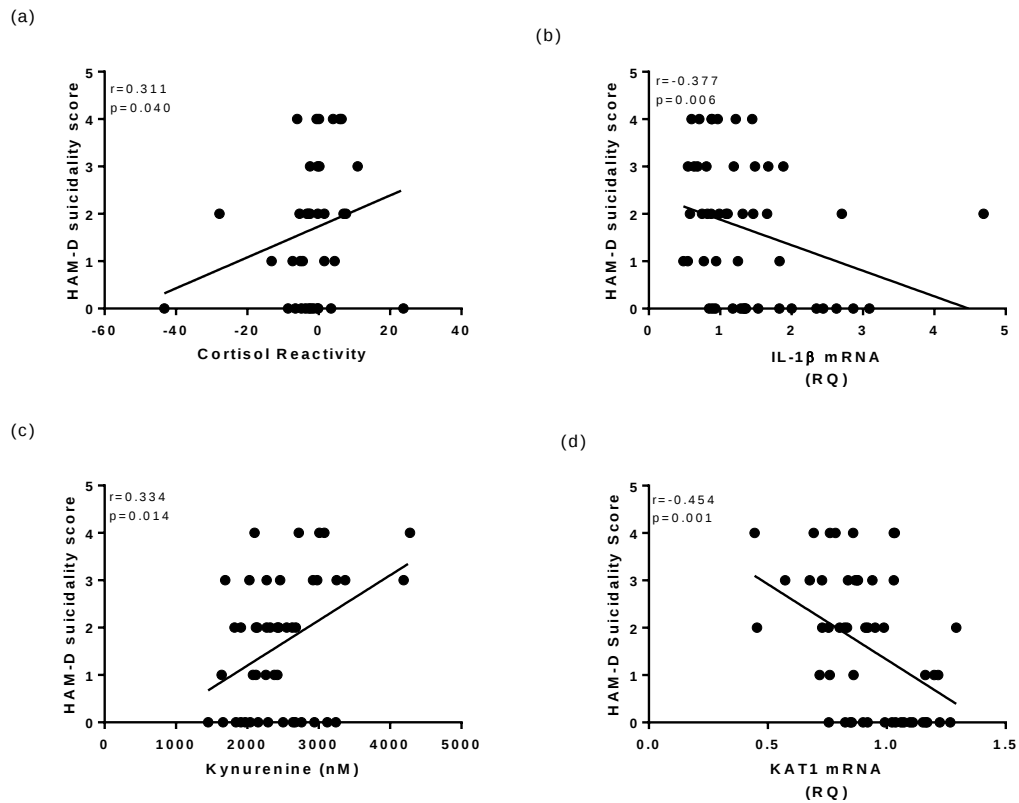


Figure 6.9 Correlational analysis between HAM-D suicidality score and biological measures in MDD patients. The HAM-D suicidality score was found to be (a) significantly positively correlated to cortisol reactivity in MDD patients and (b) significantly negatively correlated to mRNA expression of IL-1 β , (c) significantly positively correlated to kynurenine concentration, and (d) significantly negatively correlated to KAT1 mRNA expression. (Spearman's rho correlation).

6.3.3 Atypical Depression

6.3.3.1 Demographic and clinical data for depressed patients with and without atypical subtype

Depressed patients were assessed for symptoms that define the atypical subtype of MDD. Fourteen patients were found to meet the criteria for atypical depression. These patients were found to have no significant differences in age, gender, BMI, education, or smoking compared to depressed patients without atypical classification [Table 6.5]. There were also no differences in HAM-D, CES-D, PSQI, and CTQ global scores between atypical and non-atypical depressed patients [Table 6.5]. However, a Chi squared analysis revealed a significant difference in the number of participants who experienced ELA or not between groups (Atypical: 21.43% vs Not Atypical: 73.68%), indicating that the atypical group had a much lower rate of ELA than the non-atypical depressed group.

Table 6.5 Demographic and clinical data for MDD patients with and without atypical subtype. Depicted are demographic variables and several clinical measures for depressive symptoms, sleep, and childhood trauma.

	Atypical (n=14)	Not atypical (n=57)	Statistics (p-value)
Age (years)	25.57 (6.85)	28.19 (8.81)	Z=-0.927, p=0.354
Gender (female/male)	10/4	37/20	$\chi^2=0.213$, p=0.644
BMI	25.40 (6.67)	24.23 (5.61)	Z=-0.578, p=0.563
ISCED Education	3.62 (1.39)	3.37 (1.47)	Z=-0.710, p=0.478
Smoking (Yes/No)	5/9	24/33	$\chi^2=0.190$, p=0.663
HAM-D	23.36 (2.34)	22.81 (5.49)	Z=-0.523, p=0.601
CES-D	37.43 (7.65)	39.65 (9.81)	Z=-0.979, p=0.328
PSQI	11.75 (4.07)	13.51 (3.35)	Z=-1.323, p=0.186
CTQ Global Score	42.62 (15.91)	45.17 (16.68)	Z=-0.484, p=0.628
Early Life Adversity (Yes/No)	3/11	42/15	$\chi^2=13.339$, p<0.001***
Antidepressant (Yes/No)	5/9	36/21	

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Mann Whitney U test (Age, BMI, Education, HAM-D, CES-D, PSQI, CTQ Global Score), and Chi squared (χ^2) test (Gender, Smoking, ELA).

6.3.3.2 Altered cortisol awakening response in atypical depression subtype

A Mann-Whitney U test conducted on log transformed cortisol data revealed several significant differences in HPA axis parameters between patients with atypical depression and those without. The atypical group had significantly lower wakening salivary cortisol concentrations compared to the non-atypical group ($Z=-2.145$, $p=0.031$) [Figure 6.10]. They also had significantly lower salivary cortisol concentrations at T60 ($Z=-2.513$, $p=0.011$) [Figure 6.10]. In accordance with those findings, the average morning cortisol concentration of patients with atypical subtype was significantly lower than the non-atypical group ($Z=-2.786$, $p=0.005$). Lastly, the AUCg of the atypical group was significantly lower than that of the non-atypical depressed group ($Z=-2.050$, $p=0.0040$). These results strongly indicate differing activation of the HPA axis between atypical and non-atypical depression.

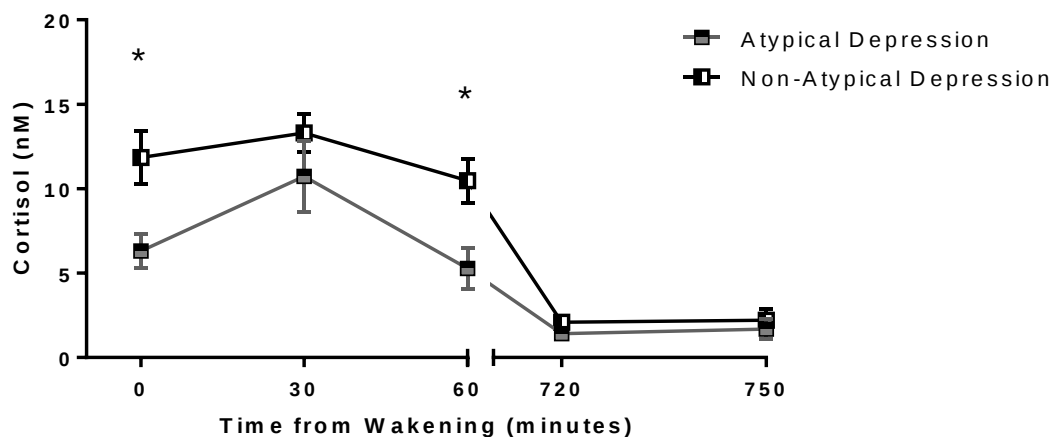


Figure 6.10 Differences in cortisol awakening response between atypical and non-atypical MDD patients. Data expressed as mean and SEM. Salivary cortisol concentrations at five time points from waking in depressed patients with atypical subtype ($n=9$) and non-atypical depression ($n=43$). Atypical depressed patients exhibited significantly lower cortisol concentrations at waking and at 60 minutes from waking. (Mann-Whitney U test). * $p\leq 0.05$ vs non-atypical MDD patients.

6.3.3.3 Alterations in kynurenine and QUIN/KYN ratio in atypical depression subtype

A Mann-Whitney U test revealed significant differences in KP expression between depressed patients with atypical subtype and those without. Kynurenine concentrations were significantly higher in patients with atypical depression compared to those without ($Z=-2.083$, $p=0.037$) [Figure 6.11a]. There were also significantly lower quinolinic acid/kynurenine ratios in the atypical subtype compared to non-atypical depressives ($Z=-2.071$, $p=0.038$) [Figure 6.11b].

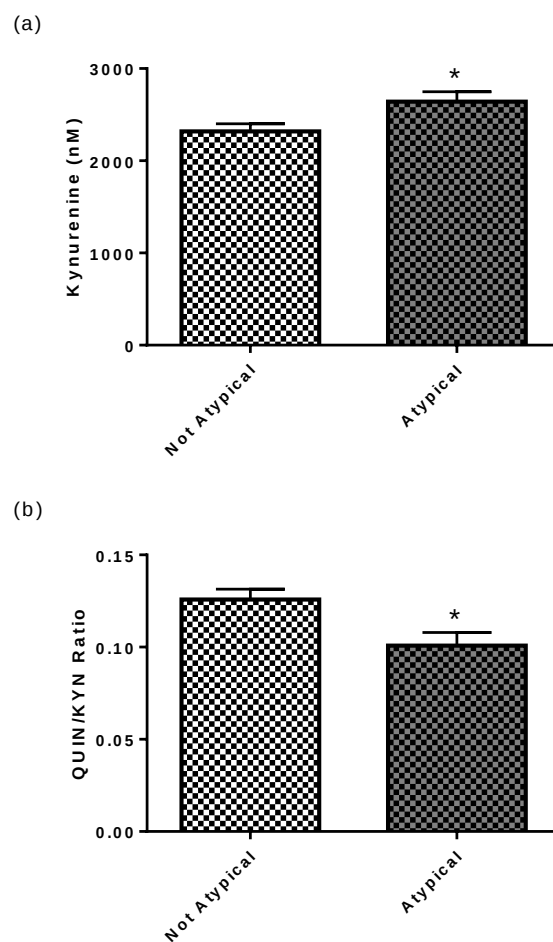


Figure 6.11 Kynurenine pathway differences in atypical depression subtype compared to non-atypical MDD. Data depicted as means and SEM. (a) Circulating kynurenine in patients with the atypical subtype ($n=10$) and non-atypical depressed patients ($n=44$). (b) Quinolinic acid to kynurenine ratio in patients with Atypical subtype ($n=10$) and without ($n=44$). (Mann-Whitney U test). * $p \leq 0.05$ vs non-atypical MDD patients.

6.3.4 First Presentation versus Recurrent MDD

6.3.4.1 Demographic and clinical data for patients with first presentation depression, recurrent depression and healthy controls

Patients recruited to the study were considered either “first presentation” or “recurrent” MDD patients. Of the depressed patients, 48 were considered first presentation depressed (FPD), while 24 were considered recurrently depressed (RD). There was a significant difference in age between groups [Table 6.6]. There was also a difference between the gender of each group, with the RD group having a much greater percentage of females compared to FPD (FPD: 58.33% vs. RD: 83.33%) [Table 6.6]. Analysis revealed a significantly higher BMI and higher level of education in RD compared to FPD. There were significantly higher HAM-D, CES-D, PSQI, and CTQ global scores in both depressed groups compared to healthy control, and the RD group exhibited higher CTQ global scores than the FPD group [Table 6.6].

6.3.4.2 Inflammatory markers in first presentation depression, recurrent depression, and healthy controls

Kruskal-Wallis analysis revealed no significant differences in concentrations of circulating inflammatory cytokines or CRP across healthy control, FPD, and RD groups. However, observing the means of each group reveals that the mean concentration of IL-6 and CRP are highest in the RD group, and lowest in healthy controls [Figure 6.12a and b]. A significantly higher whole blood transcriptional expression of IL-1 β was observed in RD patients compared to FPD patients ($Z=-2.251$, $p=0.024$) [Figure 6.13].

Table 6.6. Demographic data for patients with FPD, RD, and all healthy controls. Depicted are demographic variables and several clinical measures for depressive symptoms, sleep, and childhood trauma.

	HC (n=60)	FPD (n=48)	RD (n=24)	Statistics comparing 3 groups	Statistics comparing FPD and RC
Age (years)	28.02 (5.95)	23.81 (6.35)	35.46 (6.48)	H(2)=39.161, p<0.001***	Z=-5.420, p<0.001***
Gender (female/male)	37/23	28/20	20/4	$\chi^2=4.718$, p=0.095	$\chi^2=4.500$, p=0.034*
BMI	23.32 (3.54)	23.09 (5.45)	26.24 (5.81)	H(2)=5.449, p=0.073	Z=-2.026, p=0.043*
ISCED Education	3.43 (1.43)	2.93 (0.71)	4.30 (1.69)	H(2)=62.453, p<0.001***	Z=-3.485, p<0.001***
Smoking (Yes/No)	12/48	22/26	8/16	$\chi^2=8.234$, p=0.016*	$\chi^2=1.029$, p=0.310
HAM-D	2.76 (2.68)	22.69 (4.55)	23.39 (5.97)	H(2)=94.949, p<0.001***	Z=-0.870, p=0.384
CES-D	6.43 (6.29)	38.48 (9.23)	40.86 (9.83)	H(2)=92.771, p<0.001***	Z=-1.325, p=0.185
PSQI	4.16 (2.49)	12.71 (3.32)	14.10 (3.94)	H(2)=83.867, p<0.001***	Z=-1.594, p=0.111
CTQ Global Score	30.14 (7.49)	41.65 (14.96)	50.30 (17.92)	H(2)=31.846, p<0.001***	Z=-1.967, p=0.049*
Early Life Adversity (Yes/No)	11/46	27/18	18/5	$\chi^2=29.607$, p<0.001***	$\chi^2=2.268$, p=0.132
Medication (free/SSRI/SNRI/other)		24/14/7/3	6/12/2/3		

Data expressed as mean with SD in parentheses. Statistical analysis was performed using a Kruskal Wallis test (Age, BMI, HAM-D, CES-D, PSQI, CTQ Global Score), and Chi squared (χ^2) test (Gender, Smoking, ELA). A Mann-Whitney U test was conducted between FPD and RD groups test (Age, BMI, HAM-D, CES-D, PSQI, CTQ Global Score), and Chi squared (χ^2) test (Gender, Smoking, ELA) between FPD and RD groups. *p<0.05; ***p<0.001.

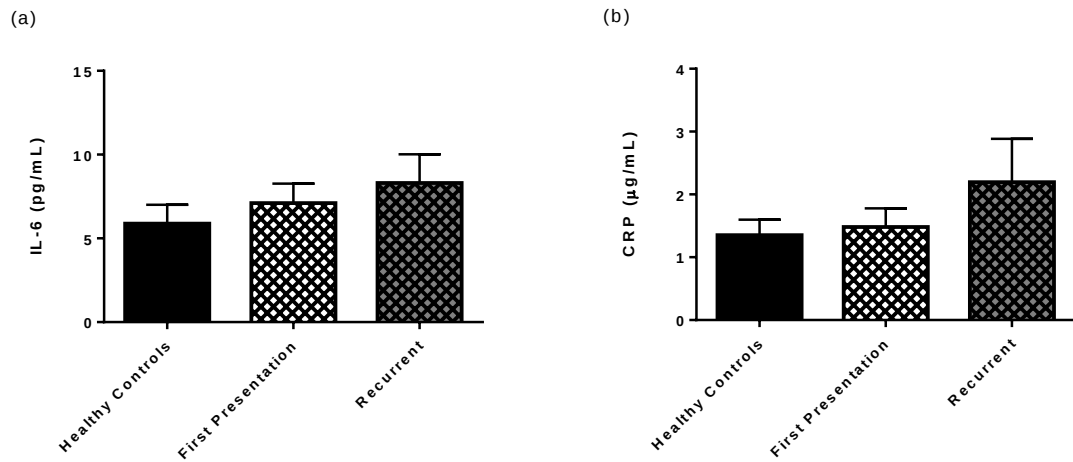


Figure 6.12 Inflammatory responses in healthy controls, first presentation depressives and recurrent depressives. Data are expressed as mean and SEM. Concentrations of circulating plasma (a) IL-6 between HC (n=28), FPD (n=35), and RD (n=18) groups and (b) CRP between HC (n=39), FPD (n=37), and RD (n=18) groups. (Kruskal-Wallis test).

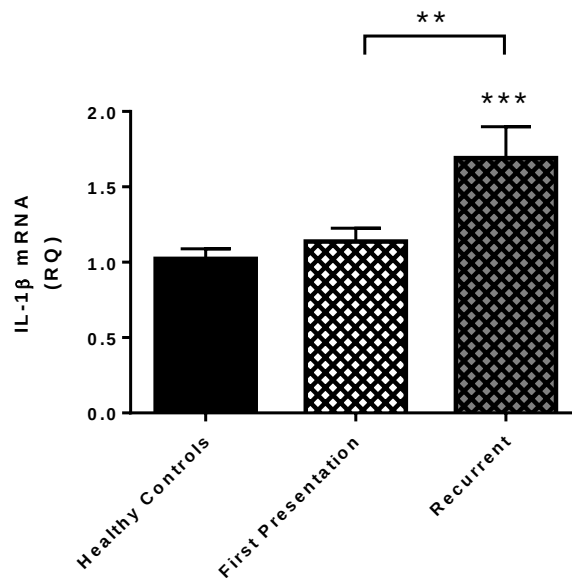


Figure 6.13 Whole blood mRNA expression of IL-1β in healthy controls, first presentation and recurrent depressives. Data are expressed as mean and SEM. Whole blood transcriptional expression of IL-1β in healthy controls (n=38), first presentation (n=33) compared to recurrent depression patients (n=22). Analysis was performed on relative quantification data by Mann Whitney U test. **p<0.01 compared to first presentation MDD patients.

6.3.4.3 Altered circulating kynurenine pathway metabolites in recurrent depression compared to first presentation MDD

A Kruskal-Wallis test between FPD and RD patients and healthy controls revealed significant differences in KP activity across groups. Post-hoc analysis revealed higher concentrations of quinolinic acid in the RD group compared to FPD ($Z=-2.922$, $p=0.003$) [Figure 6.14]. Similarly, the quinolinic acid/kynurenine ratio was significantly higher in RD than in healthy controls or FPD patients ($Z=-2.866$, $p=0.004$ and $Z=-2.117$, $p=0.034$ respectively) [Figure 6.15a], and kynurenic acid/quinolinic acid ratios were significantly lower in the RD group compared to HC ($Z=-2.433$, $p=0.015$) [Figure 6.15b].

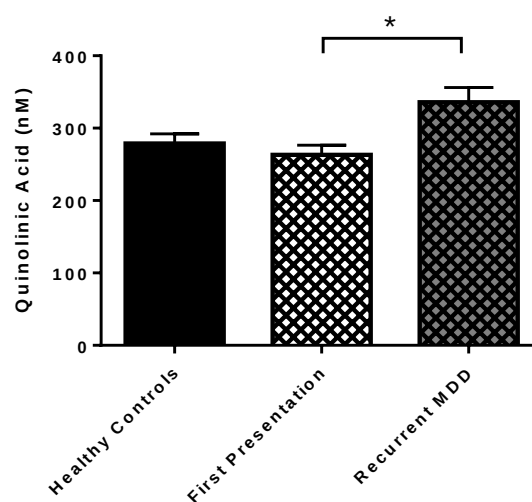


Figure 6.14 Circulating quinolinic acid in healthy controls, first presentation depressed patients and recurrently depressed patients. Data expressed as mean and SEM. Circulating plasma concentration of quinolinic acid in healthy controls ($n=34$), first presentation depressed patients ($n=38$), and recurrently depressed patients ($n=22$). (Kruskal-Wallis test). * $p<0.05$ compared to first presentation MDD patients.

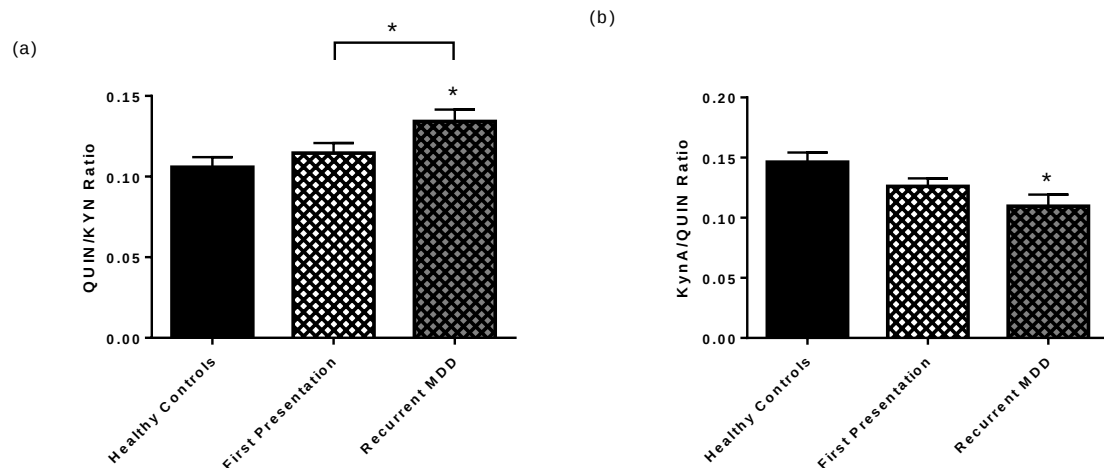


Figure 6.15 Quinolinic acid ratios in healthy controls, first presentation and recurrently depressed patients. Data expressed as mean and SEM. (a) Quinolinic acid/kynurenine ratio and (b) kynurenic acid/quinolinic acid ratio in healthy controls (n=34), first presentation depressed (n=35), and recurrently depressed (n=20) patients. (Kruskal-Wallis test). *p<0.05 compared to healthy controls or first presentation MDD.

6.3.5 Measurement of biological variables in medicated and un-medicated depressed patients

6.3.5.1 Demographic and clinical data for medicated and un-medicated depressed patients

Of the depressed patients recruited to the study, 42 were being treated with medication for their depression, and 30 were un-medicated. Between these groups, analysis revealed anti-depressant treated patients were significantly older than un-medicated patients [Table 6.5]. There were no differences in gender, BMI, education level, or smoking between medicated and un-medicated patients. There were also no differences in HAM-D, CES-D, CTQ global scores, or early life adversity between medicated and un-medicated groups [Table 6.5]. However, the medicated group exhibited significantly higher scores on the PSQI indicating greater sleep disruption in medicated patients than un-medicated patients [Table 6.5].

Table 6.7 Demographic data for medicated and un-medicated MDD patients.

	Anti-depressant treated patients (n=42)	Un-medicated patients with MDD (n=30)	Statistics (p-value)
Age (years)	29.52 (8.30)	25.13 (8.03)	t=-2.243, p=0.028*
Gender (female/male)	29/24	19/11	$\chi^2=0.257$, p=0.612
BMI	24.79 (5.57)	23.93 (6.21)	t=-0.499, p=0.620
ISCED Education	3.54 (1.30)	3.27 (1.61)	t=-0.737, p=0.464
Smoking (Yes/No)	18/24	12/18	$\chi^2=1.290$, p=0.256
HAM-D	23.34 (5.73)	22.33 (3.87)	t=-0.834, p=0.407
CES-D	40.54 (9.84)	37.47 (8.66)	t=-1.353, p=0.181
PSQI	14.23 (3.19)	11.80 (3.57)	t=-2.941, p=0.005**
CTQ Global Score	45.65 (16.77)	43.41 (16.23)	t=-0.545, p=0.588
Early Life Adversity (Yes/No)	28/11	17/12	$\chi^2=1.290$, p=0.256

Data expressed as mean with SD in parentheses. Statistical analysis was performed using an independent samples t-test (Age, BMI, HAM-D, CES-D, PSQI, CTQ Global Score), and Chi squared (χ^2) test (Gender, Smoking, ELA).

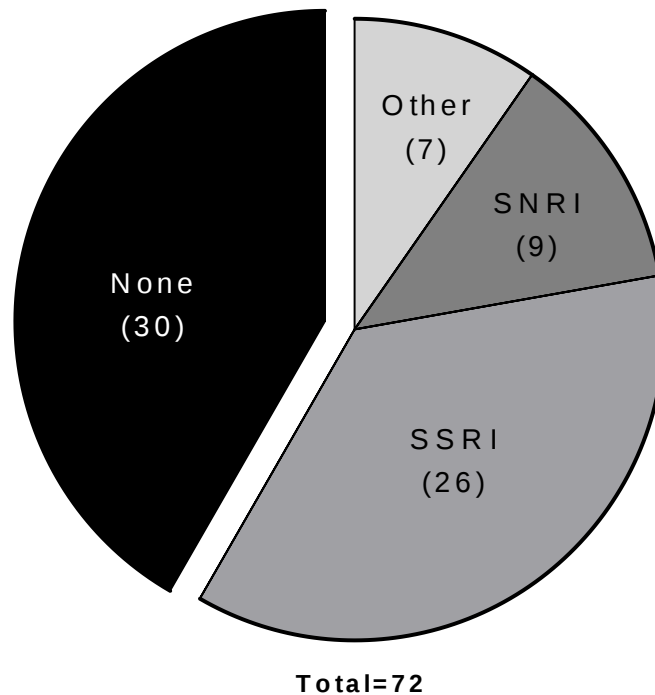


Figure 6.16 Medication use in depressed patients. Pie chart indicating types of antidepressant medication and number of depressed patients using each. The “other” medication category includes benzodiazepines, melatonergics, anti-epileptics or a combination of medications.

6.3.5.2 Biological variables in medicated and un-medicated depressed patients

A Mann-Whitney U test was carried out to determine any differences between expression of biological variables between medicated and un-medicated depressed patients. Kynurenic acid concentration was lower in the medicated group compared to the un-medicated group [Figure 6.17] [Table 6.6]. In tandem with the decrease in kynurenic acid concentration, the medicated group expressed a significantly lower kynurenic acid/kynurenine ratio and significantly lower kynurenic acid/quinolinic acid ratio compared to un-medicated depressed patients [Figure 6.18a, b] [Table 6.6]. No differences were found in whole blood mRNA expression of KP enzymes between groups.

While there was no difference in concentrations of circulating cytokines between groups, there was a higher concentration of circulating CRP in the medicated group compared to the

un-medicated group [Figure 6.19] [Table 6.6]. Analysis also revealed no differences in whole blood transcriptional expression of cytokines including IFN- γ , IL-1 β , or TNF- α .

Analysis revealed no difference in morning cortisol concentrations or CAR parameters between medicated and un-medicated depressed patients [Table 6.6]. However, medicated depressed patients exhibited lower average PM cortisol concentrations compared to un-medicated patients [Figure 6.20].

Table 6.8 Analysis of biological variables in medicated and un-medicated depressed patients.

	Anti-depressant treated patients	Un-medicated patients with MDD	Statistics (p-value)
Tryptophan (nM)	45400.00 (1640.85)	50869.57 (2479.09)	Z=-1.665, p=0.096
Kynurenine (nM)	2416.56 (134.76)	2538.26 (134.76)	Z=-0.777, p=0.437
3-HK (nM)	34.35 (1.90)	34.59 (3.03)	Z=-0.750, p=0.453
Quinolinic acid (nM)	288.68 (16.54)	291.52 (17.21)	Z=-0.357, p=0.721
Kynurenic acid (nM)	30.38 (2.05)	37.35 (2.00)	Z=-2.547, p=0.011*
KYN/TRP ratio	0.0541 (0.0028)	0.0508 (0.0022)	Z=-0.563, p=0.573
QUIN/KYN ratio	0.1235 (0.0067)	0.1193 (0.0072)	Z=-0.171, p=0.865
KynA/KYN ratio	0.0130 (0.0008)	0.0154 (0.0009)	Z=-2.065, p=0.039*
KynA/QUIN ratio	0.1109 (0.0068)	0.1347 (0.0090)	Z=-2.060, p=0.039*
SLC6A4 (RQ)	1.18 (0.22)	0.95 (0.29)	Z=-1.311, p=0.190
IDO1 (RQ)	0.65 (0.14)	0.96 (0.22)	Z=-1.664, p=0.096
IDO2 (RQ)	1.16 (0.25)	1.58 (0.45)	Z=-0.865, p=0.387
KMO (RQ)	1.25 (0.18)	1.12 (0.14)	Z=-0.283, p=0.777
Kynureninase (RQ)	1.19 (0.28)	1.24 (0.21)	Z=-1.295, p=0.195
KAT1 (RQ)	0.90 (0.04)	0.93 (0.03)	Z=-0.336, p=0.737
IFN- γ (ng/ml)	11.86 (2.89)	13.09 (3.96)	Z=-0.193, p=0.847
IL-1 β (ng/ml)	4.05 (1.08)	6.94 (2.38)	Z=-0.629, p=0.530
IL-6 (ng/ml)	8.43 (1.27)	6.11 (1.44)	Z=-1.282, p=0.200
TNF α (ng/ml)	15.66 (2.13)	18.55 (4.80)	Z=-0.378, p=0.706
CRP (μ g/ml)	2.26 (0.047)	0.95 (0.25)	Z=-1.282, p=0.041*
IFN- γ (RQ)	1.74 (0.93)	2.10 (0.93)	Z=-0.400, p=0.689
IL-1 β (RQ)	1.49 (0.15)	1.11 (0.10)	Z=-1.310, p=0.190
TNF- α (RQ)	0.91 (0.06)	0.87 (0.05)	Z=-0.202, p=0.840
Cortisol T0 (nM)	10.40 (1.47)	11.54 (2.38)	Z=-0.220, p=0.826
Cortisol T30 (nM)	12.02 (1.14)	14.35 (1.94)	Z=-0.559, p=0.576
Cortisol T60 (nM)	9.48 (1.65)	9.71 (1.51)	Z=-0.029, p=0.977
Cortisol T720 (nM)	1.72 (0.30)	3.79 (1.53)	Z=-1.820, p=0.072
Cortisol T750(nM)	1.43 (0.36)	3.08 (1.27)	Z=-1.777, p=0.082
Cortisol AM Ave (nM)	11.41 (1.48)	11.52 (1.35)	Z=-0.296, p=0.767
Cortisol PM Ave (nM)	1.57 (0.25)	3.48 (1.27)	Z=-1.434, p=0.015*
Cortisol AUCg	643.51 (70.73)	733.22 (101.06)	Z=-0.225, p=0.822
Cortisol AUCi	48.50 (54.93)	2.68 (129.90)	Z=-0.211, p=0.833
Cortisol PK (nM)	14.62 (1.52)	16.44 (2.43)	Z=-0.157, p=0.875
CAR Reactivity	-1.85 (1.41)	-1.55 (2.64)	Z=-0.176, p=0.860
CAR Slope	-0.045 (0.05)	-0.035 (0.02)	Z=-0.249, p=0.803
CAR Intercept	11.55 (1.30)	13.67 (2.32)	Z=-0.391, p=0.696
HSD11 β 1 (RQ)	1.00 (0.18)	0.96 (0.23)	Z=-0.070, p=0.944

Data expressed as means with SEM in parenthesis. Statistical analysis was performed using a Mann-Whitney U test. Cortisol data was log transformed for analysis. mRNA expression is represented as relative quantification values.

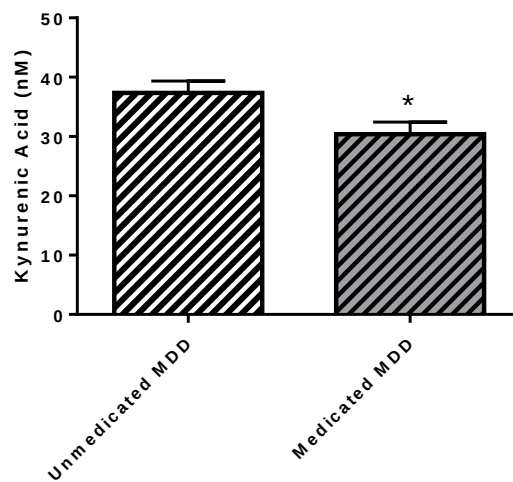


Figure 6.17 Circulating kynurenic acid concentration in medicated and un-medicated MDD patients. Data expressed as mean and SEM. Plasma kynurenic acid concentrations in medicated (n=37) and un-medicated (n=23) depressed patients. (Mann-Whitney U test). *p<0.05 compared to un-medicated MDD patients.

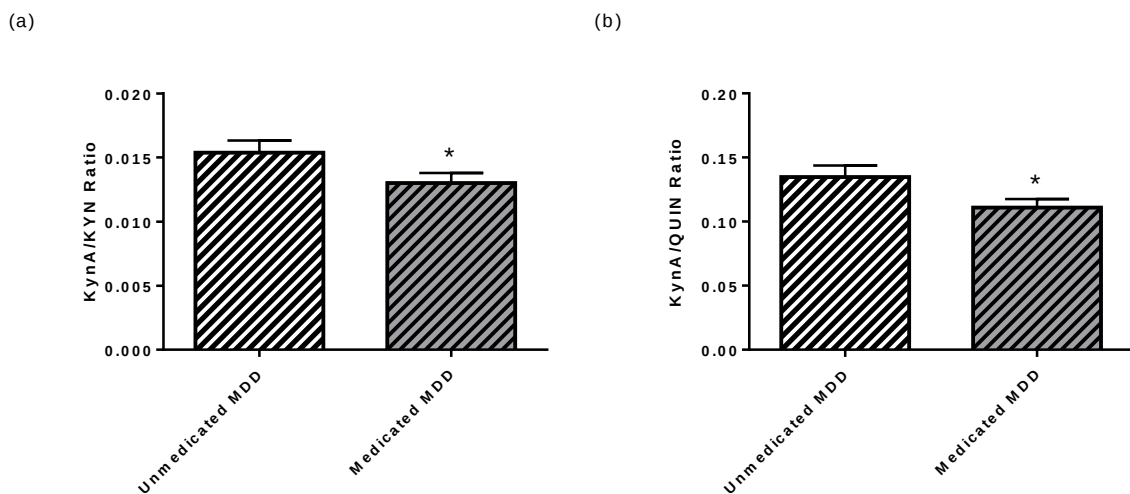


Figure 6.18 Kynurenic acid ratios in medicated and un-medicated MDD patients. Data expressed as mean and SEM. (a) Kynurenic acid/kynurenine concentration ratio and (b) kynurenic acid/quinolinic acid concentration ratio in medicated depressed patients (n=32) compared to un-medicated depressed patients (n=23). (Mann-Whitney U test). *p<0.05 compared to un-medicated MDD patients.

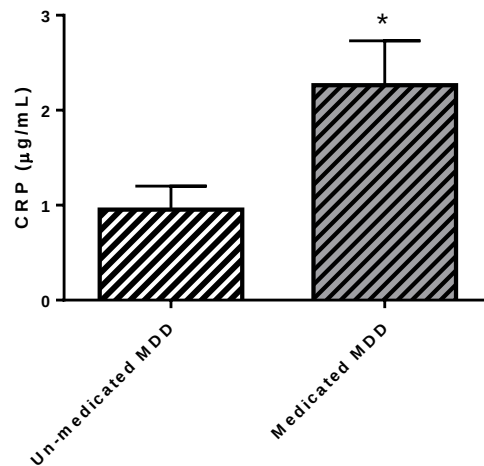


Figure 6.19 Circulating CRP concentrations in anti-depressant medicated MDD patients compared to un-medicated MDD patients. Circulating plasma CRP concentrations in medicated (n=32) and un-medicated depressed patients (n=23). Data are displayed as mean and SEM. (Mann-Whitney U test) * $p \leq 0.05$ vs un-medicated patients.

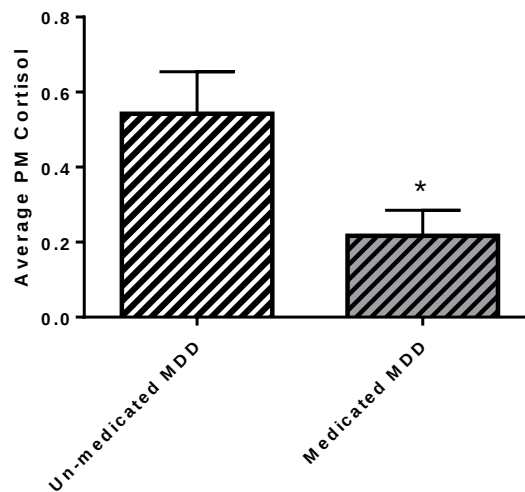


Figure 6.20 Evening salivary cortisol concentrations in anti-depressant medicated and un-medicated MDD patients. Average evening concentrations of salivary cortisol in medicated (n=31) compared to un-medicated (n=22) MDD patients. (Mann-Whitney U test). * $p < 0.05$ compared to un-medicated depressed patients.

6.4 Discussion

Depression is a heterogeneous disorder, as patients may present with low mood accompanied by different or even opposite symptoms. HPA axis, immune system, and kynurenine pathway dysregulation were assessed between healthy controls and depressed patients as a whole in the previous chapters, but the present study dissected the differences in activation of these pathways between depression subtypes. Early life adversity, HAM-D subscale scores, atypical subtype, recurrence, and antidepressant medication use were each examined to gain further insight. Analysis revealed increased measures of inflammation in patients who had experienced early life adversity and higher anxiety scores. Subtypes of depression exhibited differing activation of the kynurenine pathway, too. One of the major findings of this analysis was that recurrently depressed patients exhibited higher quinolinic acid to kynurenine ratio indicative of the neurotoxic branch of the kynurenine pathway compared to controls. First presentation patients showed no differences compared to controls, suggesting that differences in quinolinic acid exist solely in recurrent patients of this cohort. Distinction of first presentation versus recurrent as well as acknowledgement of the atypical depression subtype have not been addressed in research published about kynurenine pathway dysfunction in depression to date. Differences in KP activation were present in the subtypes identified, and future studies should be designed with specific intention to assess differences between these groups. A greater understanding of the unique biological profiles that correspond to specific depression subtypes has the potential to assist in developing antidepressant treatments targeted toward specific subsets of patients.

6.4.1 Biological differences between depressed patients with and without early life adversity

Childhood trauma is known to be a risk factor in the development of depression (Kendler et al., 1998). It is also associated with greater severity of depression (Lu et al., 2008) and higher rates of suicide in depressed patients who are being treated with anti-depressants (Singh et al., 2013). For these reasons, it is essential that the biological differences between patients with and without this risk factor are assessed.

This study showed that depressed patients had experienced more childhood trauma and early life adversity than healthy controls. Depressed patients scored significantly higher in the CTQ categories of emotional and physical abuse, and emotional and physical neglect

[Table 6.2]. However, there was no difference in sexual abuse scores between patients and controls. Studies have shown that childhood trauma is a risk factor in developing depression in adulthood (Felitti et al., 1998; Edwards et al., 2003; Chapman et al., 2004), and results from this study are consistent with this.

Within the depressed group, expression of clinical and biological variables was compared between those who had and had not experienced ELA. There were no differences in clinical measures between depressed patients with and without ELA, suggesting that patients who have experienced ELA did not have significantly greater severity of depression symptoms or sleep disruption than those without ELA [Table 6.3], despite the existence of a significant correlation between HAM-D and CTQ scores of depressed patients. Previous studies have reported that the degree of childhood trauma a depressed patient has experienced is correlated to depressive symptoms (Hayashi et al., 2015), and our findings are in accordance with this.

Analysis of biological variables revealed significantly higher levels of circulating IL-1 β , and significantly higher whole blood transcriptional expression of IL-1 β in the group who had experienced ELA [Figure 6.1]. This adds to the growing evidence base that inflammatory cytokines are elevated in depressed patients with childhood trauma compared to those without trauma (Danese et al., 2007; Carpenter et al., 2010; Lu et al., 2013).

It was also determined that depressed patients with ELA exhibited greater left hippocampal CA3 volumes [Figure 6.2b]. This finding is at odds with the notion that increased inflammatory cytokine activity is associated with decreased hippocampal volumes (Kesler et al., 2013), suggesting that this is an area of research that requires further investigation.

It would be worthwhile to assess these variables in healthy controls with and without ELA, however, there were not enough controls in this study that had experienced ELA to consider this comparison.

6.4.2 Associations between Hamilton depression scale symptom cluster scores and biological variables in depressed patients

Heterogeneity of symptom profiles of patients diagnosed with MDD is evident (Lux and Kendler, 2010; Hybels et al., 2011; O’Keane et al., 2012). For this reason, the present study sought to assess correlations between symptom cluster scores of depressed patients and the biological variables that were measured. Several relationships were revealed.

6.4.2.1 Core depression symptom cluster

Core depression score was negatively correlated to whole blood mRNA expression of SLC6A4 [Figure 6.3a], indicating reduced expression of the serotonin transporter in patients with more severe core depression scores. Core depression score was positively associated with mRNA expression of IFN- γ and IDO1 [Figure 6.3b,c], indicating that severity of core depression scores is related to increased inflammation, and induction of IDO which occurs via inflammation. When observing relationships between core depression and kynurenine pathway parameters, it was determined that the kynurenic acid to kynurenine (KynA/KYN) ratio was negatively associated with symptom severity [Figure 6.3d]. Reduced KynA/KYN ratio in conjunction with increased core depression scores supports a theory of depletion of neuroprotective factors becoming more severe with core depression severity. Increased inflammation and IDO activity have been proposed as a mechanism of kynurenine pathway induction and possibly kynurenic acid reduction (Capuron et al., 2003; Lopresti et al., 2014), therefore these findings give strength to the hypothesis.

6.4.2.2 Insomnia or sleep difficulty symptom cluster

The HAM-D insomnia subscale was found to be positively correlated to SLC6A4 expression [Figure 6.4], implying that participants with less sleep disruption experience greater reduction of serotonin transporter expression. The insomnia subscale scores of depressed patients were also positively correlated to left and right hippocampal subiculum volumes, and right whole hippocampal volumes [Figure 6.5a-c]. These findings suggest that greater hippocampal volume reductions occur in depressed patients without insomnia symptoms. Sleep disruptions including primary insomnia within PTSD patients has not been found to be associated with hippocampal volume loss (Winkelman et al., 2010; Mohlenhoff et al., 2014). Hippocampal volume reduction witnessed in depressed patients in the current study (Chapter 4), is more likely related to depression diagnosis than insomnia due to the repeated findings of brain volume reduction in depression and not insomnia (Grieve, 2013 et al.).

6.4.2.3 Anxiety subscale

Anxiety subscale scores were positively correlated to increased concentrations of circulating TNF- α , decreased mRNA expression of KMO in whole blood, and increased KynA to quinolinic acid ratio (KynA/QUIN) [Figure 6.6a-c]. Increased inflammatory cytokines have

been reported in anxiety disorders (Bankier et al., 2008; Hoge et al., 2009; von Kanel et al., 2007), but the decrease in expression of KMO and increase in KynA/QUIN ratio in conjunction with increased anxiety scores was not an anticipated finding. Since this study found decreased KynA/QUIN ratios in depressed patients compared to healthy controls (Chapter 5), decreased KynA/QUIN ratios would have been anticipated with increased subscales of HAM-D scores, too. This finding suggests that cases of depression with more prevalent anxiety features may be represented by a different form of kynurenine pathway dysfunction than in depressed patients without anxiety symptoms. These findings show that while inflammation is associated with increased anxiety scores, reduction of the kynurenic acid arm and induction of the quinolinic acid arms of the kynurenine pathway are seemingly not a consequence of that inflammation.

6.4.2.4 Somatic or vegetative symptom cluster

Previous studies have argued that the elevated inflammation witnessed in depressed patients is mainly driven by somatic symptoms (Dantzer et al., 2008; Duivis et al., 2013). However, the somatic/vegetative HAM-D subscale scores of depressed patients, indicative of gastrointestinal symptoms, heaviness of limbs, and sexual dysfunction, were not associated with any inflammatory markers measured in this study. The somatic/vegetative score was negatively associated with left hippocampal CA3 volumes [Figure 6.7]. While the somatic/vegetative HAM-D subscale scores were not significantly associated with any inflammatory cytokine measures, reduced brain volume could indicate immune activity (Marsland et al., 2008; Kesler et al., 2013). Furthermore, functional neuroimaging studies have shown that patients with somatic-vegetative depression exhibit altered functional activity in reward system structures such as the pre-genual anterior cingulate cortex (Heinzel et al., 2009). These findings would suggest that the development of a neuroimaging model of somatic/vegetative depression might be helpful in understanding the biological differences between patients with and without symptoms of this type.

6.4.2.5 Suicidality score

The HAM-D suicidality scores of depressed patients were positively correlated to cortisol reactivity [Figure 6.9a]. Increased dysfunction of the HPA axis has been observed in depressed patients with higher suicidality symptoms (Westrin and Nimeus, 2003; Jokinen

and Nordstrom, 2008; Pompili et al., 2013), and the present study contributes further evidence in support of this. Suicidality scores in depressed patients were negatively correlated to whole blood transcriptional expression of IL-1 β [Figure 6.9b], which was surprising since IL-1 β mRNA expression was found to be increased in depressed patients compared to healthy controls (Chapter 4). However, it is possible that within the depressed group, inflammation is more prevalent among patients that do not exhibit suicidality symptoms. Concerning the kynurenine pathway, suicidality score was positively correlated to concentrations of circulating kynurenine and negatively associated with whole blood mRNA expression of KAT1 [Figure 6.9c,d]. Since kynurenine pathway induction is evident among depressed patients with increased suicidality scores in this study and others (Sublette et al., 2011; Bryleva and Brundin, 2016), it might be that depressed patients with higher suicidality have a more active HPA axis which induces the KP through TDO, rather than increased inflammation inducing tryptophan catabolism via IDO. The significant relationships identified within the depressed cohort between suicidality score and biological measures reveal that changes in kynurenine pathway may be enhanced in depressed patients with suicidality. Given that suicide is the most serious outcome of a depressive episode, future research should focus on elucidating the biological distinction between patients with and without suicidal ideation.

6.4.3 HPA axis and kynurenine pathway activity in patients with and without atypical subtype of depression

Atypical depression is marked by mood reactivity, hypersomnia, and hyperphagia (American Psychiatric Association, 2000). According to the STAR-D trial, patients with atypical depression have lower rates of treatment response with SSRIs than non-atypical patients (Stewart et al., 2010). Differential biological pathways accounting for atypical and non-atypical profiles of depression have been proposed though no established mechanism has been confirmed (Antonijevic, 2006; Karlovic et al., 2012; O'Keane et al., 2012).

This study found that patients with atypical depression had lower cortisol at wakening and at T60 compared to non-atypical depressives [Figure 6.10]. Consequently, atypical depressives exhibited lower average morning cortisol. These results are in accordance with literature stating that decreased CRH and cortisol have been observed in atypical depression (Nemeroff, 1996; Anisman et al., 1999; O'Keane et al., 2005), though results from studies have tended to vary, with many finding no differences between depressed patients with and

without atypical subtype (Young et al., 2001; Stewart et al., 2009; Vreeburg et al., 2009). The results of the present study suggest decreased HPA hyperactivity in atypical depressives compared to non-atypical patients, but these findings are based on a small sample size (Atypical: n=9, Non-atypical: n=43).

In the present study, depressed patients with the atypical subtype exhibited significantly higher concentrations of circulating kynurenine, and lower quinolinic acid to kynurenine ratios [Figure 6.11a,b]. While this indicates the possibility of increased kynurenine pathway induction in the atypical group, it shows that non-atypical patients had higher levels of quinolinic acid relative to its precursor. Kynurenine pathway activity has not been reported in the atypical subtype of depression prior to this study, therefore this pathway should be explored in larger sample sizes in the future.

6.4.4 Inflammation and kynurenine pathway alterations in first presentation depression and recurrent depression

At least 45% of people with depression experience recurrences, and among those with recurrent depression, an average of seven or eight episodes over the course of a lifetime has been reported (Kruijshaar et al., 2005). The severity of depressive and somatic symptoms increases with number of episodes of depression (Roca et al., 2011), and recurrent depression is associated with more numerous comorbid medical conditions than first presentation depression (Gili et al., 2010). Increased inflammation has been associated with recurrence of depression (Valkanova et al., 2013). However, according to a thorough literature search, there is no study to date that has examined kynurenine pathway differences between first presentation and recurrent depressives, thus the analysis performed in the present study is novel.

Analysis revealed differences in immune system and kynurenine pathway parameters between recurrent and first presentation depressed patients. Whole blood transcriptional expression of IL-1 β was significantly higher in recurrent depressives compared to first presentation depressives [Figure 6.13], confirming the inflammatory response that accompanies recurrence of depression. Circulating quinolinic acid was higher in recurrent depression than in first presentation patients, indicating the presence of more neurotoxic product in this patient group [Figure 6.14]. The quinolinic acid to kynurenine ratio was significantly higher in recurrent depression compared to healthy controls and first

presentation patients, while kynurenic acid to quinolinic acid ratio was significantly lower in recurrent depression than in healthy controls. These findings suggest that the recurrent depression patient group exhibited an imbalance between kynurenine pathway metabolites favouring the potentially harmful arm of the pathway. The lack of statistical difference in activation of the kynurenine pathway between the first presentation depression group and healthy controls suggests that KP alterations may be a result of chronic illness rather than a state marker of depression. While this analysis is based on a small population, it gives motivation for future research designed to specifically investigate kynurenine pathway parameters between first presentation and recurrently depressed patients.

6.4.5 Differences in biological variables between medicated and un-medicated depressed patients

About half of depressed patients achieve remission with two trials of antidepressant medication (Huynh and McIntyre, 2008). More than half of the depressed patients in this study were taking anti-depressant medication at the time of participation, but had not achieved remission. For this reason, it is essential to assess the differences in activity of the immune system and kynurenine pathway between patients being treated pharmacologically and those who are not. Increased circulation of inflammatory cytokines has been shown in depressed patients being treated with antidepressant medication (Hannestad et al., 2011; Vogelzangs et al., 2014). In the present study, analysis of biological variables between medicated and un-medicated depressed patients revealed significantly higher circulating CRP concentrations in patients receiving antidepressant treatment [Figure 6.19]. This indicates that despite treatment, the immune system was activated more severely than in medicated than un-medicated patients. These results also mirror previous findings that currently-depressed patients taking antidepressant medication exhibit to higher immune activation (Vogelzangs et al., 2014).

Assessment of the kynurenine pathway between treatment naïve and currently depressed antidepressant medicated patients has rarely been reported, but in the few studies conducted, results have been mixed. Mackay et al. (2009) reported increased circulating tryptophan in depressed patients after 6 weeks of treatment with either medication or counselling, and no differences in kynurenine pathway metabolite concentrations or ratios after treatment or between groups. Myint et al. (2007) reported similar findings that circulating levels of kynurenic acid were no different before and after 6 weeks of

antidepressant treatment and that levels were no different in medicated patients compared to treatment naïve patients. However, in the present study, differences in kynurenine pathway metabolism were detected.

Results from the current study showed that kynurenic acid was significantly lower in medicated patients [Figure 6.17], along with lower KynA/KYN and KynA/QUIN ratios [Figure 6.18a,b]. Similar to the finding of increased inflammation, this suggests that the medicated group, despite receiving treatment, have even greater dysregulation of a major pathway involved in depression pathophysiology. From these results, it would appear that antidepressant treatment had not resolved the dysfunctions of the immune system and kynurenine pathway, nor the depression symptoms, of patients that were medicated. Due to the fact that pre-treatment measures were not collected from medicated patients in this study, a conclusion is difficult to make and the effect of antidepressant medication on kynurenine pathway activity and how this relates to treatment response is still largely unknown.

6.4.7 Limitations and future directions

This investigation into depression subtypes is preliminary, but indicates that future studies should be designed with subtypes of depression in mind. One improvement on the current study's methods would be to implement the use of a designated suicidality scale that assesses suicidal ideation with a greater range. There are many validated scales for assessment of suicidal risk, including the Suicide Intent Scale (Beck et al., 1974).

The atypical subtype of depression was not well represented in our cohort, and results were based on a small n-size of atypically depressed patients compared to non-atypical. Despite the small group size, significant differences were found in HPA axis activity and kynurenine pathway related parameters. This is the first reporting of kynurenine pathway alterations in atypical depression according to extensive literature searches, therefore replication of these results on a larger scale are essential.

While we attempted to assess the different biological pathways of medicated and un-medicated depressed patients, it would be beneficial to look at the same measures in patients before and after starting medication. With this method, all patients would have a baseline measure of HPA axis, immune, and KP activity that could be re-assessed after a time on medication. Following up with patients after a certain time from commencement of

antidepressant treatment would be beneficial in assessing which patients had remitted and which had not. Then, to observe the differences between remitted patients and those with ongoing depression would provide insight into the underpinnings of treatment resistance in depression. This should be a goal for future research of this type, as the results of this study have indicated a greater inflammatory profile in currently depressed patients on antidepressant medication compared to those who were untreated.

6.4.8 Conclusions

Early life adversity, recurrence of depressive episodes, atypical features and other symptom profiles are factors that play a role in HPA axis, immune, and kynurenine pathway activation in patients with depression. Each of the symptom profiles investigated in this study should be assessed more thoroughly in future studies. The findings of this research, while preliminary, indicate that viewing depression as a heterogeneous disorder is necessary. Establishing a better understanding of the biological basis of depression depends on recognition of the varied presentations within the disorder.

Chapter 7

Discussion and Conclusions

Major depressive disorder is a debilitating mood disorder and is one of the leading causes of disability worldwide (WHO). About 450,000 people in Ireland are affected by this common condition at any given time (Aware Ireland). There are more than 500 deaths by suicide each year in Ireland (HSE), of which depression is the leading cause, therefore research seeking to better understand the biological mechanisms underlying this disease are imperative. In this thesis, several biological systems hypothesised to play a role in depression pathophysiology were assessed in patients with MDD, and the relationship between dysregulation of each of these pathways was observed in the depressed group. The hypothalamic-pituitary-adrenal (HPA) axis, immune system, limbic structure volumes, and kynurenine pathway were each found to be altered in depressed patients in ways that could lead to negative biological outcomes. Furthermore, this study revealed differences in the activation of the aforementioned pathways between patients with differing depressive symptom profiles and subtypes, asserting the importance of acknowledging the heterogeneity of MDD in future research.

7.1 HPA axis hyperactivity and glucocorticoid resistance in depression

The first objective of this study was to assess the role of the HPA axis in a cohort of depressed patients. This was accomplished by measuring cortisol and cortisone concentrations in saliva samples taken from participants at several time points throughout a single day relative to wakening. The primary finding in this study was the significant elevation of wakening cortisol concentrations in the depressed cohort. When assessing parameters of the cortisol awakening response (CAR), it was evident that the stress response systems of the depressed patients were different to that of healthy controls. The reactivity of the CAR was reduced in depressed patients, and a regression line fitted through morning cortisol data was shown to have a smaller slope and higher intercept in depressed patients compared to controls. These findings were in accordance with previous studies that have suggested an elevated yet blunted CAR in depressed patients (Stetler and Miller, 2005; Vreeburg et al., 2009). It has been suggested that persistent stress results in sustained hyperactivity of the HPA axis and leads to glucocorticoid resistance, the impaired inhibitory response of the glucocorticoid receptor (Carroll, 1982; Calfa et al., 2003). Evidence supporting this theory has been demonstrated by the lack of suppression of cortisol secretion following administration of dexamethasone, an exogenous steroid that binds to

the glucocorticoid receptor and normally results in decreased cortisol release (Gold et al., 1986; Holsboer et al., 1987). Glucocorticoid resistance would perpetuate hyperactivity of the HPA axis and may explain the role of hypercortisolemia in the onset of depression.

Cortisone, an inert glucocorticoid, was also measured in saliva and no differences in concentrations were detected between depressed patients and controls at any time point. However, the increase in cortisol and parity in cortisone concentrations resulted in a significantly higher cortisol/cortisone ratio in depressed patients at wakening. Moreover, whole blood transcriptional expression of HSD11 β 1, the gene encoding for the enzyme that converts cortisone to cortisol, was significantly positively correlated to peak cortisol concentration, and the cortisol/cortisone ratio at the wakening time point. This finding suggests the possibility of involvement of the 11 β -HSD1 enzyme in HPA axis dysregulation. These results support the theory of HPA axis alterations in MDD, while indicating that the involvement of this system may be more complex than elevated cortisol alone.

Glucocorticoids such as cortisol and cortisone exert a range of anti-inflammatory and immunosuppressive activities after binding to the glucocorticoid receptor. Because of this function, synthetic glucocorticoids are used to effectively treat autoimmune and inflammatory diseases such as arthritis and asthma. Meanwhile, endogenous corticosteroids are believed to protect against the harmful effects of an overactive immune system (Tuckermann et al., 2005; Lim et al., 2007). The glucocorticoid resistance witnessed in depressed may result in reduced sensitivity of immune cells to glucocorticoids, ultimately resulting in a dysfunctional feedback mechanism between the HPA axis and immune system (Stark et al., 2002; Marques et al., 2009; O'Farrell and Harkin, 2015).

7.2 Inflammatory response in depression

Typically, inflammatory cytokines are released by damaged or infected cells to signal the immune system. Depressed patients have displayed an activated immune responses compared to the healthy population through elevated levels of inflammatory cytokines (Raison, 2006), and concentrations of these cytokines have been shown to increase with severity of depressive symptoms (Thomas et al., 2005). On the other hand, some studies have failed to find associations between depressive symptoms and inflammation, or found that associations were negated when analysis included covariates such as BMI and gender (Bouhuys et al., 2004). Moreover, it has been suggested that since a wide range of inflammatory activity is often observed within studies of depressed subjects, relationships

between inflammation and depressed populations may be accounted for by subsets of individuals with particular symptom profiles (Tiemeier et al., 2003).

In this study, inflammatory cytokines were measured to assess immune activation in depressed patients. Plasma concentrations of circulating cytokines were measured, and the relative gene expression of some of those cytokines was quantified in whole blood. There were no significant differences detected in circulating concentrations of IL-6, IL-1 β , IFN- γ , TNF- α , or CRP between depressed patients and healthy controls. However, relative gene expression of IL-1 β was significantly higher in the depressed cohort. Previous studies have reported more pronounced increases in a range of inflammatory cytokine expression (Raison et al., 2006), yet others have failed to detect a significant relationship between depression and inflammation in cytokines other than IL-1 β (Levine et al., 1999). The results of this study suggest that immune activation was present in this group of depressed patients, but was subtle. The theory that inflammation contributes to some, but not all, cases of depression may explain the results at hand (Raison et al., 2006).

Some clinical features were found to be associated with inflammatory markers in this study. Sleep disturbance and childhood trauma have been suggested to be more involved in the elevated inflammatory response than depression diagnosis. It has been proposed that increased inflammatory responses are partially a result of sleep disturbance in addition to depression (Motivala et al., 2005; Krueger, 2008), and childhood trauma has been shown to be significantly associated with increased IL-1 β (Levine et al., 2015). In the current study, gene expression of IL-1 β was significantly positively associated with both sleep disturbance and childhood trauma among the entire cohort. When assessing inflammation between depressed patients with and without early life adversity, both the circulating levels and the expression of IL-1 β were significantly elevated in those with adversity, strongly supporting the theory that early life adversity may trigger inflammatory activation, leading to depression later in life. Whole blood mRNA expression of IL-1 β was significantly higher in recurrently depressed patients than patients experiencing their first episode of depression, providing additional evidence that inflammation is involved in specific cases of depression but not all. Moreover, whole blood mRNA expression of IFN- γ was positively associated with core depressive symptoms within the depressed cohort, and circulating TNF- α was significantly positively associated with anxiety symptoms within the depressed group, suggesting specific symptom profiles exhibit stronger inflammatory activation.

7.3 Hippocampal subfield volume reduction in depression

The immune system and inflammation may play an important role in mediating brain tissue damage (Calabrese et al., 2015). Loss of grey matter is apparent in natural aging, yet volume reduction occurs at a higher rate in depressed patients than in healthy controls (Grieve et al., 2013). Associations between inflammatory cytokines including IL-6 and TNF- α with brain volume reduction have been reported in past studies (Marsland et al., 2008; Kesler et al., 2013; Braskie et al., 2014), suggesting that alterations of the immune system and brain matter integrity may exist in tandem and contribute to depression pathogenesis. This study evaluated brain volume differences between depressed patients and controls in regions of the limbic system believed to be altered in mood disorders such as MDD, and discovered significant reductions in subfields of the hippocampus in depressed patients. Moreover, elevated inflammation was significantly associated with smaller hippocampal subfield volumes.

Hippocampal subfield analysis revealed significantly smaller left and right hippocampal CA3 volumes and a significantly smaller right CA4 volume in depressed patients compared to controls, suggesting structural alterations had occurred in these specific areas of the hippocampus, or that neurogenesis had been disrupted in these regions. It is believed that the hippocampal CA3 plays a role in associative memory recall and memory sequence (Jensen and Lisman, 1996; Nakazawa et al., 2002). Reduced neurogenesis in this region is consistent with the fact that depressed patients often experience cognitive deficits such as reduced memory function (Lee et al., 2012). The exact function of the CA4 region has not yet been established, but neurogenesis in the CA region in general has been shown to be suppressed during stress (McEwen and Magarinos, 2001), and another recent study showed lower CA1-3 volumes in medicated MDD patients compared to healthy controls (Huang et al., 2013), implying that the CA region as a whole is altered in depression. Moreover, reduction of hippocampal subfields was significantly associated with increased whole blood mRNA expression of IL-1 β in a correlation of all subjects, confirming the relationship between inflammation and brain volume in this cohort. Reduced volume of the CA3 and CA4 in depressed patients in this study is in line with the hypothesis that the hippocampus is damaged in depression (MacQueen and Frodl, 2011), and significant associations with immune markers provided further evidence of the link between inflammation and altered brain matter integrity in MDD.

It has been established that the hippocampus plays a role in stress and the HPA axis. Glucocorticoid and mineralocorticoid receptors exist in the human hippocampus and chronic exposure to high levels of stress or stress hormones have been associated with structural changes in the CA3 (Feldman and Conforti, 1980; Sapolski et al., 1986; McEwen and Magarinos, 2001; Lupien and Lepage, 2001). Additionally, stress has been shown to impair neurogenesis in the hippocampus, and alter learning and memory abilities in human and animal models (Diamond et al., 1996; Gould et al., 1997; Baker and Kim, 2002). The present study found relationships between cortisol concentrations at waking and altered hippocampal volumes, including an increased hippocampal fissure in association with elevated waking cortisol, reinforcing the link between the hippocampus and the HPA axis.

7.4 Induction of the kynurenine pathway by IDO and TDO

It has been hypothesised that the reduction of serotonin availability observed in depression is due to depletion of its precursor, the amino acid tryptophan, which is also the precursor to kynurenine production. The kynurenine pathway of tryptophan metabolism is induced by HPA axis activity and by immune activation. The rate-limiting enzymes that catalyse the conversion of tryptophan to kynurenine are tryptophan 2,3 dioxygenase (TDO) and indolemine 2,3 dioxygenase (IDO). TDO is upregulated in the presence of elevated glucocorticoids including cortisol and IDO is upregulated in response to immune activation (Maes, 2011). Inflammatory cytokines such as IFN- γ , TNF- α , IL-1 β , and IL-6 have each been shown to induce IDO activity (Carlin et al., 1989; Fujigaki et al., 2006; Zunszain et al., 2012). Studies have shown that IDO plays a role in immune tolerance and suppression by inhibiting pathogenic protein synthesis in host defence (Taylor and Feng, 1991). However, increased IDO activity as a result of inflammation has been shown to induce depressive like behaviour in animal models (O'Connor et al., 2009). The results of this study have indicated the presence of elevated cortisol at waking, and increased mRNA expression of inflammatory cytokine IL-1 β , which posed a question as to how kynurenine pathway metabolism might be influenced as a consequence. While this study was unable to measure transcriptional levels of TDO, and found no difference in whole blood transcriptional expression of IDO1 or IDO2 between depressed patients and healthy controls, IDO expression was significantly correlated to whole blood mRNA expression of enzymes and kynurenine pathway induction, such as KMO and kynureninase, as well as quinolinic acid concentration in patients with MDD. Also, IDO2 was negatively associated with right whole hippocampal volumes. While

there were no overt differences between depressed and healthy controls groups in terms of kynurenine pathway inducing enzymes, the findings revealed significant relationships between IDO and downstream KP activity.

7.5 The imbalance of potentially neurotoxic and neuroprotective kynurenine pathway metabolites in depression

There has been growing interest in the kynurenine pathway and its involvement in the pathogenesis of depression, as induction of the kynurenine pathway could be the link between the monoamine hypothesis of depression and disruption of the immune system. In this study, several catabolites of the kynurenine pathway were measured in depressed patients and healthy controls, revealing significant differences in the balance of circulating pathway metabolites. First, this study replicated the robust finding that tryptophan concentrations are depleted in depression (DeMyer et al., 1981; Cowen et al., 1989; Maes et al., 1991). Tryptophan depletion is one mechanism by which serotonin depletion is hypothesised to occur in depression (Maes et al., 2011). While there was no difference in kynurenine/tryptophan ratio between depressed patients and controls, a significant difference in concentrations of downstream pathway metabolites was detected.

In the present study, the ratio of neurotoxic quinolinic acid to its precursor kynurenine was found to be significantly higher in depressed patients, indicating that depressed patients exhibited a relative increase in a potentially harmful component of the KP in relation to its precursor. Subsequently, a significantly lower concentration of kynurenic acid was detected in depressed patients. If these ratios in the periphery reflect what is occurring in the central nervous system, it would suggest that depressed patients may be left more vulnerable to the excitotoxicity and oxidative stress that occurs as a result of the presence of quinolinic acid. Furthermore, the whole blood mRNA expression of the enzyme that converts kynurenine to kynurenic acid, KAT1, was found to be negatively associated with HAM-D scores amongst depressed patients, showing that these adverse biological effects may increase with depression symptom severity. Quinolinic acid is an NMDA receptor agonist, while kynurenic acid is an NMDA receptor antagonist. These two products of the kynurenine pathway function in opposing ways, and the significantly diminished kynurenic acid/quinolinic acid ratio seen in depressed patients in this study is indicative of an imbalance of this pathway in depressed patients.

7.6 A biological model of depression encompassing HPA axis, immune, and KP dysregulation

The HPA axis, immune system, and kynurenine pathways are functionally linked and have each been shown to be altered in depression. Moreover, measures of each pathway were shown to be significantly associated, and greater dysfunction within some of these pathways was determined to be associated with greater depression symptomatology. It is known that HPA axis activation and inflammation induce metabolism of tryptophan, yet the relationship between these pathways and expression of downstream kynurenine pathway metabolites is poorly understood in both health and depression, thus the findings in this study are novel. Contradictory to the hypotheses, this study found that elevated morning cortisol concentrations were associated with reduction of circulating kynurenine pathway metabolite concentrations. However, this study did find that induction of the kynurenine pathway, in particular the quinolinic acid branch of the pathway, was associated with an increase of circulating inflammatory marker CRP. With the additional measure of hippocampal subfield volumes revealing a relationship between alterations in brain morphometry and indices kynurenine pathway activation, this study was the first of its kind. The finding of reduction of right whole hippocampal volume in conjunction with whole blood IDO2 mRNA expression and increased left whole hippocampal volume in association with whole blood KAT1 mRNA expression provide supportive evidence that neuroactive kynurenine products may affect the integrity of limbic structures, which are already known to be influenced by inflammation (Hamidi et al., 2004; Calabrese et al., 2015). The results of this study asserted that the dysregulation of the kynurenine pathway in depression exists concurrently with alterations of the HPA axis and immune system, suggesting that the biological causes and consequences of depression may be explained by a network that encompasses all of these pathways [Figure 7.1].

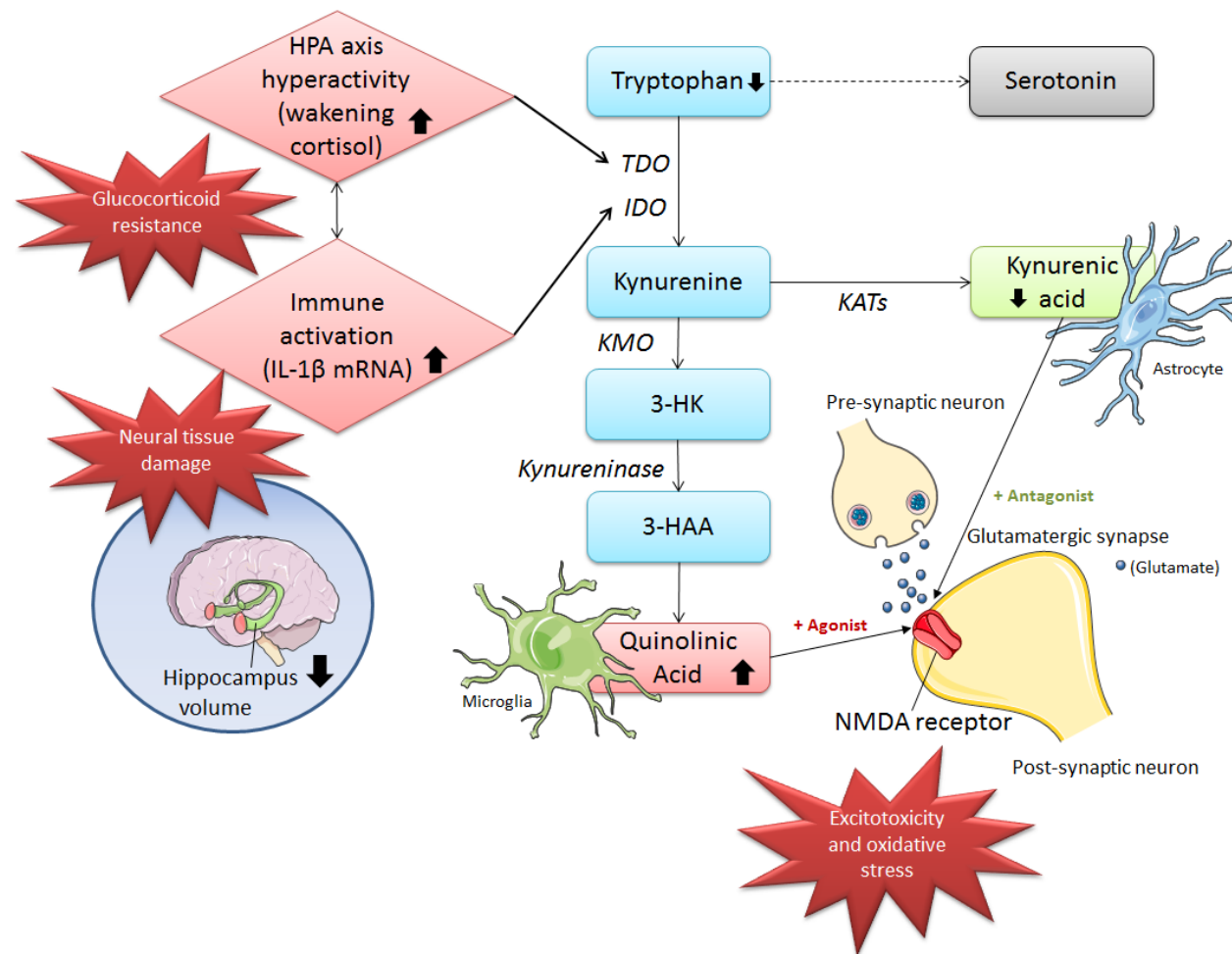


Figure 7.1 Thesis summary. This study found elevated wakening cortisol concentrations and increased transcriptional expression of IL-1β, indicating alterations of the HPA axis and immune system respectively. Significantly reduced hippocampal subfield volumes occurred in CA3 and CA4 of depressed patients, indicating compromised brain tissue integrity. Depletion of tryptophan was evident in depressed patients, and significant reduction of neuroprotective kynurenic acid was also revealed. KAT1 which converts kynurenine to kynurenic acid was negatively associated with HAM-D scores within depressed patients. A significant increase in quinolinic/kynurenine ratio in depressed patients was also detected by analysis. Quinolinic acid agonises the NMDA receptor which leads to excitotoxicity and oxidative stress. Serotonin depletion and negative neurobiological outcomes are hypothesised to occur in depression because of these alterations in the HPA axis, immune system, and kynurenine pathway.

7.7 Heterogeneity of depression: factors that play a role in HPA axis, immune, and KP alterations

It has been established that depression is a heterogeneous disorder with several subtypes and varying symptom profiles (Carragher, 2011; Hybels et al., 2011; O’Keane et al., 2012). In Chapter 6, measurements of the HPA axis, immune system, brain morphometry, and kynurenine pathway were assessed among depression subtypes. Early life adversity, HAM-D subscale scores, atypical subtype, recurrence, and antidepressant medication use were each examined to gain further insight into depression aetiology.

This study determined that greater expression of inflammatory cytokines was associated with anxiety and with early life adversity. Increased measures of inflammatory cytokines have been shown in patients with anxiety disorders (Bankier et al., 2008; Hoge et al., 2009; von Kanel et al., 2010). It has also been widely reported that elevated concentrations of inflammatory cytokines exists in depressed patients with childhood trauma compared to those without (Danese et al., 2007; Carpenter et al., 2010; Lu et al., 2013). The findings of this study add to the growing literature that suggests distinctive immune profiles between depressive subtypes.

Subtypes of depression exhibited differing activation of the kynurenine pathway in this study as well. Distinction of first presentation versus recurrence and acknowledgement of the atypical depression subtype have not yet been addressed in existing literature concerning kynurenine pathway activation in MDD. One of the major findings of the subtype analysis was the that recurrently depressed patients exhibited higher activation of the neurotoxic branch of the kynurenine pathway compared to controls, while first presentation patients showed no differences compared to controls. This finding indicates that KP dysfunction is more prominent in the recurrent patients of this cohort. In regard to atypical depression subtype, it was found that the non-atypical patients exhibited lower kynurenine concentrations and higher quinolinic acid to kynurenine ratios. The atypical group also exhibited lower morning cortisol concentrations. This might suggest that the pathology of atypical depression is not as related to HPA and KP dysregulation as non-atypical depression. Future studies should be designed with the specific intention of assessing differences in kynurenine pathway activation between depression subtypes and symptom profiles. A greater understanding of the unique biological profiles that correspond to specific depression subtypes has the potential to assist in the development of antidepressant treatments targeted toward specific subsets of patients.

7.8 Future directions

Inflammatory cytokines such as those reported to be elevated in major depressive disorder, and those measured in this study, are produced by macrophages (Dinarello and Charles, 2007). Of the cytokines measured, only mRNA expression of IL-1 β was found to be elevated in the depressed cohort of this study despite a large body of evidence suggesting inflammation plays a role in depression pathophysiology (Dowlati et al., 2010; Miller, 2010). Chemokines have also been implicated in the pathogenesis of depression (Suarez et al., 2003; Eyre et al., 2016), thus measurement of a panel of immune markers that included chemokines may provide additional insight in this cohort. It is possible that if a larger range of immune markers had been measured, a more complete understanding of the inflammatory response occurring in depression would have been obtained. Another possibility for measuring brain inflammation directly would be in post mortem studies of depression in which inflammation is measured in the tissue via immunohistochemical methods. Inflammation is currently not possible to measure directly in the brains of living humans in clinical studies, but indirectly with imaging techniques and blood plasma or serum measurements (Vezzani and Friedman, 2011).

Given that depression is experienced in more women than men, with nearly a 2:1 ratio (Kendler et al., 1993), future studies should aim to investigate the differences in kynurenine pathway activation between males and females. While this study included a representative number of females to males, there were no differences detected between measurements of biological markers between sexes. The disparity between the number of men and women that experience depression could be due to several factors including increased psychological stressors for females (Shively et al., 2005; Kendler and Gardner, 2014), or hormonal changes brought on by pregnancy, use of hormonal contraceptive pill, or menopause (Cohen et al., 2006; Cheslack-Postava et al., 2015), but the biological underpinnings of increased vulnerability to depression in females remain elusive.

To date, few studies have been published regarding circulating concentrations of kynurenine pathway metabolites quinolinic acid and kynurenic acid before and after an episode of major depression in humans. It would be of interest to see if the metabolic pathways of patients who have experience remission from a depressive episode exhibit a balance of kynurenic acid and quinolinic acid resembling that of non-depressed people. One study of rat glial cultures showed that kynurenic acid to 3-hydroxykynurenine ratio increased after treatment with several antidepressant medications including fluoxetine and citalopram in a time-

dependent fashion, but this is the only evidence to date indicating that SSRIs have a positive effect on kynurenine pathway metabolism (Kocki et al., 2012). Mackay et al. (2009) found that fluoxetine had no effect on concentrations of tryptophan, kynurenine and KYNA in depressed patients, but did not measure quinolinic acid.

Going forward, participants of this study will be invited to participate in a follow-up investigation. The same markers of HPA axis, immune activity, brain morphometry, and kynurenine pathway metabolism will be assessed in patients after a year from their initial recruitment. This future project hopes to assess the changes in biological variables upon remission, indicating whether some of the biological differences witnessed were state or trait markers of depression. It will also be crucial to examine which participants remitted and which have either not remitted or relapsed into another depressive episode. Assessing the differences between patients who are able to achieve remission with use of an SSRI or other antidepressant medication will aid in the investigation of treatment resistance in depression. Since just under half of depressed patients experience recurrence (Kruijsaar et al., 2005), elucidating the biological differences between patients who are able to achieve remission with pharmacological treatment is a necessity of future research. While previous research has suggested that treatment resistant patients have greater inflammatory alterations (Miller, 2009) and greater glucocorticoid dysfunction (Sanacora et al., 2012), the findings of this study point to the possibility that recurrently depressed patients have a more exaggerated dysfunction of the kynurenine pathway than those presenting with depression for the first time (Chapter 6). It is possible that the ineffectiveness of pharmacological treatment is due to the fact that antidepressant drugs do not directly target the negative biological effects of reduced kynurenic acid and increased quinolinic acid relative to its precursor. Since the results of the present study clearly point to involvement of the kynurenine pathway in depression pathophysiology, the focus of future treatments should be directed toward preventing or counteracting the negative effects of quinolinic acid.

In-vitro models would also be highly useful in exploring dysfunction of the HPA axis, inflammation, and the KP in a cohort of depressed patients. In-vitro models, such as isolation and stimulation of peripheral blood mononuclear cells (PBMCs) of depressed patients and healthy controls, have the potential to provide information about the functionality of cells. PBMCs include T cells, B cells, natural killer (NK) cells, and monocytes, therefore their function in in-vitro experiments is believed to be representative of the physiological response of the immune system (Sullivan et al., 2000). Since the literature supports a theory

of depression involving immune activation, the functionality of these cells is of interest. Isolating PBMCs of depressed patients and controls and then treating them with different conditions including unstimulated, stimulated with lipopolysaccharide (LPS), anti-CD3, or dexamethasone would be helpful in assessing the responses of these cells to different types of activation (Jansky, et al., 2003). Stimulation of PBMCs with LPS would represent activation of the innate immune system via monocytes, whereas anti-CD3 promotes T-cell and adaptive immune system activation. Stimulation of PBMCs with dexamethasone would represent a state of increased glucocorticoid receptor sensitivity. After stimulation in these various conditions at different intervals of incubation, cell supernatants from these stimulations could be preserved and used in assays that would assess HPA, immune, and KP activation (Jones et al., 2015). Measurement of proteins, metabolites, and mRNA expression using ELISA, LC-MS/MS, and PCR could be performed on supernatants to determine activation of each of these pathways. Then, comparisons could be made between the cells' responses to each of the stimulation challenges. Moreover, the results of in-vitro studies could be assessed in relation to the findings observed in this thesis, in plasma and whole blood of patients and controls, to see if the findings were replicated or differed, thus giving insight into the cell types that drive the apparent differences in HPA, immune, and KP activation in depression. For example, these experiments could give insight into whether stimulation of PBMCs of depressed patients results in greater imbalances of kynurenine metabolites via T cell driven stimulation or otherwise, and how this may differ between the cells of depressed patients and healthy controls (Jones et al., 2015). These experiments have the potential to provide insight into the molecular drivers of disorders that relate to immune dysfunction, including depression, and have shown to be a reliable model of a cell's physiological and metabolic activity. Finally, determination of the cells types that drive the alterations in biological pathways of depression could give indication of which cell types should be targeted by future pharmacological treatments of depression. In-vitro models could be very useful as the next step in exploring the network of biological alterations in depression that is proposed by this thesis.

One drug being investigated for its potential use as an anti-depressant is the dissociative anaesthetic, ketamine. Ketamine is an antagonist of NMDA receptor which could counteract the effects of quinolinic acid. It has been shown to exert antidepressant effects in humans and in animal models (Berman et al., 2000; Zarate et al., 2005). A recent clinical trial reported that in 97 treatment resistant depressed patients, ketamine infusions were safe and well tolerated, and had a 67% response rate based on 50% improvement of symptoms

(Wan et al., 2015). The fact that most antidepressant medications such as SSRIs may take two weeks to show an effect, usually require six weeks to achieve the full antidepressant response, and that patients commencing SSRIs may be at a higher risk of suicide or acts of self-harm during the first month of treatment, suggest that future medications should aim to target pathways other than the monoaminergic system (Berton and Nestler, 2006; Conwell and Heisel, 2012). It has been reported that the antidepressant effect of ketamine occurs within 24 hours of an infusion (Kohler and Betzler, 2015). However, the effects of treatment with ketamine are not long lasting, and about 70% of patients treated with one infusion relapse within two weeks (Zarate et al., 2005; Kohler and Betzler, 2015), indicating that multiple infusions would be required to achieve long term therapeutic action (Sisti et al., 2014). Nonetheless, the mounting evidence suggesting that the imbalance of neurotoxic and neuroprotective kynurenines is highly involved in the onset of depression would point to the use of a drug such as ketamine as a promising treatment to counteract the biological consequences of reduced kynurenic acid.

It is largely accepted that there are mental health benefits to physical activity (Lawlor and Hopker, 2001). Recent studies suggest that the mechanism by which exercise could positively impact mood could be by counteracting the alterations of the kynurenine pathway that were witnessed in depressed patients in this thesis. Agudelo et al. (2014) showed that skeletal muscle-PGC-1 α 1 transgenic mice were resilient against stress induced depression. PGC-1 α 1 is a transcriptional coactivator known to induce expression of KATs and increase kynurenic acid production, and normally accumulates within skeletal muscle during exercise. Increases in exercise training were shown to induce production of KATs via PGC-1 α 1 (Agudelo et al., 2014). Moreover, increased conversion of kynurenine to kynurenic acid in the periphery may reduce availability of kynurenine to cross the BBB and exert its effects on the brain (Harkin, 2014). This was also supported by the observation of increased levels of PGC-1 α 1 and KATs enzyme expression in biopsied muscle tissue of humans who had performed a 3-week training program that involved aerobic exercise (Agudelo et al., 2014). These findings support a mechanism by which exercise induces the kynurenic acid branch of the kynurenine pathway in the periphery, leading to improved mood. It suggests that exercise could be useful in treatment of depression or in preventing depression, and provides a possible target for new treatments. Based on the results of this thesis, a search for future treatments of depression should focus on mechanisms that promote production of kynurenic acid and its functions.

7.9 Conclusions

This study demonstrated the existence of a complex biological network in depression involving hypercortisolemia, inflammation, altered hippocampal subfield volumes, and reduction of potentially neuroprotective products of the kynurenine pathway [Figure 7.1]. Persistently elevated cortisol levels in depressed patients may result in glucocorticoid resistance, which would lead to reduced immune suppression and increased inflammatory responses. In turn, inflammation may stifle neurogenesis which is evidenced by volume reduction of hippocampal subfields in depressed patients. Both HPA axis and immune system activation encourage degradation of tryptophan into kynurenine, and in depressed patients, this pathway favours the production of metabolites such as quinolinic acid over the production of kynurenic acid. The relative imbalance is believed to represent an altered environment in the CNS indicative of increased oxidative stress and excitatory transmission, reinforcing the negative biological outcome that may be responsible for depressive symptomatology. Each of the systems assessed were found to be altered in depression, and the search for a biological mechanism responsible for MDD must acknowledge the contribution of the dysfunctions of each of these pathways in the pathophysiology of the disease.

Chapter 8

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Appendix

Appendix 1: Publications and poster presentations

Published papers

Tozzi, L., **K. Doolin**, C. Farrel, S. Joseph, V. O'Keane and T. Frodl (2016). "Functional magnetic resonance imaging correlates of emotion recognition and voluntary attentional regulation in depression: A generalized psycho-physiological interaction study." *J Affect Disord*.

Tozzi, L., A. Carballedo, G. Lavelle, **K. Doolin**, M. Doyle, F. Amico, H. McCarthy, J. Gormley, A. Lord, V. O'Keane and T. Frodl (2016). "Longitudinal functional connectivity changes correlate with mood improvement after regular exercise in a dose-dependent fashion." *Eur J Neurosci* 43(8): 1089-1096.

Tie, Y., L. Rigolo, A. Ozdemir Ovalioglu, O. Olubiyi, **K. L. Doolin**, S. Mukundan, Jr. and A. J. Golby (2015). "A New Paradigm for Individual Subject Language Mapping: Movie-Watching fMRI." *J Neuroimaging* 25(5): 710-720.

Manuscripts submitted for publication

Jairaj C., C.M. FitzSimons, F. McAuliffe, N. O'Leary, N. Joyce, A. McCarthy, E. Cassidy, E. Healy, J. Donnelly, E. Tully, J. Sheehan, J. Burke, M. Imcha, M. Ni Bhuinnean, J. Austin, **K. Doolin**, C. Farrell, V. O'Keane (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., C. Farrell, **K. Doolin**, L. Booij, F. Pomares, J. Chiarella, V. O'Keane, T. Frodl (2017). Role of demethylation of the FKBP5 gene in major depressive disorder: related imaging and stress hormone changes. Manuscript submitted for publication.

Conference poster presentations

Doolin, K., Harkin, A., O'Keane, V. Significant relationships between wakening cortisol concentrations, inflammatory cytokines, and suicidality in first presentation depressives. 29th ECNP Congress of Applied and Translational Neuroscience; 2016, September 17-20; Vienna, Austria.

***Doolin, K.,** Harkin, A., O'Keane, V. Tryptophan pathway depletion and hyperactivity of the HPA axis in Major Depressive Disorder. Poster session presented at: Trinity College Health Science Faculty Research Day; 2016, September 15; Dublin, Ireland.

Doolin, K., Harkin, A., O'Keane, V. An investigation of kynurenine pathway activation and inflammatory cytokines in first presentation depression. Poster session presented at: "Stress, inflammation and depression: focus on novel psychotropic drug targets". Thematic meeting of the International College of Neuropsychopharmacology; 2015, June 4 – 6.

Doolin, K. and Farrell, C., O'Keane, V. First onset depression and the hypothalamic-pituitary-adrenal axis: an investigation of state and trait biomarkers. Poster session presented at: Young Neuroscientists Symposium. Symposium of Neuroscience Ireland; 2014, Sept 20.

***Won the "Best Poster Prize" in the Neuroscience category.**

Appendix 2: REDEEM study information and consent pack

a.



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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Tel.: 01-896-1000



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 3: 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: _____
Age: _____

Date of Assessment: _____
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)
Laden 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)? | NO | YES * |
| IF YES TO EITHER, CODE YES. | | | |
| b | Did you have trouble sleeping nearly every night (difficulty falling asleep, waking up in the middle of the night, early morning waking 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?

NO YES *

**MAJOR DEPRESSIVE
EPISODE, CURRENT**

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? | → NO | YES |
|------|---|------|-----|

- | | |
|---|---|
| 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?	NO	YES
IF NO: When something good happens does it fail to make you feel better, even temporarily?			
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
<i>Major Depressive Episode with Melancholic Features Current</i>	

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 symptomatology.

e.

Pittsburgh Sleep Quality Index (PSQI)

Name: _____

Age: _____

Date: _____

Gender: _____ Male _____ Female

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,

1. When have you usually gone to bed? _____
2. How long (in minutes) has it taken you to fall asleep each night? _____
3. When have you usually gotten up in the morning? _____
4. 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 (≤ 15 min=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.					

Appendix 4: More demographic and clinical data

a. Whole blood mRNA expression

Demographic data for patients with MDD and healthy controls whose whole blood mRNA expression was measured.

	Patients (n=55)	Controls (n=38)	Statistics (p-value)
Age (years)	28.25 (8.63)	28.92 (6.84)	Z=-0.591, p=0.555
Gender (female/male)	38/17	24/14	$\chi^2=0.356$, p=0.551
BMI	24.93 (6.09)	23.49 (3.79)	Z=-0.909 p=0.364
Smoking (Yes/No)	23/32	5/33	$\chi^2=8.772$, p=0.003**
ISCED Education	3.49 (1.50)	6.24 (1.79)	Z=-5.759, 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).

p $\leq$ 0.01 vs. control, *p $\leq$ 0.001 vs. controls.

Clinical data for patients with MDD and healthy controls whose whole blood mRNA expression was measured.

	Patients (n=55)	Controls (n=38)	Statistics (p-value)
HAM-D	22.62 (5.02)	1.79 (1.99)	Z=-8.204, p<0.001***
CES-D	39.68 (9.59)	5.42 (5.37)	Z=-8.058, p<0.001***
PSQI	12.93 (3.74)	3.68 (2.06)	Z=-7.826, p<0.001***
CTQ Global Score	43.40 (14.68)	28.47 (5.66)	Z=-5.120, p<0.001***
Early Life Adversity (Yes/No)	36/19	3/35	$\chi^2=30.578$, p<0.001***
Antidepressant (Yes/No)	36/19	0/38	

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). ***p $\leq$ 0.001 vs. controls.

b. Structural MRI scans

Demographic data for patients with MDD and healthy controls who completed structural brain scans.

	Patients (n=47)	Controls (n=35)	Statistics (p-value)
Age (years)	30.21 (8.62)	28.51 (6.93)	Z=-0.892, p=0.373
Gender (female/male)	32/15	23/12	$\chi^2=0.051$, p=0.821
BMI	24.69 (5.37)	23.33 (3.20)	Z=-0.969, p=0.333
Smoking (Yes/No)	19/28	5/30	$\chi^2=6.621$, p=0.010**
ISCED Education	3.64 (1.56)	6.11 (1.73)	Z=-5.177, 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).

p $\leq$ 0.01 vs. control, *p $\leq$ 0.001 vs. controls.

Clinical data for patients with MDD and healthy controls who completed structural brain scans.

	Patients (n=47)	Controls (n=35)	Statistics (p-value)
HAM-D	22.34 (5.48)	1.77 (2.24)	Z=-7.738, p<0.001***
CES-D	38.96 (9.81)	5.26 (5.70)	Z=-7.615, p<0.001***
PSQI	12.92 (4.07)	3.71 (2.14)	Z=-7.200, p<0.001***
CTQ Global Score	46.46 (16.40)	29.21 (6.08)	Z=-4.911, p<0.001***
Early Life Adversity (Yes/No)	32/15	5/30	$\chi^2=22.849$, p<0.001***
Antidepressant (Yes/No)	30/17	0/35	

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).